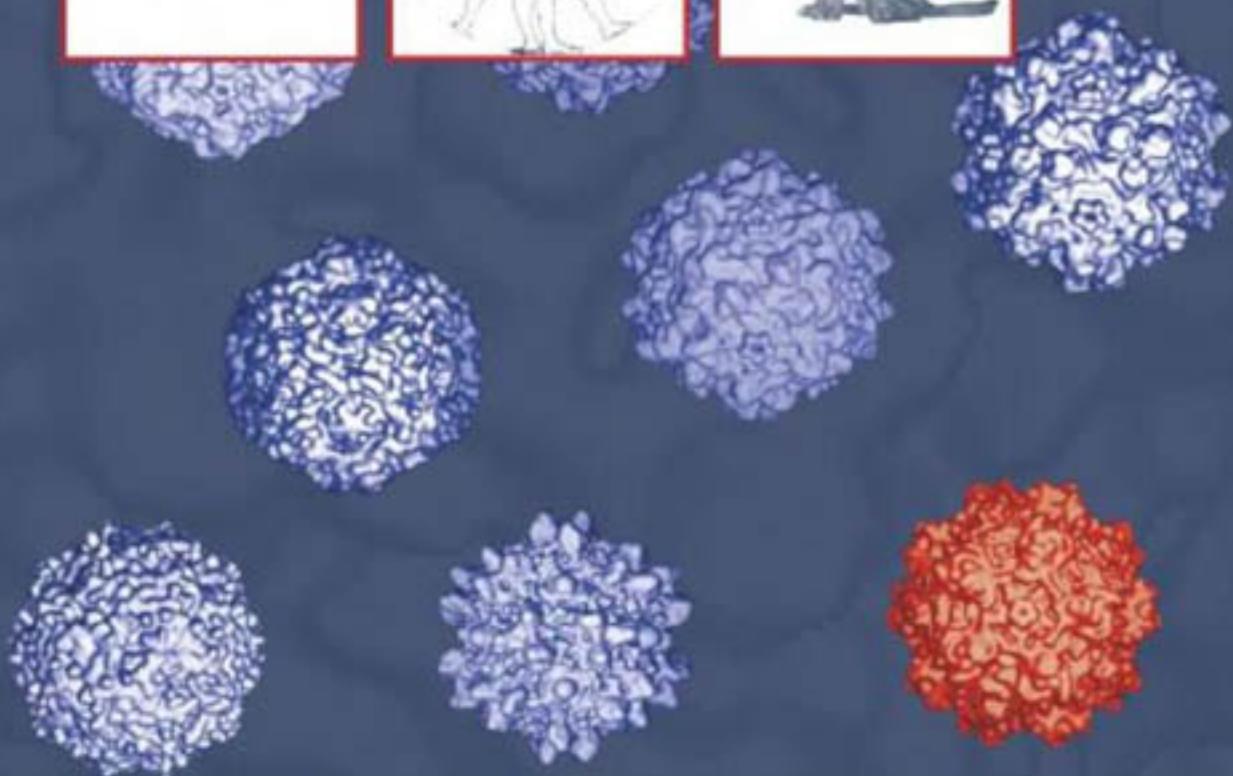


# PARVOVIRUSES



Jonathan R Kerr, Susan F Cotmore, Marshall E Bloom,  
R Michael Linden and Colin R Parrish

# Parvoviruses

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# Parvoviruses

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# Dedication

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## Jacov Tal (1940–2005): An Appreciation

As this book is being readied for press we have received the news of the sudden and untimely passing in February of our friend and colleague Jacov Tal. It was a profound shock for us all to hear of the dreadful circumstances of Jacov's illness, made even more so because he was in such good health and fine spirit at the Xth Parvovirus Workshop in Florida last September 2004.

Jacov was a long-time member and contributor, and a very integral part of, the world wide parvovirus community from the time when this was quite a small group. He will be greatly missed but very fondly remembered. We all have wonderful memories of Jacov, and many will remember the parvovirus workshop that he organized in Israel in 1989. I personally have many wonderful memories of him as a scientist and friend, and particularly the two years he spent with us at NIH.

Jacov's initial training in Israel was in biochemistry, first as an undergraduate at The Hebrew University in Jerusalem and then as a graduate student at the Weitzman Institute in Uri Littauer's lab. However, following his studies on tRNA with Littauer and obtaining his PhD in Biochemistry in 1971, he set out on a career as a virologist and worked on several classes of viruses, although he ultimately spent most of his career devoted to parvoviruses. He first studied adenovirus RNA transcription as a postdoc with Heschal Raskas in St Louis. He then went to the laboratory of Michael Bishop and Harold Varmus at The University of California, San Francisco, to study retroviruses where he analyzed the structures of avian tumor viruses genes and their cellular counterparts. Jacov then returned to Israel to the fledgling Ben Gurion University of the Negev in Beer Sheva and established studies in virology and retained a faculty position there for the rest of his career as Head of Virology.

Upon his return to Israel, Jacov took up the study of parvoviruses and initially was interested in MVM and its transcription in studies he did with the late Yossi Aloni. He also began to study the biology of MVM and its host cell tropism and, in many of these studies over the years, he collaborated with Peter Tattersall at Yale or Jean Rommelaere in Europe. In the mid-1980s he spent a 2-year period in my

laboratory at NIH in Bethesda and contributed to some of the initial studies on the *trans*-regulation of AAV transcription by the AAV rep gene.

In addition to his accomplishments as a scientist, the enduring memory and impact of Jacov as a friend, colleague, collaborator, and mentor to his students, is his love of interaction with others, his extraordinary positive impact upon them, his perpetual questions, his great sense of humor and his wonderful story-telling. This emanated from an inner philosophy that he had about life, that we are all here to achieve useful things and that we can best do this by constantly asking questions and seeking the answers together. Based upon these beliefs, Jacov had a highly developed sense of community and fairness, so that he held strong but well-reasoned opinions that he never failed to express. Nevertheless, his opinions were always presented



in a most constructive way and we are all individually and collectively the better for hearing them.

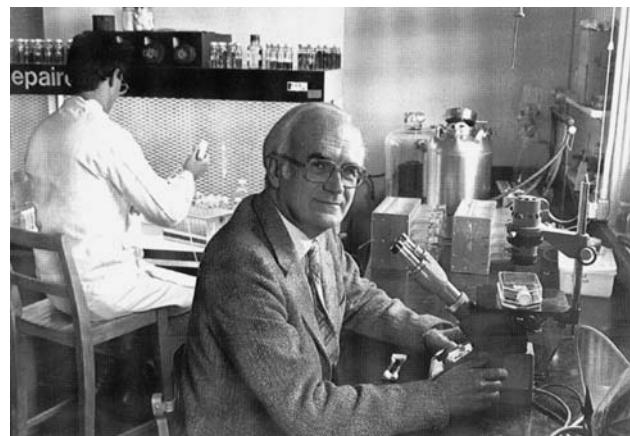
The photo accompanying these comments beautifully captures the essence of Jacov, the man, and the occasion of the publication of this book is a wonderful opportunity to memorialize him. So, although we feel a profound sadness at Jacov's passing, we also celebrate his life and the wonderful community that he created around him.

*Barrie Carter, March 2005*

#### **David A. J. Tyrrell (1925–2005)**

David Tyrrell began his research into the common cold shortly after the end of the Second World War at a time when knowledge of its causes was inaccurate and conflicting, and went on to reveal virtually all of its mysteries: that it was caused by several hundred viruses which were transmitted by aerosol droplets, and how the viruses actually cause disease; interactions with other diseases; and the effect of certain predisposing factors. He also tested several candidate treatments, but as more and more 'cold viruses' were revealed, it became clear why no single treatment could be expected to work consistently.

David Arthur John Tyrrell was born on 19 June 1925 and graduated with distinction in Medicine at the University of Sheffield in 1948 and upon the encouragement of Professor (later Sir) Charles Stuart-Harris, became interested in the novel area of virus research, and joined a group studying influenza viruses. In 1951, he began a three-year attachment to the Rockefeller Institute for Medical Research in New York, working with Frank Horsfall on influenza viruses, and returned to Sheffield University in 1954. In 1957, he was invited to run the MRC Common Cold Unit (CCU) in Salisbury, by Sir Harold Himsworth, then Secretary of the MRC. He accepted and took up the post on 1 April 1957.



**Figure 1** David checking cell cultures using a light microscope in the virus laboratory.

The CCU was housed in Harvard Hospital, which was a gift from the USA to the UK in time of war. It was built in 1940 from pre-fabricated materials shipped from the USA, and was equipped and staffed by Harvard University in Boston and the American Red Cross. Harvard Hospital was designed and run as an infectious disease hospital, and after the war ended, it was given to the MRC and became the CCU in 1946. The creation and the continuation of the CCU was due critically to the support at the MRC of Sir Christopher Andrewes, an influenza virus researcher, who had greatly inspired David.

Under his direction, the CCU began to uncover the specific viral causes of the common cold, its global epidemiology, some possible treatments and the important role played by emotional stress in the genesis of symptomatic infection. The involved the use of human volunteers (a total of 20 000), many of whom visited the unit on an annual basis in return for a 10 day holiday on Harnham Hill, a small stipend and a dose of cold virus, delivered intranasally.

An important dimension of the work was David's skill in motivating and bringing people together towards a common goal, and this applied at local, national and international levels in the initiation and maintenance of collaborations with the World Health Organization (WHO), National Institutes of Health (NIH) and others which led to an international network of friends and collaborators.

In the early 1960s, David was involved in planning the Clinical Research Centre which was closely integrated with Northwick Park Hospital in Harrow in north-west London and opened in stages between 1970 and 1975. As Head of the Division of Communicable Diseases and Deputy Director, he was later involved in developing and running the general medical and infectious disease services of the CRC.

He was elected FRS in 1970 and appointed CBE in 1980. In the early 1980s, he hosted the seminal studies of parvovirus B19 infection in human volunteers, a collaboration with Professor (later Sir) John Pattison, which provided some firm



**Figure 2** Aerial view of the Common Cold Unit, Salisbury, Wiltshire.

foundations in our knowledge of the way this virus causes disease, and upon which much further work was based.

David retired from the CCU in 1990 but continued to contribute in new ways. He chaired the government's BSE committee (1989–1995) and the Taskforce on Chronic Fatigue Syndrome (CFS)/myalgic encephalomyelitis (ME) (1994). From 1994, he served as Vice-Chairman of the Research Committee of the CFS Research Foundation and became its Chairman in 2000. During this time, he was involved in studies outlining the role of parvovirus B19 as a trigger for CFS and in setting up a program of research in to the ongoing physical causes of CFS using gene expression and proteomics. This work continues today, with the ultimate aim of developing a diagnostic test and effective treatments for CFS sufferers.

David was a refined and elusive man who was sparkling with ideas, a wonderful sense of humor and who, despite his

success, never lost the common touch. In his undertakings, the best interests of people was always foremost in his motivation; patients, colleagues, friends, family and community at all levels.

David was also a devoted husband and father to three children; he enjoyed gardening and read very widely. He lived a Christian faith, and was the President of both the Christian Medical Fellowship and the Friends of the Christian Medical College at Ludhiana, India. He was also the organist and choirmaster at All Saints Church, Whiteparish, Wiltshire.

He is survived by his wife, Moyra, and two daughters, Frances and Sue.

*Jonathan Kerr, June 2005*

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# Foreword

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The first book devoted to parvoviruses that I remember was the result of a meeting on the molecular biology of mammalian parvoviruses held at Cold Spring Harbor in the spring of 1977. All of the authors, who constituted a majority of the meeting participants, received free copies. Actual sales were reputed to have totaled eight, a new record for the Cold Spring Harbor Press. This signal lack of success in achieving public interest in the topic was likely a major reason that no subsequent meetings have occurred in the same venue. Despite the initial lack of widespread interest in the parvoviruses, perseverance by many of the participants in that early meeting, as well as by new investigators in the field, has led to significant appreciation of the importance of this virus family with respect to the pathogenicity of many members. More unexpectedly, the adeno-associated viruses (AAV) have attained widespread interest as potential vectors for human gene therapy. Three developments in these areas have given particular impetus to the study of parvoviruses:

- The relationship of dogs to people has been an important component historically of human psychology and sociology. Thus, when large numbers of puppies suddenly began dying simultaneously in three widely separated locations, Australia, Hawaii, and the United Kingdom, there was widespread interest; i.e. it was a true 'human' interest story. Epidemiologically, the outbreak was unusual because of the wide separation of the original sites and even more unusual because all three were islands with very strong quarantine regulations to prevent just such events. There was a burst of investigatory activity, which showed that the etiological agent was a parvovirus that had 'jumped' species boundaries; one of the early examples of such an event to be recognized and characterized. Interestingly, the source of canine parvovirus has never been determined definitively.
- The second boost to the popularity of parvoviruses was the discovery by John Pattison and his co-workers that a parvovirus, B19, was the causative agent of aplastic crises in children with sickle cell disease. Until that point AAV had been the only known human parvovirus and it was of interest only to aficionados of its unusual biology. Once there was a proven human

parvovirus pathogen a great deal of work was done by the clinical virologists who were able to demonstrate that not only children with sickle cell disease were affected by B19: infection with the virus occurred nearly universally in children and caused the exanthem known as fifth disease.

- Additional interest in B19 arose from its ability to cause miscarriage in the second trimester of pregnancy. Initially the unusual biology of AAV was attributed to the fact that it was considered defective since it required a helper virus co-infection in order to multiply in cells in culture. Viruses are by their very nature defective, since they are obligate intracellular parasites. But a virus that required not only a permissive cell, but also a helper virus, and which was not known to cause disease in any host species, people in particular, was bound to be of interest to only a narrow slice of the virology community.

Interestingly, AAV has become a good example of the nature and value of basic research. We now appreciate that all nuclear DNA animal viruses cause persistent infections. In the case of AAV, the persistent or latent phase of the life cycle of the virus is the dominant phase. Because of its inability to replicate in cell culture in the absence of a helper virus, AAV has become one of the best studied models of viral latency. Just as important is the consequence of the lack of human pathogenicity. About 80 percent of adults are seropositive, yet the virus has never been associated with the cause of any human disease.

What once was considered a reason for lack of interest is now appreciated as a very desirable attribute in the search for useful vectors for human gene therapy. As appreciation of the importance of genetics grows and with the elucidation of the human genome, the desire to be able to treat diseases with a genetic basis has grown. For a fair number of diseases the gene involved is known and has been isolated, cloned and characterized. The therapeutic challenge is to be able to introduce a normal gene into the cell to replace or overcome the consequences of a defective gene. What is required is a vector to be able to insert the new gene. Although various types of vectors have been tried, those based on viruses have remained in the forefront because viruses have been designed by nature to deliver genes into cells. The first

problem, of course, is that viruses also cause disease, so most have to be modified so that they can still function without leading to disease. Here AAV has an inherent advantage with its long-term persistence and lack of pathogenicity. Other parvoviruses are also being studied to develop different systems for gene delivery, although these studies are still at a more fundamental stage. Thus parvovirus research has gone from basic biological research to the applied level, both with respect to the characterization and prevention of diseases caused by these viruses, and to the therapeutic use of the viruses to treat genetic diseases.

One last word at the level of human disease; because of their biology the autonomous parvoviruses require dividing cells to replicate. This has led to consideration of their use as anticancer agents. For somewhat different reasons, AAV also has been considered as an anticancer agent. The notion of microbes as therapeutic agents has certainly been an intriguing one. In this respect parvoviruses are on the leading edge of the curve.

I have not said anything about those parvoviruses that infect arthropods. They serve to demonstrate the ubiquitous nature of these agents. Although in many respects their overall structure and their genome sequence arrangements are comparable to those of the vertebrate species, the genetic organization of their genomes displays in some instances a complexity that goes beyond that seen in the vertebrate viruses. Clearly we have just scratched the surface in identifying the insect parvoviruses. Since arthropods represent a large fraction of the biological kingdom, their viruses constitute an important area of study.

In summary, we have begun to appreciate in some detail the complexity and significance of the *Parvoviridae*. This book represents our current knowledge and the promise of the future. I am confident that it should attract a somewhat larger readership than the predecessor, which I mentioned in beginning.

*Kenneth I. Berns*

# Introduction

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The purpose of this introductory essay is to outline, very briefly, the scope of our volume – an ambitious project that sought to bring together our current understanding of relationships within the virus family *Parvoviridae*, and to provide a comprehensive review of recent advances in our knowledge of their structure and molecular biology, the interactions they establish with their host organisms, and the clinical consequences, or clinical potential, of those interactions. To this end, we have divided the book into four Parts, dealing first with the structures and mechanisms that these viruses exploit in interacting with their host at the level of the individual infected cell. Next we examine interactions at the level of the whole organism, exploring factors that determine host range and tissue tropism, establish patterns of pathology, interactions with the host's immune system, or mediate these viruses' manifest ability to specifically suppress the growth of oncogenically transformed cells. In the third Part we review individual viruses, and the patterns of infection and/or pathology that they establish in their specific hosts; and, finally, we explore the medical potential of these viruses as vectors that are able to transencapsidate and deliver therapeutic genes to the nuclei of specific cell populations, while impinging only minimally on other aspects of host physiology. This volume does not include summaries of the technologies used to generate or purify virus stocks. For these, the interested reader is referred to previous reviews (P. Tijssen *Handbook of Parvoviruses*, Boca Raton, FL: CRC Press, 1990), or the emerging literature, where methods for optimizing and scaling-up production of virus vectors carrying therapeutic transgenes, most notably from the various adeno-associated virus (AAV) species, with an eye to their potential clinical use are constantly being refined.

## BIOLOGY OF THE VIRUS FAMILY

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In the first section of this volume, parvovirus diversity is explored from a variety of perspectives. These range from the emergence of the closely related clades and quasi-species that are now becoming apparent within the vertebrate *Parvovirinae* subfamily and that reflect the unexpectedly

high mutation and recombination rates seen in this family of single-stranded DNA viruses, to the massive sequence jumps, reflecting divergence over vast expanses of evolutionary time, which differentiate genera in the arthropod-infecting *Densovirinae* subfamily. In an upcoming report of the International Committee for Virus Taxonomy (ICTV, Tattersall *et al.*, 2005) classification of the *Parvoviridae* moves firmly away from older taxonomic criteria such as viral life style or the numbers of specific structural or functional elements in the genome, to a predominantly sequence-based phylogenetic approach. This should allow the creation of taxa that more perfectly reflect divergent evolution from common ancestors, and to this end several new genera have already been defined, namely the vertebrate *amoviruses*, *bocaviruses*, and the *pefudenviruses* of the *Densovirinae*, each representing distinct phylogenetic branches.

Following a discussion of the new taxonomic criteria (Chapter 1), individual chapters in Part 1A discuss characteristics and diversities that unite and divide species in each of the major virus genera.

The hallmark of parvoviruses is their diminutive size, which defines and limits their parasitic strategies. They are among the smallest of the isometric viruses, with virion diameters of just around 250 Å, containing a simple tightly-packed single-stranded DNA genome of 5 kilobases that encodes just two genes. This makes them supremely dependent on their host cell, and in some cases on helper viruses from various, more complex, virus families to supply the vast panoply of synthetic machinery required to ensure their existence. Thus they have become masters at usurping host signals for their own preferential amplification, and at stealth, sometimes establishing latent infections in which they are barely detected, and certainly ignored by, their host. Accordingly, members of this family can be significant pathogens, as seen for the human parvovirus B19 or the feline panleukopenia virus serotype, while others are widespread in the human population, but efficiently establish asymptomatic persistent but latent relationships that make them ideal candidates as gene therapy vectors. In Part 1B, the structure, function, and specific enzymatic activities of the viral particle are dissected, with particular focus on how these structures mediate interactions with the host. Following a discussion of their unique single-stranded

genomes, which possess short hairpin telomeres that mediate essential steps in the viral replication scheme (Chapter 7), the phospholipase A2 domains present in the VP1 subset of viral capsid proteins is reviewed (Chapter 8). Essential for a step in viral infectivity that follows internalization into the host cell but precedes escape from that cell's endosome, this type of invasion machinery was first discovered in the *Parvoviridae*, and is the subject of much current research interest. In recent years the application of X-ray crystallographic techniques to the viral particles has allowed dramatic insight, at molecular and atomic levels, into how these structures protect the viral genome and mediate the necessary interactions with the transport and defense machinery of their host cells, ultimately delivering their delicate payload to the replication machinery of the host nucleus. These advances are reviewed in Chapters 9 and 10.

Part 1C traces virus–host cell interactions involved in every step of the viral life cycle at the level of the single cell, and including both mechanisms of latency and productive infection. Overviewed in Chapter 11, specific steps in the infectious process from cell entry and trafficking to the nucleus (Chapters 12 and 13), through viral DNA replication (Chapters 14 and 15), integration (Chapter 16), latency and its disruption by helper viruses (Chapter 17), and RNA processing strategies (Chapter 18) are explored. This Part then moves on to look at the viral gene products in detail, examining how their myriad roles in the life cycle are modulated by their relative rates of synthesis, post-translational modification and trafficking (Chapters 19 and 20), and finally how the non-structural and capsid proteins come together to mediate vectorial encapsidation of newly-displaced progeny single-stranded genomes into preformed capsid shells, driven by the virally encoded SF3-helicase motor (Chapter 21).

## PATHOGENESIS AND BIOLOGY OF PARVOVIRUS INFECTIONS

In Part 2, four chapters outline our knowledge of the pathogenesis and biology of parvovirus infections or the ways in which parvoviruses cause disease and the factors that determine the phenotype of each virus infection. Host range and cell tropism of the parvoviruses (Chapter 22) are considered first with a particular emphasis on studies of canine (CPV) and feline (FPV) parvoviruses, minute virus of mice, porcine parvovirus, and Aleutian mink disease parvovirus. This chapter provides detailed discussion of the acquisition of extended host range by FPV through the multistep adaptation of a new receptor and the emergence of CPV, and what is known of the controls of cell and tissue tropism. This is followed by a detailed review of the pathogenesis of parvovirus infections (Chapter 23) including host immunity, virulence determinants (of both the virus and the host in the context of parvovirus infection), virulence mechanisms

(e.g. lytic infection, apoptosis and cytotoxicity, immune phenomena, etc.), and aspects of acute versus persistent infection. Aspects of the immune response to erythrovirus infection in humans is then discussed in Chapter 24 in terms of humoral and cellular immunity. Chapter 25 deals with parvovirus oncosuppression which is an *in vivo* phenomenon and manifest as the prevention of tumor establishment, reduction or arrest of tumor growth, regression of established tumors, diminished take of transplantable tumor cells, or prolongation of the life of tumor-bearing animals.

## SPECIFIC PARVOVIRUS INFECTIONS AND ASSOCIATED DISEASE

Parvovirus infection has been associated with disease of primates, animals, and insects. In primates and animals certain clinical manifestations are characteristic of parvovirus infection including fetal infection, congenital features such as myocarditis in puppies, encephalopathy, enteritis, hepatitis, anemia, leucopenia, thrombocytopenia, and immune complex disease. In Part 3 is a collection of chapters describing the individual parvovirus species and their infections and associated disease in respective hosts. Specific chapters are devoted to human dependoviruses (Chapter 26), human and primate erythroviruses (Chapter 27), rodent parvoviruses (Chapter 28), feline and canine parvoviruses (Chapter 29), porcine parvovirus (Chapter 30), waterfowl parvoviruses (Chapter 31), Aleutian mink disease parvovirus (Chapter 32), minute virus of canines (Chapter 33), bovine parvovirus (Chapter 34), and shrimp parvoviruses (Chapter 35).

## USE OF PARVOVIRUSES AS GENE THERAPY AND VACCINE DELIVERY VECTORS

In Part 4, the potential for use of parvoviruses as vectors for gene delivery is explored. A number of properties have led to the exploitation of AAV as a gene delivery vector including its small size, chemical definition, the lack of a detectable inflammatory response, and a ready ability to transduce both dividing and non-dividing cells followed by persistence for the lifetime of the cell. A singular and specialized area of basic science has grown out of the considerable expansion in the study of both AAV vectors and AAV biology. Two introductory chapters review the biology of gene delivery (Chapter 36) and clinical development (Chapter 37), respectively. Chapter 37 reviews those diseases for which gene delivery vectors have been developed, for example, cystic fibrosis, hemophilia, alpha-1-antitrypsin deficiency, muscular dystrophy, Canavan disease, Parkinson disease, and rheumatoid arthritis, and also discusses applications to treat cancer and infectious disease. This is

followed by discussions of the mechanism of recombinant AAV transduction (Chapter 38), extending the (limited) capacity of AAV vectors (Chapter 39), tailoring of the AAV capsid (Chapter 40) to specifically target a narrow range of cell types, and gene targeting (Chapter 40) to correct the gene defect in the genomic DNA to achieve a sustained benefit and to reduce the theoretical possibility of insertional

mutagenesis. There follows a discussion of recombinant AAV delivery to the liver (Chapter 42) and retina (Chapter 43). Finally, the role of helper-independent parvoviruses as gene therapy and vaccine delivery vectors is considered (Chapter 44), with a particular focus on the exploitation of the oncotropism of autonomous parvoviruses to facilitate antitumor therapy.

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# Abbreviations

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AAAV	avian adeno-associated virus	BFPV	blue fox parvovirus
AADC	aromatic amino acid decarboxylase	BFU-E	burst-forming units – erythroid
AaeDNV	<i>Aedes aegypti</i> densovirus	BgDNV	<i>Blattella germanica</i> densovirus
AalDNV	<i>Aedes albopictus</i> densovirus	BHK	baby hamster kidney
AAT	alpha-1-antitrypsin	BIH	benign intracranial hypertension
AAV	adeno-associated virus	BIRC4	baculoviral IAP repeat-containing protein-4
AAV2	adeno-associated virus 2	BLE	B19-related lupus erythematosus
AAVSI	adeno-associated virus integration site I	BLOM	bubbleless left flop minus
ABCR	ATP-binding cassette transport	BLOP	bubbleless left flop plus
aCL	anticardiolipin	BMD	Beck muscular dystrophy
Ad	adenovirus	BmDNV	<i>Bombyx mori</i> densovirus
AD	Aleutian disease	bp	basepair
Ad-DBP	adenovirus single-strand binding protein	BPL	beta-propiolactone
AdDNV	<i>Acheta domesticus</i> densovirus	BPV	bovine parvovirus
ADE	antibody-dependent enhancement	BudR	bromodeoxyuridine
ADRP	autosomal dominant retinitis pigmentosa	CAAV	canine adeno-associated virus
AFP	alphafetoprotein	CARE	cis-acting replication element
AIDS	acquired immunodeficiency syndrome	CaMV	minute virus of canines
ALL	acute lymphoblastic leukemia	CAR	coxsackie virus and adenovirus receptor
AMD	age-related macular degeneration	CBC	cap-binding complex
AMDV	Aleutian mink disease virus	CD	Canavan disease
AML	acute myeloblastic leukemia	CD/CD	cesarean-derived, colostrum-deprived
AMP	adenosine monophosphate	CeDNV	<i>Casphalia extranea</i> densovirus
ANA	antinuclear antibody	CF	cystic fibrosis
ANCA	antineutrophil cytoplasmic antibody	CFS	chronic fatigue syndrome
AP2	adapter protein 2	CFTR	cystic fibrosis transmembrane regulator
APAR	autonomous parvovirus replication	CFU-E	colony-forming units – erythroid
APC	antigen-presenting cells	CFU-GM	colony-forming units – granulocyte-macrophage
aPL	anti-phospholipid	cGMP	current good manufacturing practice
APS	antiphospholipid syndrome	CHO	Chinese hamster ovary
Asp	aspartic acid	ChpPV	chipmunk parvovirus
ApDNV	<i>Aedes pseudoscutellaris</i> densovirus	CIE	counter immune electrophoresis
APT	activated partial thromboplastin time	CMCRC	Comparative Medicine Clinical Research Center
ASPA	aspartoacylase	CMV	cytomegalovirus
ATM	ataxia-telangiectasia mutated	CnMV	canine minute virus
ATP	adenosine triphosphate	CNTF	ciliary neurotrophic factor
ATR	ATM-Rad3 related	CNV	choroidal neovascularization
BAAV	bovine adeno-associated virus	CNS	central nervous system
B-CLL	B-cell chronic lymphocytic leukemia	CpDNV	<i>Culex pipiens</i> densovirus
BDNF	brain-derived neurotrophic factor	CPE	cytopathic effect
BDPV	Barbarie duck parvovirus	CPV	canine parvovirus
BEI	binary ethyleneimine	CRE	cAMP-responsive element
BFL	bovine fetal lung		

CRFK	Crandell feline kidney	GAD	glutamic acid decarboxylase
CRM	chromosome region maintenance	GCA	giant cell arteritis
CRM1	chromosome maintenance region 1	GDNF	glial cell line-derived neurotrophic factor
CsCl	cesium chloride	GFP	green fluorescent protein
CSF	cerebrospinal fluid	GM-CSF	granulocyte-macrophage colony stimulating factor
CTD	carboxy terminal domain	GmDNV	<i>Galleria mellonella</i> densovirus
CTfr	canine transferrin receptor	GMEB	glucocorticoid-modifying element-binding protein
CTL	cytolytic T-cell	GPV	goose parvovirus
D-BP	d-region binding protein	GTPase	guanosine triphosphatase
DBP	DNA binding protein	H-1PV	H-1 parvovirus
DBS	deep brain stimulation	HA	hemagglutination
DC	dendritic cells	HADEN	hemadsorbing enteric [strain]
DCM	dilated cardiomyopathy	HAI	hemagglutination inhibition
DEAF	deformed enhancer activating factor	HaPV	hamster parvovirus
DHS	DNAseI hypersensitive site	HBPV	HB parvovirus
DI	defective interfering	HBsAg	hepatitis B antigen
DMD	Duchenne muscular dystrophy	HCI	hydrochloric acid
DN	dominant negative	HCM	hypertrophic cardiomyopathy
DNAPKcs	DNA-dependent protein kinase catalytic subunit	HD	histidine-aspartic acid
DNV	densovirus	HePV	hepatopancreatic parvovirus
DPV	duck parvovirus	HI	hemagglutination inhibition
DRP	Dnase-resistant particles	His	histidine
ds	double stranded	HIV	human immunodeficiency virus
dsAAV	double-stranded AAV	HLA	human leukocyte antigen
DSBs	double-strand breaks	HMG	high mobility group
dsDNA	double-stranded DNA	HPV	human papillomavirus
DsDNV	<i>Diatraea saccharalis</i> densovirus	HR	homologous recombination
DSE	downstream element	HSP	heat shock protein
DSMB	delta safety monitoring board	HSP90	heat shock protein 90
EAAV	equine adeno-associated virus	HSPG	heparan sulfate proteoglycan
EaDNV	<i>Euxoa auxiliaris</i> densovirus	HSV	herpes simplex virus
EBD	Evans blue dye	HU	hydroxy urea
EDS	egg drop syndrome	ICE	interleukin-1beta-converting enzyme
EGF	epidermal growth factor	ICTV	International Committee on the Taxonomy of Viruses
EGFP	enhanced green fluorescent protein	IFA	immunofluorescence assay
EGFR PTK	epidermal growth factor receptor protein tyrosine kinase	IFA	indirect immunofluorescence
EI	erythema infectiosum	IFN	interferon
ELISA	enzyme-linked immunosorbent assay	IEF	isoelectric focusing
EM	electron microscopy	IEM	immune electron microscopy
EnaC	epithelial sodium channel	IES	intronic splicing enhancer
Eps8	epidermal growth factor receptor pathway substrate 8	IF	immunofluorescence
ERG	electroretinogram	IgE	immunoglobulin E
ES	embryonic stem [cells]	IgG	immunoglobulin G
ESE	exon splicing enhancer	IgM	immunoglobulin M
ESK	embryonic swine kidney cell line	IGF-1	insulin-like growth factor-1
ETS	epitope-type specificity	IHC	immunohistochemistry
FDC	follicular dendritic cells	IHHNV	infectious hypodermal and hematopoietic necrosis virus
FGF	fibroblast growth factor	IL	interleukin
FGFR1	fibroblast growth factor receptor 1	IRES	internal ribosomal entry site
FISH	fluorescence <i>in situ</i> hybridization	IRS	internal replication sequence
FITC	fluorescein isothiocyanate	ISH	<i>in situ</i> hybridization
FPV	feline panleukopenia virus	ITR	inverted terminal repeat
GABA	γ-aminobutyric acid		

IU	infectious units	NPC	nuclear pore complex
IVIG	intravenous immunoglobulin	NS	non-structural [protein]
JcDNV	<i>Junonia coenia</i> densovirus	N-SA	N-linked sialic acid
kb	kilobases	NTP	nucleotide triphosphate
KRV	Kilham rat virus	NV	neovascularization
LCA	Leber congenital amaurosis	OAAV	ovine adeno-associated virus
LCR	locus control region	OIR	oxygen-induced retinopathy
LdiDNV	<i>Lymantria dispar</i> densovirus	OR	odds ratio
LDL-R	low-density lipoprotein receptor	ORF	open reading frame
L-DOPA	L-dihydroxyphenylalanine	PBMC	peripheral blood mononuclear cells
LduDNV	<i>Leucorrhinia dubia</i> densovirus	PC4	positive coactivator 4
LEH	left-end hairpin	PcDNV	<i>Planococcus citri</i> densovirus
LGMD	limb girdle muscular dystrophies	PCNA	proliferating cell nuclear antigen
LH	luteinizing hormone	PCR	polymerase chain reaction
LHR	luteinizing hormone receptor	PCV2	porcine circovirus 2
LMB	leptomycin B	PD	Parkinson disease
LPS	lipopolysaccharide	PDGF	platelet-derived growth factor
LPV	lapine parvovirus	PEDF	pigment epithelium-derived factor
m.u.	map units	PfDNV	<i>Periplaneta fuliginosa</i> densovirus
MAb	monoclonal antibody	PFT	pig fallopian tube
MACRIA	m-anitobody capture radioimmunoassay	PID	post-inoculation day
MAP	mitogen-activated protein	PI-3K	phosphatidylinositol-3 kinase
MDCF	modified direct complement-fixation	PiDNV	<i>Pseudoplusia includens</i> densovirus
MDCK	Madin Darby canine kidney	PIF	parvovirus initiation factor
MDPV	Muscovy duck parvovirus	PIM	phosphorylation inhibitory motif
MEV	mink enteritis virus	PK	pig kidney
MHC	major histocompatibility complex	PKA	protein kinase A
MLC	myosin light chains	PKC	protein kinase C
MLCK	myosin light chain kinase	PLA <sub>2</sub>	phospholipase A <sub>2</sub> (secretory, sPLA <sub>2</sub> ; cytosolic, cPLA <sub>2</sub> ; independent, iPLA <sub>2</sub> s; PLA <sub>2</sub> receptor, PLA <sub>2</sub> R)
MLN	mesenteric lymph nodes	PML	promyelocytic leukemia
MNU	N-methyl-N-nitrosourea	PmPV	pig-tailed macaque parvovirus
M1DNV	<i>Mythimna loreyi</i> densovirus	PMWS	postweaning multisystemic wasting syndrome
MLV	modified-live virus	PODs	promyelocytic leukemia oncogenic domains
mob	mobilization protein	PPGSS	papular-purpuric gloves and socks syndrome
MOI	multiplicity of infection	PPV	porcine parvovirus
MP	myosin phosphatase	PR	photoreceptor cell
MpDNV	<i>Myzus persicae</i> densovirus	PRCA	pure red cell aplasia
Mr	molecular mass	PrDNV	<i>Pieris rapae</i> densovirus
MRCK	myotonic dystrophy kinase-related	Prm	phosphorylation inhibitory motif
mRNA	messenger RNA	Pro	proline
MSCs	mesenchymal stem cells	PRRSV	porcine reproductive and respiratory syndrome virus
MVM	minute virus of mice	PstDNV	<i>Penaeus stylostris</i> densovirus
MVMi	immunosuppressive strain of MVM	RA	rheumatoid arthritis
MVMp	prototype strain of MVM	rAAV	recombinant adeno-associated virus
MYPT1	myosin phosphatase target subunit 1	rAAV2	recombinant adeno-associated virus 2
NAA	N-acetyl-aspartate	RBE	rep-binding element
NBs	nuclear bodies	RBS	rep-binding site
NER	nucleotide excision repair	RCM	restrictive cardiomyopathy
NES	nuclear export signal	RCR	rolling-circle replication
NeuGC	N-glycol neuraminic acid	RCS	Royal College of Surgeons
NF <sub>1</sub>	nuclear factor 1	RCV	replication-competent viruses
NHEJ	non-homologous end-joining	RDO	RNA-DNA oligonucleotide
NIHF	non-immune hydrops fetalis		
NK	natural killer		
NLM	nuclear localization motif		
NLS	nuclear localization sequence or signals		

rds	retinal degeneration slow	SN	virus neutralization with antiserum
RDS	runt-deformity syndrome	snRPs	small nuclear riboproteins
REF	rat embryo fibroblast	SPF	specific-pathogen free
REH	right-end hairpin	SPLV	serum parvovirus-like particle
RF	replicative-form	SPV	simian parvovirus
RF	rheumatoid factor	SRV	simian retrovirus
RFC	replication factor C	ss	single stranded
RF DNA	double-stranded replicating DNA	SSc	systemic sclerosis
RFLP	restriction fragment length polymorphism	SSCP	single-stranded conformational polymorphism
RGCs	retinal ganglion cells	SSD-BP	single-strand-D-sequence binding protein
RGD	arginine-glycine-aspartic acid	ssDNA	single-stranded DNA
RHR	rolling-hairpin replication	SSO	single-stranded oligonucleotide
RI	replicative intermediate	ST	swine testis
RmPV	Rhesus macaque parvovirus	STN	subthalamic nucleus
RMV-1	rat minute virus 1	SV	simian virus
RNA	ribonucleic acid	SvDNV	<i>Simulium vittatum</i> densovirus
RNER	arginine, asparagine, glutamic acid, arginine	TAA	tumor-associated antigens
ROP	retinopathy of prematurity	TAC	transient aplastic crisis
ROPV	rat orphan parvovirus	tar	transactivation region
RP	retinitis pigmentosa	TBP	TATA-binding protein
RPA	replication protein A	TC-PCP	T-cell-derived phosphatase
RPE	retinal pigment epithelium	TEC	transient erythroblastopenia of childhood
RPV-1	rat parvovirus 1	TEM	transmission electron microscopy
RRS	rep recognition sequence	TEPD	transmembrane epithelial potential difference
RSV	Rous sarcoma virus	TFO	triplex-forming oligonucleotide
RT-PCR	reverse transcription polymerase chain reaction	TfR	transferrin receptor
RTPV	RT parvovirus	TGF	transforming growth factor
RV	rat virus	TLC	thin-layer chromatography
SAABs	SMN-associated APAR bodies	Tn1	troponin 1
SAIDS	simian acquired immunodeficiency syndrome	TnC	troponin C
SC	splenocyte	TNF	tumor necrosis factor
scAAV	self-complimentary AAV	TnT	troponin T
SCID	severe combined immuno deficient	TR	terminal repeat
SCW	streptococcal cell wall	TRS	terminal resolution site
sc-rAAV	self-complimentary rAAV	TsDNV	<i>Toxorhynchites splendens</i> densovirus
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis	TVX	tumor virus X
SF3	super family III	USE	upstream element
SfDNV	<i>Sibine fusca</i> densovirus	UV	ultraviolet
sFv	single-chain fragment variable region	VAHS	virus-associated hemophagocytic syndrome
SGT	small glutamine-rich TPR	VEGF	vascular endothelial growth factor
SISPA	sequence-independent single primer amplification	VLPs	virus-like particles
SIV	simian immunodeficiency virus	VP1	viral protein 1
SLE	systemic lupus erythematosus	VP2	viral protein 2
SMaRT	spliceosome-mediated mRNA <i>trans-splicing</i>	WBCT	whole blood clotting time
SMC	smooth muscle myofibres	WFPs	waterfowl parvoviruses
SMEDI	stillbirth, mummification, embryonic syndrome death, and infertility	WSSV	white spot syndrome virus
SMN	survival motor neuron [protein]	Wt	wild type
SMV	spawner isolated mortality virus	wtAAV	wild-type AAV
		ZF	zinc finger
		ZFN	zinc finger nucleases

PART **1**

# Biology of the Virus Family

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# SECTION A

## Virus Groups and Evolution of the Family

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# The evolution of parvovirus taxonomy

PETER TATTERSALL

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## THE PAST

The recorded history of the parvoviruses begins in 1959, when Kilham and Olivier reported the isolation of a small, stable virus, initially named rat virus (RV) and subsequently known as Kilham rat virus (KRV), from lysates of an experimental rat tumor (Kilham and Olivier, 1959). Over the next 10 years, a number of other physically similar agents were isolated, some, such as Toolan's H1 virus, as contaminants of laboratory tumors that were being serially transplanted in immunosuppressed rodents, and others, such as Hallauer's LuIII virus and Crawford's minute virus of mice (MVM), as contaminants of cell cultures or virus stocks in routine laboratory use (Toolan, 1961; Crawford, 1966; Hallauer *et al.*, 1971). Although the natural host species of some of these viruses was unknown, when isolates were injected into rodents they caused a broad spectrum of pathology, which varied with the virus serotype and age of the host, from apparently asymptomatic viremia to teratogenesis and fetal or neonatal death (reviewed in Tattersall and Cotmore, 1986). That these viruses might not be confined to vertebrate hosts started to become apparent in 1964, when Meynadier reported the isolation of a physically similar virus from larvae of the greater wax moth, *Galleria mellonella* (Meynadier *et al.*, 1964). Over the next 40 years similar arthropod viruses would be isolated from each of the major orders of Insecta and even one, so far, from the Crustacea. The mid-1960s also saw the discovery of a group of physically similar, but defective viruses, which were dubbed 'adenovirus-associated viruses' (subsequently shortened to 'adeno-associated viruses' – AAV) because they were found to be dependent upon adenovirus co-infection for their own replication (Atchison *et al.*, 1965; Hoggan *et al.*,

1966). At about the same time, an infectious agent responsible for epidemics of enteritis, panleukopenia and congenital cerebellar ataxia in domestic cats was identified, called feline panleukopenia virus (FPV), and it soon became apparent that this shared many characteristics with the small rodent viruses (Gorham *et al.*, 1965; Kilham *et al.*, 1967).

Carlos Brailovsky first introduced the name 'parvovirus' to the literature in a 1966 paper that denoted RV as *Parvovirus ratti*, in an early attempt to establish a latinized binomial nomenclature system for viruses (Brailovsky, 1966). In the late 1960s, a somewhat rancorous debate broke out over the name with which to collectively describe these minute DNA viruses. Two camps emerged, those who proposed Brailovsky's 'parvovirus', derived from the Latin *parvus* meaning small, and those who championed the name 'picodnavirus', in parallel with the 'picornavirus' label, recently adopted for the supposedly similar small, RNA-containing viruses. Realizing that a single typographical 'mutation' could interconvert what turned out to be two distinctly different virus families, reason and clarity prevailed, and the name parvovirus was adopted. In the 1970s, the taxonomic family *Parvoviridae* was established and approved by a working group of the International Committee on the Taxonomy of Viruses (ICTV), which then set out to construct rational taxonomic rules for the further subdivision of this rapidly expanding family. This process was initially confounded by an ongoing debate as to whether the viruses in question contained double- or single-stranded DNA. A careful study of the genome encapsidated in MVM, led Crawford and colleagues to conclude that the viral DNA was single-stranded (Crawford *et al.*, 1969), and led them to propose that parvoviral particles were too small to encapsidate a double-stranded DNA molecule 5 kilobases

in length as had been suggested for the AAVs. Crawford and colleagues further suggested that the AAV duplexes might arise from postextraction annealing of plus and minus strands that had been packaged into separate virions. While they characterized their explanation as ‘rather unlikely’ or even ‘highly improbable’, this prescient suggestion was quickly shown to be correct by the elegant density labeling experiments of Rose, Berns and colleagues (Rose *et al.*, 1969; Berns and Rose, 1970). Subsequent analysis of duplex forms of the viral DNA by restriction endonuclease cleavage showed it to be both linear and non-permuted, filling out our current view of the parvoviral genome.

## THE PRESENT

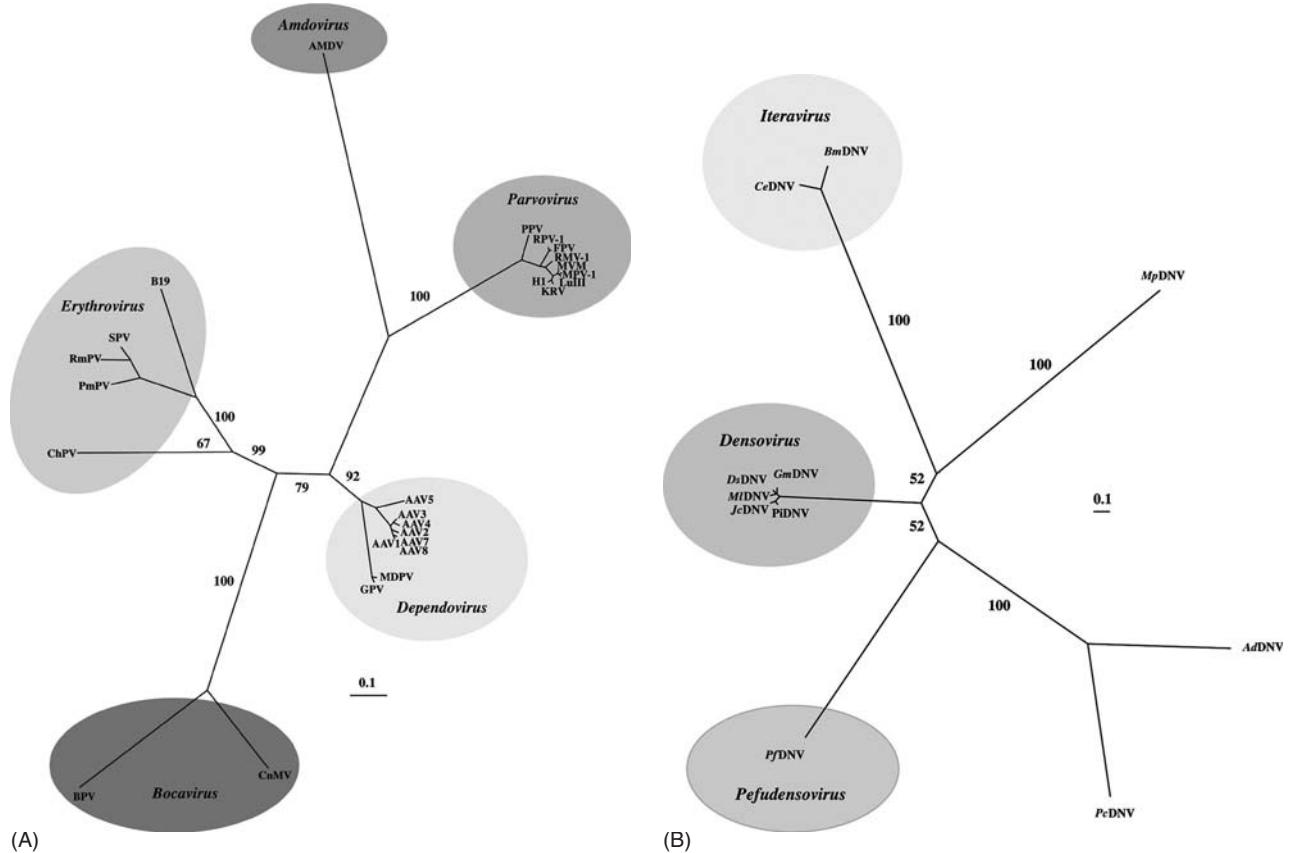
The family descriptor *Parvoviridae* now encompasses all small, isometric, non-enveloped DNA viruses that contain linear, single-stranded genomes. Indeed, in the known biosphere, only parvoviruses have DNA genomes that are both single-stranded and linear. Each parvoviral virion contains a single genomic molecule between 4 and 6 kilobases (kb) in length, which terminates in short palindromic sequences that can fold back on themselves to create duplex hairpin telomeres. The hairpins at each end of the genome can be different from one another, both in sequence and predicted structure, or they can each be part of an inverted terminal repeat (ITR). These terminal structures render the genome self-priming for complementary strand synthesis, which can be accomplished with all DNA polymerases tested to date. The terminal hairpins are essential for these virus’ unusual replication strategy, and hence serve as an invariant hallmark of the family.

In general, parvoviruses are exceptionally stable and are antigenically and structurally quite simple. A combination of protein analysis, electron microscopy and, latterly, X-ray crystallography, has established that the virion is an icosahedral structure exhibiting  $T = 1$  symmetry. Typically, several species of structural protein, encoded in the genome as a nested sequence set, are used to form the capsid shell, which comprises 60 copies of the common 60–70 kDa C-terminal region of the polypeptide set (Tattersall *et al.*, 1977). Resistance to inactivation by organic solvents indicates that parvoviral virions lack essential lipids. None of the viral proteins is known to be glycosylated, but they may be modified post-translationally by important phosphorylation events. In most parvoviruses, the N-terminal region of VP1 that is not included in the other structural proteins contains a functional phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzymatic core (Zadoni *et al.*, 2001), whose activity is essential for an early step in viral entry.

The ICTV only governs the taxonomic classification of viruses at the levels of Order, Family, Genus and Species. Incidentally, only one grouping of viruses at a level higher than Family has been approved to date, that of the Order

*Mononegavirales*, a superfamily that contains the *Paramyxoviridae*, *Rhabdoviridae* and *Filoviridae*. Following the establishment of the family Parvoviridae, the ICTV approved its division into two subfamilies on the basis of host range – the *Parvovirinae*, which infect vertebrate hosts, and the *Densovirinae*, which infect insects and other arthropods – recognizing that the two groups of viruses infect host species that were themselves separated at the much higher level of Phylum. Although genome sequences were not then available to describe phylogenetic relationships between the members of the *Parvoviridae*, the DNA sequence data amassed since that time confirm that all viruses in the family share a common evolutionary history, and that the viruses cluster together into two groups, corresponding to the subfamilies, which are very distant from one another. In the 30 years following the initial classification of these viruses into a single family, continued attempts have been made to further divide each subfamily rationally, into genera and species, so as to reflect their probable evolutionary relationships. Initially the criteria for doing this mirrored those used in classical taxonomic systems, in lumping together those members exhibiting similar biological or structural characteristics in individual genera. Thus criteria used for defining a genus have included whether the virus is capable of autonomous replication or is dependent upon a helper virus, whether the virion contains a single-strand sense or packaged strands of both senses in separate virions, and whether the virus has one, two or three transcriptional promoters. As the sequences of more and more viral genomes became available, and tools for DNA and protein sequence comparison more sophisticated, it has become increasingly apparent that the demarcations based on these criteria may not strictly reflect divergent evolution from common ancestors and computer-based phylogenetic analysis has become the predominant method for assessing their evolutionary relatedness (Lukashov and Goudsmit, 2001). Thus a genus can now be identified as a monophyletic group of species that represents a single branch of a phylogenetic tree, as shown for each subfamily in Figure 1.1.

The next rung on the taxonomic ladder is the category of species. As with all taxa, species is a concept rather than a physical entity, thus viral isolates should be regarded as ‘belonging to a particular species’, rather than defining an individual representative virus as ‘the species’. Accordingly, the ICTV definition of species is ‘a polythetic class of viruses that constitutes a replicating lineage and occupies a particular ecologic niche’, indicating that no single characteristic has to be shared by all individuals in order for them to be collectively considered a species. This recognition of the inherent variability that may exist between members of the same virus species is particularly important, since it has become apparent that viruses considered to be members of the same species can be genotypically quite distinct, forming ‘clades’ within distinct geographically areas, or even quasispecies within the same host.



**Figure 1.1** (A) Phylogenetic relationship between the non-structural genes of members of the subfamily Parvovirinae. (B) Phylogenetic relationship between the non-structural genes of members of the Densivirus and Iteravirus genera of the subfamily Densovirinae. The genus Brevidensovirus is not shown because of its great evolutionary distance from the other Densovirinae members. Phylogenetic trees were constructed, by Z. Zadori and P. Tijssen, using the programs included in the PHYLIP package (Felsenstein, 2004). For bootstrap analysis, the SEQBOOT program was run first. For distance matrix analysis, the aligned sequences were processed first with the PROTDIST (using Dayhoff's PAM 001 scoring matrix) and then with the FITCH program (global rearrangements). The most probable tree was calculated with the CONSENSE program, and the resulting trees visualized using TREEVIEW. (Redrawn from original trees kindly provided by Z. Zadori and P. Tijssen). See also Color Plate 1.1.

Although phylogenetic analysis is becoming a central technique in taxonomic classification, it is less reliable when applied at the species level for viruses in general, and parvoviruses in particular. This is because the phylogenetic approach predominantly depends upon the assumption that genetic information is only transmitted vertically over generational time, an assumption that does not always hold for viruses, which frequently re-assort genetic information by horizontal mechanisms. In particular, viruses evolve by recombination with other viruses or with the much larger pool of sequences represented by their host genomes. An extreme example of this is the finding of a functional homologue of the AAV2 *Rep* gene within the genome of human herpesvirus type 6 (Thomson *et al.*, 1991), which represents what might be described as a dramatic 'pleaching' of the branches of two very disparate phylogenetic trees. So-called molecular clock measurements of evolutionary distance, implicit in the phylogenetic approach, may also be distorted

for viruses by accelerated evolution, at least at the nucleotide level, following host species 'jump' or target tissue switch, or even following human intervention. An example of the latter would be the survival of a tiny fraction of a high titer virus population following a highly mutagenic viral clearance technique, such as UV irradiation, where subsequent infection might expand a novel virus clade that would appear to be evolutionarily far removed from the original virus population. These considerations suggest that it would be wise to take into account other characteristics that may be constrained within a single replicating lineage, and not just DNA or protein sequence, when criteria for species demarcation are decided.

One criterion that serves as a useful complement to sequence-based phylogenetic analysis is serotype. Just two antigenic sites, defined by mutations that confer resistance to neutralization by monoclonal antibodies, have been identified for canine parvovirus (CPV). Despite this antigenic

simplicity, individual serotype, defined by neutralization of infectivity in cell culture, hemagglutination-inhibition or specific ELISA, is a remarkably stable property when assessed with polyclonal antiviral sera. Generally, only a single parvovirus serotype is found circulating in its natural host population. When more than one serotype can be isolated from a particular host species, phylogenetic comparison has invariably shown each serotype to be quite distinct at the level of genome sequence, allowing them to be assigned to different species, or even different genera. Additionally, when FPV ‘jumped’ host species, into mink in the 1940s and into dogs in the 1970s, it remained serotypically indistinguishable from its FPV parent in cross-protection studies. Indeed, this serotypic identity with FPV has persisted through the subsequent global replacement of the original CPV with a more extensively dog-adapted strain. This exceptional stability within a replicating lineage has allowed parvovirus serotype to be used as a major criterion for taxonomic species demarcation, at least for the parvoviruses of vertebrates (Tattersall *et al.*, 2005). Whether the antigenic properties of members of the *Densovirinae* are as stable is not known, but the absence of an adaptive immune response in insects would suggest that their virions are under even less selective pressure to shift antigenically than are those of the *Parvovirinae*. Interestingly, despite their differences in capsid serology, the non-structural proteins of most species within a single genus show significant antigenic cross-reactivity, so that genus-specific infections can often be diagnosed serologically.

Novel parvoviral virus isolates are initially regarded as unassigned strains or variants, and it is the responsibility of a group of working parvovirologists, constituting the ICTV Parvovirus Study Group, to decide whether such isolates should constitute a new species, using the criteria outlined above. Having decided that a new species is warranted, this group submits a set of formal taxonomic proposals to the ICTV, which is then posted on a web page, accessible to other ICTV members, for a period of comment and discussion. The ICTV Executive Committee then votes to approve, or disapprove, the new species. It should be pointed out that the rigor of this procedure makes the reverse processes, of removing a species or changing its genus assignment, even more onerous. This becomes a particular problem when species are dealt with that were assigned to genera in the pregenomic era, and for which there is still no DNA sequence information. Unfortunately, some of these ‘grandfathered’ species may no longer even exist as virus isolates, from which such data could be derived.

The process for creating a new genus is similar, but more complicated, and involves the identification of a ‘type species’, which is usually the best characterized member of the new genus. It should be emphasized that any individual virologist who wishes to propose a change in the taxonomic status or nomenclature of any parvovirus is encouraged to contact members of the parvovirus study group ([www.danforthcenter.org/iltab/ictvnet/asp/\\_ MainPage.asp](http://www.danforthcenter.org/iltab/ictvnet/asp/_ MainPage.asp)),

so that an open and balanced deliberation can take place, and the resulting proposal be forwarded to the ICTV. Adhering to their guidelines for nomenclature and taxonomy, hopefully, will allow the parvovirus field to avoid the confusion engendered by competing alternative classification schemes. Every few years, the ICTV gathers the current taxonomic data for each virus family, and publishes them in an official report. The most recent version of this, the VIIth Report of the ICTV, is currently in press. In the parvovirus section of the report (Tattersall *et al.*, 2005), members of the *Parvovirinae* subfamily are divided into five genera and those of the *Densovirinae* into four genera, as detailed in Box 1.1. These nine genera comprise 34 accepted species, with 20 species tentatively assigned to individual genera and a further nine virus isolates currently unassigned within the subfamily *Densovirinae*.

In the following sections, the accompanying tables show the current classification of species within each genus, dividing them into three categories that reflect the processes discussed above. First, there are those currently ‘accepted’ by the ICTV, whose inclusion in the genus has been substantiated by phylogenetic analysis of both non-structural and capsid gene sequences. These are followed by species ‘grandfathered’ into the genus on the basis of previous rounds of classification, but for whom there is no DNA sequence information yet available. Given the recent reclassification of the duck and goose parvoviruses, discussed below, and the creation of two new genera from former members of the *Parvovirus* genus, the current assignment of these grandfathered species to particular genera should be considered provisional. Finally, there are members currently designated ‘tentative’. Most of these clearly fall into the genus by phylogenetic analysis, but the process of acceptance by the ICTV has not yet been completed, either because their sequences have only been reported recently, or, for the vertebrate viruses, because definitive evidence of their antigenic uniqueness is still lacking.

**Box 1.1** The taxonomic structure of the family Parvoviridae

Subfamily	<i>Parvovirinae</i>
• Genus	<i>Parvovirus</i>
• Genus	<i>Dependovirus</i>
• Genus	<i>Erythrovirus</i>
• Genus	<i>Amdovirus</i>
• Genus	<i>Bocavirus</i>
Subfamily	<i>Densovirinae</i>
• Genus	<i>Densovirus</i>
• Genus	<i>Iteravirus</i>
• Genus	<i>Brevidensovirus</i>
• Genus	<i>Pefudensovirus</i>

## THE SUBFAMILY PARVOVIRINAE

### Genus *Parvovirus*

Species within the genus *Parvovirus* are listed in Box 1.2. In the majority of these, the mature virion contains a negative-strand DNA of ~5 kilobases (kb), although in parvovirus LuIII, both strands are packaged, in separate particles, in an approximately equimolar ratio. The genome has hairpin structures of dissimilar sequence and predicted structure at each terminus. At the 3'-end of the

#### Box 1.2 Species within the genus *Parvovirus*

##### Accepted members

- *Minute Virus of Mice* (MVM) – type species  
Several strains: designated MVMp [J02275], MVMi [M12032], MVMc [U34256]
- *Feline panleukopenia virus* (FPV)  
Several strains: FPV (feline) [M75728], CPV (canine) [M19296], RPV (raccoon) [M24005] and MEV (mink enteritis virus) [D00765]
- *H-1 parvovirus* (H-1PV) – one strain sequenced [X01457]
- *Kilham rat virus* (KRV)  
Several strains: H-3, X-14, HER1, RV-13 [AF321230], RV-UMass[U79033, AF036711], RV-Y [AF317513]
- *LuIII virus* (LuIIIV) – one strain reported [M81888]
- *Mouse parvovirus 1* (MPV-1)  
Several isolates: designated MPV-1, [U12469] MPV-1b [U34253], MPV-1c [U34254]
- *Porcine parvovirus* (PPV)  
Several strains: NADL-2 [L23427], Kresse [U44978]

##### Grandfathered members

- *Chicken parvovirus*
- *HB parvovirus* (HBPV)
- *Lapine parvovirus* (LPV)
- *RT parvovirus* (RTPV)
- *Tumor virus X* (TVX)

##### Tentative members

- Hamster parvovirus (HaPV) – one strain reported [U34255]
- Rat minute virus 1 (RMV-1)  
Several isolates: designated RMV-1a [AF332882], RMV-1b [AF332883], RMV-1c [AF332884]
- Rat parvovirus 1 (RPV-1) – one strain reported [AF036710]

negative strand, by convention the left-hand end of the genetic map, the terminal hairpin is 115–121 nucleotides (nt) long, while for the 5', or right-hand, hairpin reported structures vary between 200 and 248 nucleotides in length. There are two transcriptional promoters, at map units ~4 and ~40, from the left-hand end, and transcripts coterminate at a single polyadenylation site near the right-hand end of the genome. Characteristic cytopathic effects are induced by these viruses during replication in cell culture. Many species hemagglutinate red blood cells of one or more species, but not necessarily of their presumed natural host. Under experimental conditions, the host range may be extended to a large number of vertebrate species, and transplacental transmission has been detected for several species. While canine and raccoon parvoviruses were originally classified as separate species, the work of Parrish and colleagues (Chapter 2) has clearly shown that these viruses, along with mink enteritis virus, are all cross-neutralizing host range variants and they have now been re-classified as strains within the feline panleukopenia virus species.

### Genus *Dependovirus*

Originally, as the name *Dependovirus* implies, the genus comprised only helper-dependent adeno-associated viruses, but phylogenetic analysis now places the autonomously replicating goose (GPV) and duck parvoviruses firmly within this genus, where they cluster with the recently sequenced avian adeno-associated virus (AAAV). The duck parvovirus species currently comprises two closely related strains, Muscovy duck parvovirus (MDPV) and Barbarie duck parvovirus (BDPV). The currently accepted members of the *Dependovirus* genus are listed in Box 1.3. Except for the duck and goose viruses, all of these are dependent upon helper adenoviruses or herpes viruses for efficient replication. For some of the AAVs, however, under certain conditions, such as the treatment of host cells with mutagens or hydroxyurea, replication can be detected in the absence of helper viruses. Infections without helper virus frequently result in a persistent latent infection. Under these conditions in cultured human cells AAV2 can integrate site-specifically, into 13.4q-qter on chromosome 19. AAV2 and AAV3 are endemic in the human population, whereas AAV5 has been isolated from humans only once. While AAV1 is probably a monkey parvovirus, AAV4, AAV7 and AAV8 are clearly of simian origin. AAV6, originally classified as a separate species, appears to be a recombinant between AAV2 and AAV1 that is serologically identical to AAV1, and has been reclassified as a strain belonging to the latter species. Currently, the virions of all members of this genus contain equivalent numbers of positive or negative DNA strands, between 4.7 and 5.1 kb in size. AAV genomes have ITRs of ~145 nucleotides, the first ~125 nucleotides of which form a palindromic hairpin sequence. The autonomously replicating avian parvovirus species have

**Box 1.3 Species within the genus *Dependovirus*****Accepted members**

- *Adeno-associated virus-1* (AAV-1)  
Two strains: AAV1 [AF063497], AAV6 [AF208704]
- *Adeno-associated virus-2* (AAV-2) – one strain reported [J01901]
- *Adeno-associated virus-3* (AAV-3) – one strain reported [AF028705]
- *Adeno-associated virus-4* (AAV-4) – one strain reported [U89790]
- *Adeno-associated virus-5* (AAV-5) – one strain reported [AF085716]
- *Avian adeno-associated virus* (AAAV) – one strain reported [AY186198]
- *Bovine adeno-associated virus* (BAAV) – one strain reported [AY388617]
- *Duck parvovirus* (DPV)
- Two strains: Barbarie DPV (BDPV) [U22967] and Muscovy DPV (MDPV) [X75093]
- *Goose parvovirus* (GPV) – one strain reported [U25749]

**Grandfathered members**

- *Canine adeno-associated virus* (CAAV)
- *Equine adeno-associated virus* (EAAV)
- *Ovine adeno-associated virus* (OAAV)

**Tentative members**

- Adeno-associated virus-7 (AAV-7) – one strain reported [AF513851]
- Adeno-associated virus-8 (AAV-8) – one strain reported [AF513852]

**Possible member\***

- Bovine parvovirus type 2 (BPV-2) [AF406966]

\* This genome has not yet been isolated as a virus.

much larger ITRs, of between 444 and 457 nucleotides, and differ from the AAVs in exhibiting marked pathogenicity in their natural hosts, causing epidemics of significant agricultural impact in commercial flocks of domestic fowl.

By DNA sequence analysis, members of the genus *Dependovirus* all appear to have three transcriptional promoters, at ~5, ~19 and ~40 map units, although their activities have yet to be demonstrated directly for many viruses. Functional polyadenylation sites occur at the right-hand end and, in some cases, also in the middle of the genome. Each species is antigenically distinct, as assessed by neutralization using polyclonal antisera, and natural infection is usually confined to a single host species. Individual species differ by more than 5 percent in their non-structural gene DNA sequence.

**Genus *Erythrovirus***

Human parvovirus B19, the only known human pathogenic member of the *Parvoviridae*, was discovered serendipitously in 1975 as an antigen present in a serum sample bearing the laboratory code ‘B19’, from an apparently healthy donor whose blood was being screened for hepatitis reactivity (Cossart *et al.*, 1975). B19 has been shown to be responsible for transient aplastic crisis in patients with a variety of hemolytic anemias, and has been linked seroepidemiologically to erythema infectiosum (EI), a widespread childhood rash-like disease also referred to as fifth disease (Anderson *et al.*, 1983). Intrauterine transmission of B19 from an infected mother to her fetus can sometimes lead to hydrops fetalis, particularly in the second trimester of pregnancy. The B19 genome was characterized, cloned and sequenced some 20 years ago (Summers *et al.*, 1983; Cotmore and Tattersall, 1984; Shade *et al.*, 1986), firmly placing it in the *Parvovirinae* subfamily. However, these analyses revealed that B19 was phylogenetically distant from viruses belonging to the *dependoviruses* or *parvoviruses*, the only two genera existing at that time, so that the new genus *Erythrovirus* was created, with B19 as its type species. Recently it has become apparent that there are at least three distinct genotypes of B19, circulating in different human subpopulations (Servant *et al.*, 2002). It is not yet clear how these clades arose, but it appears that they are all serotypically equivalent to the index strain B19-Au, and thus should all be considered members of the same species. Their existence has led to some confusion in the literature, since it appears to have prompted some authors to rename human parvovirus B19 as ‘human erythrovirus’, even though this nomenclature is not currently sanctioned by ICTV. As seen in Box 1.4, additional erythroviruses have been identified in other primate species, which appear to share with B19 an exquisite specificity for erythroid progenitor cells and accordingly give rise to similar disease spectra. A more distantly related *Erythrovirus* species has also been isolated from Manchurian chipmunks (Yoo *et al.*, 1999).

Populations of mature B19 virions contain equivalent numbers of positive and negative sense DNA strands, ~5.5 kb in size. The genome has inverted terminal repeats of 383 nucleotides, the first 365 nucleotides of which form a palindromic sequence. There is a single transcriptional promoter at map unit 6 and two polyadenylation signals, one near the middle of the genome, the other near the right-hand end, that terminate transcripts encoding NS1 and VP1, respectively. Two small ORFs can also be accessed by alternatively spliced mRNAs, depending upon the relative strength of their initiation codons. One of these encodes a 7.5 kDa polypeptide of unknown function, while the other encodes an 11 kDa protein containing three proline-rich regions that conform to consensus Src homology 3 (SH3) ligand sequences. Members of each species are probably antigenically distinct, and natural infection appears to be

**Box 1.4 Species within the genus *Erythrovirus*****Accepted members**

- *Human parvovirus B19* – type species

Many isolates; three major genotypes have been identified represented by B19V-Au [M13178], B19-LaLi [AY044266], and B19-V9 [AJ223617]

**Tentative members**

- Chipmunk parvovirus (ChpPV) – one strain reported [U86868]
- Pig-tailed macaque parvovirus (PmPV) – one strain reported [AF221123]
- Rhesus macaque parvovirus (RmPV) – one strain reported [AF221122]
- Simian parvovirus (SPV) – (cynomolgus monkey) one strain reported [U26342]

**Possible member\***

- Bovine parvovirus type 3 (BPV-3) [AF406967]

\* This genome has not yet been isolated as a virus.

confined to a single host species. Individual species diverge by more than 5 percent in their non-structural gene DNA sequences.

**New genera within the *Parvovirinae***

As discussed above, the taxonomy of the *Parvoviridae* has recently been rationalized to more closely reflect their evolution, by clustering viruses using phylogenetic analysis of encoded structural and non-structural genes and their predicted products. This has led to the creation of two new genera within the subfamily, to accommodate some disparate autonomously-replicating viruses previously classified in the genus *Parvovirus*. The ICTV's Code for the Taxonomy of Viruses directs that new names shall be selected such that they do not convey a meaning for the genus that may subsequently appear to exclude viruses that are clearly members of the taxon, but lack the particular characteristic embodied in the name. This sensible principle avoids the unfortunate implication that, for example, the name *Dependovirus* now conveys, of a biological property that only applies to some members of the genus. Names of the new genera were, therefore, designed to reflect their founding members in a non-pejorative fashion. The new genus *Amdovirus* contains a single outlying species, Aleutian mink disease virus (AMDV), while the genus *Bocavirus* was christened with a sigla formed from the names of the two current member species, *bovine parvovirus* and *canine minute virus*.

**Box 1.5 Species within the genus *Amdovirus*****Accepted member**

- *Aleutian mink disease virus* (AMDV) – type species  
Several strains: ADV-G [M20036], ADV-Utah, ADV-Pullman, etc.

**Box 1.6 Species within the genus *Bocavirus*****Accepted members**

- *Bovine parvovirus type 1* (BPV-1) – type species  
One strain reported: BPV [M14363]
- *Canine minute virus* (CnMV) – one strain reported [AF495467]

Despite their phylogenetic isolation, most features of these viruses are shared with members of the *Parvovirus* genus.

**Genus *Amdovirus***

Mature AMDV virions contain a negative strand DNA of 4748 nucleotides, with different palindromic sequences at each terminus. Permissive replication has only been observed in cell culture for one isolate, AMDV-G, which replicates in Crandell feline kidney cells. Restricted replication is also observed in cells bearing Fc receptors, such as macrophages, where the virus' ability to infect may be antibody-dependent (Dworak *et al.*, 1997). There are several highly pathogenic strains of AMDV, and evidence of infection with this virus has been detected in most mustelids, skunks, and racoons. Virion structure differs slightly from members of the *Parvovirus* genus. Apart from its divergent DNA sequence, the major distinguishing feature of AMDV is its VP1 N-terminus, which is much shorter than those of other members of the *Parvovirinae*, and lacks a phospholipase 2A enzymatic core (Box 1.5).

**Genus *Bocavirus***

The NS1 and VP1 genes of the *bocaviruses*, BPV and CnMV, are 34–41 percent similar to one another, but their genomes are quite distinct from all other viruses in the *Parvovirinae* subfamily. Like the *parvoviruses* and *am dovirus*, the *bocaviruses*, have disparate termini, although, at ~5.5 kb, their genomes are somewhat larger. BPV is also notable because it packages 90 percent negative-sense and 10 percent positive-sense DNA and because it encodes a 22.5 kDa nuclear phosphoprotein, NP-1, that is distinct from any other parvovirus-encoded polypeptide (Box 1.6).

**Box 1.7 Species within the genus *Densovirus*****Accepted members**

- *Junonia coenia* densovirus (JcDNV) – type species  
One strain reported: JcDNV [S17265]
- *Galleria mellonella* densovirus (GmDNV) [L32896]

**Tentative members**

- *Diatraea saccharalis* densovirus (DsDNV) – one strain reported [AF036333]
- *Mythimna loreyi* densovirus (MIDNV) – one strain reported [AY461507]
- *Toxorhynchites splendens* densovirus (TsDNV) – one strain reported [AF395903]
- *Pseudoplusia includens* densovirus (PiDNV)

**Box 1.8 Species within the genus *Iteravirus*****Accepted members**

- *Bombyx mori* densovirus (BmDNV) – type species  
One strain reported: BmDNV [AY033435]
- *Casphalia extranea* densovirus (CeDNV) – one strain reported [AF375296]

**Tentative member**

- *Sibine fusca* densovirus (SfDNV)

**Box 1.9 Species within the genus *Brevidensovirus*****Accepted members**

- *Aedes aegypti* densovirus (AaeDNV) – type species  
One strain reported: AaeDNV [AY160976]
- *Aedes albopictus* densovirus (AalDNV) – one strain reported [AY095351]

**Tentative members**

- *Penaeus stylirostris* densovirus\* (PstDNV) – one strain reported [AF273215]
- *Aedes pseudoscutellaris* densovirus (ApDNV)
- *Simulium vittatum* densovirus (SvDNV)

\* Suggested name for infectious hypodermal and hematopoietic necrosis virus (IHHNV) of penaeid shrimps.

**THE SUBFAMILY DENSOVIRINAE****Genus *Densovirus***

*Densovirus* genomes are ~6 kb in length, with long inverted terminal repeats. They represent a paradigm shift for the *Parvoviridae*, since they exhibit ambisense organization. Populations of virions encapsidate equal numbers of positive and negative strands, but one strand, positive by convention, contains three open reading frames (ORFs), which are predicted to encode NS proteins on mRNAs transcribed rightwards from a promoter 7 map units from the left-hand end, while the complementary, or negative, strand encodes four structural proteins, on an mRNA transcribed leftwards from a promoter 9 map units from the right-hand end. The genome contains a >500 nucleotide long inverted terminal repeat, the first ~100 nucleotides of which can fold to form a T-shaped structure of the type found in the ITR of AAV DNA. The individual species, listed in Box 1.7, diverge by at least 5 percent in their non-structural gene DNA sequences, and their natural infection is confined to a single host species.

**Genus *Iteravirus***

The monosense *Iteravirus* genome is ~5 kb in length, and populations of virions encapsidate equal numbers of plus and minus strands, in separate particles. There are two ORFs for non-structural proteins and one ORF encoding the four structural proteins, located on the same strand, with a transcriptional promoter upstream of each ORF. The genome has an inverted terminal repeat of 230 nucleotides, the first 159 of which are palindromic and are predicted to form a 'J-shaped' hairpin structure when

folded. Separate species, listed in Box 1.8, diverge by at least 5 percent in their non-structural gene DNA sequences, and their natural infection is confined to a single host species.

**Genus *Brevidensovirus***

The *brevidensoviruses* are at least as different from other members of the subfamily Densovirinae, as these are from any member of the subfamily *Parvovirinae*. The monosense *Brevidensovirus* genome is ~4 kb in length, has disparate hairpin termini and transcriptional promoters at map units 7 and 60. The left-hand end comprises a palindromic sequence of 146 nucleotides while a different palindromic sequence of 164 nucleotides occupies the right-hand end. Both terminal sequences can fold to form a T-shaped structure. ORFs for the structural and nonstructural proteins are on the same strand. The genome does not contain any sequence recognizable as a PLA<sub>2</sub> domain. Populations of virions encapsidate positive and negative strands, but the majority of strands (85 percent) are negative-sense. The individual species within the genus, listed in Box 1.9, differ by at least 5 percent from one another in their non-structural gene DNA sequences, and

**Box 1.10 Species within the genus *Pefudensovirus*****Accepted member**

- *Periplaneta fuliginosa* densovirus (PfDNV) – type species  
One strain reported: PfDNV [AF192260]

**Box 1.11 Unassigned viruses within the subfamily Densovirinae**

- *Acheta domesticus* densovirus (AdDNV) [AX344110]
- *Blattella germanica* densovirus (BgDNV) [AY189948]
- *Culex pipiens* densovirus (CpDNV)
- *Euxoa auxiliaris* densovirus (EaDNV)
- *Leucorrhinia dubia* densovirus (LduDNV)
- *Lymantria dispar* densovirus (LdiDNV)
- *Myzus persicae* densovirus (MpDNV) [AY148187]
- *Pieris rapae* densovirus (PrDNV)
- *Planococcus citri* densovirus (PcDNV) [AY032882]

natural infection by each *Brevidensovirus* species appears to be confined to a single host species.

### Genus *Pefudensovirus*

Like the *Densovirus* genus, *pefudensoviruses* exhibit ambisense organization. The genome is ~5.5 kb in length, with a rightward promoter at 3 map units and a leftward promoter at 97 map units, located within the 201 nucleotide ITRs. The VP gene is located in the 5' half of the complementary strand, and is split into a small upstream ORF and a large downstream ORF, which appear to be spliced in order to code for the largest VP. Frame-shifting after splicing is also required in order to generate the largest VP. Unlike other members of the family *Parvoviridae*, in which the PLA<sub>2</sub> domain is expressed in the N-terminus of the largest structural protein, in *Pefudensovirus* this motif is centered 60–70 amino acids from the C-terminus of the ORF predicted to encode the small VP protein. The ORFs predicted to encode the three *pefudensovirus* non-structural proteins are organized in the same way as for those of the *Densovirus* genus, and are of similar sizes (Box 1.10).

## THE FUTURE

Clearly a priority for upcoming ICTV business is to move for the acceptance of each sequenced, tentative species listed in Boxes 1.2–1.10 into its appropriate genus, and to propose designations for the unassigned viruses in the subfamily *Densovirinae* listed in Box 1.11, a process that may require the creation of additional genera. The latter task is complicated by the great evolutionary distances that are apparent between some existing genera in the *Densovirinae*. Published studies on the phylogenetic relationship between parvoviruses have tended to use whole genome sequences (Lukashov and Goudsmit, 2001), an approach that works well for distinguishing between monophyletic clusters within the current members of the *Parvovirinae*, but has difficulty in placing all known parvovirus sequences on the same tree. One answer to this problem of scale would be to use only well-conserved regions, common to all parvoviruses, to construct phylogenetic trees that include the whole family.

Parvoviruses encode several well-recognized protein domains that are unlikely to have been perturbed by horizontal acquisition or loss, and might serve as potential linearly-descended sequence blocks. The nickase domain within NS1/Rep, which appears to have been inherited from an ancestor held in common with the *Microviridae* and *Geminiviridae* might suffice, but it is bipartite and not very extensive (Koonin and Ilyina, 1993). The PLA<sub>2</sub> domain, while highly conserved when present (Zadori *et al.*, 2001), is missing from two of the nine current genera. Another highly conserved feature in parvoviral structural proteins is the eight-membered β-barrel that forms the core of the icosahedral capsid shell (Chapman and Rossmann, 1993). However, the use of these motifs for phylogenetic comparison suffers from the dual drawbacks of their being non-contiguous and difficult to delineate exactly without structural information for every virus.

Thus, the prime remaining candidate for such an evolutionary ‘tag’ must be the helicase domain, which comprises a relatively contiguous set of well-defined functional sub-domains, called Walker boxes, that occupy a stretch of between 100 and 103 amino acids within the NS1/Rep coding sequence of all known parvoviruses. Indeed, this helicase domain belongs to the superfamily III (SF3) helicases, which themselves belong to the enormous family of related AAA + ATPases (Neuwald *et al.*, 1999). In addition to the conserved sequence within parvoviral NS1/Rep genes, SF3 helicases have also been identified within genes encoded by DNA viruses as diverse as the *Poxviridae*, *Baculoviridae*, *Papillomaviridae*, *Polyomaviridae* and *Circoviridae*, in the genomes of small RNA viruses such as the *Picornaviridae* and *Comoviridae*, as well as in bacteriophages such as P4. Significantly, other than the remnants of prophages in bacteria, no SF3 helicases have been found encoded in cellular genomes. This suggests that the SF3 lineage of AAA + ATPases, whose phylogenetic branch diverged from the rest of the tree of life before the separation of the *archaea*, *bacteria* and *eukarya*, might have originally evolved in primitive

replicons that now are only represented by viruses (Iyer *et al.*, 2004). Thus the SF3 helicase domain evident in present day parvoviruses may represent a direct, uninterrupted vertical link to the first common ancestor of this virus family.

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# The genus *Dependovirus*

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The adeno-associated viruses (AAVs), members of the genus *Dependovirus*, share a common, but not absolute requirement for an unrelated DNA virus (adenovirus, herpesvirus, papillomavirus, vaccinia virus) to aid in the completion of their otherwise replication deficient life cycle (Atchison *et al.*, 1965; Buller *et al.*, 1981; Schlehofer *et al.*, 1986; Walz *et al.*, 1997; Meyers *et al.*, 2000). Until quite recently, only seven AAV isolates (AAV1–6, including AAV3b) had been characterized (Srivastava *et al.*, 1983; Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997; Rutledge *et al.*, 1998; Bantel-Schaal *et al.*, 1999; Chiorini *et al.*, 1999b; Xiao *et al.*, 1999). Current investigation has led to the identification and cloning of 53 new AAV capsid DNA sequences from non-human primates (Gao *et al.*, 2002; 2003) and 64 from human tissues ([www.ncbi.nlm.nih.gov/entrez/query.fcgi](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi); Gao *et al.*, 2004) (Table 2.1). These recent discoveries of PCR-derived isolates of AAV have led to questions about the origins and evolution of this useful genus of parvovirus.

The evolutionary pressures placed on the AAVs are different from the autonomous parvoviruses mainly because of their need for a helper virus. Since infection of the same cell with both AAV and its helper may not always coincide, the AAVs have evolved using two different approaches that increase the chances of AAV and helper virus co-infection. The prototypic isolate, AAV2, infects a number of different cell lines, tissues, and species of animals (reviewed in Rabinowitz and Samulski, 1998). This promiscuity has enabled the virus to be positioned in many cell types awaiting the arrival of helper virus. Further, in the absence of a helper, the AAV genome may integrate into the host's chromosome or remain episomal to set up a latent infection. This quiescent viral genome may then be reactivated from

latency upon subsequent infection with a helper virus or if the cellular milieu becomes favorable for viral replication. These strategies are evidently not harmful to the host as infection with AAV is not associated with any known disease or pathology and, in fact, its presence may actually be beneficial to the host.

The origins and evolution of the adeno-associated viruses will be discussed in this chapter. Topics include an examination of the diversity of the AAV capsid variants, the methods currently used to classify these AAV variants, and the co-evolution of AAV with its helper and its host. Throughout, we will highlight some of what is known about the biology of the distinct AAV capsid variants and their potential usefulness in human gene therapy.

## DEPENDOVIRUS GENETIC ELEMENTS AND ISOLATES

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Structurally, the dependoviruses are amongst the smallest of the known viruses, approximately 20 nm in size. Genetically they are very simple, all isolates contain only three major genetic elements where evolution can occur: the inverted terminal repeats (ITR), the capsid gene (*cap*), and the replication gene (*rep*). In addition, minor genetic areas such as the promoters, introns, and polyadenylation sites can potentially be a source of diversity.

Although the majority of the dependoviruses are AAVs that have been isolated from humans and non-human primates, it is of interest to note that other mammalian AAVs have been identified in dogs, cows, horses, pigs, and birds,

**Table 2.1 All AAV isolates deposited into Genbank as of 9/04.** The capsid sequences of the currently available AAV isolates were placed in the clade groupings according to Gao et al. (2004) with the gene bank accession number placed adjacent. The AAVs were grouped into six clades of which AAV1, AAV2, AAV7, AAV8, and AAV9 are prototypical members of Clades A, B, D, E, and F, respectively. The AAV2/3 hybrids are members of Clade C. Other AAVs (AAV3a, AAV3b, AAV4, AAV5 and others) do not fit into any of the clades and are regarded as clonal isolates

	Gene bank accession number		Gene bank accession number		Gene bank accession number
<b>Complete Genomes</b>					
<b>Adeno-associated virus 1</b>	NC_002077, AF063497	Hu47 Hu51 Hu52	AY530610 AY530613 AY530614	Hu66 Hu42 Hu67	AY530626 AY530605 AY530627
<b>Adeno-associated virus 2</b>	NC_001401	Hu T41	AY695378	Hu40	AY530603
<b>Adeno-associated virus 3</b>	NC_001729	<b>Hu S17</b>	AY695376	Hu41	AY530604
<b>Adeno-associated virus 3B</b>	NC_001863	<b>Hu T88</b>	AY695375	Hu37	AY530600
<b>Adeno-associated virus 4</b>	NC_001829	<b>Hu T71</b>	AY695374	Rh40	AY530559
<b>Adeno-associated virus 5</b>	Y18065, AF085716	<b>Hu T70</b> <b>Hu T40</b>	AY695373 AY695372	Rh2 Bb1	AY243007 AY243023
<b>Adeno-associated virus 6</b>	NC_001862	<b>Hu T32</b>	AY695371	Bb2	AY243022
<b>Avian AAV ATCC VR-865</b>	AY186198, AY629583, NC_004828	Hu T17 Hu LG15 <b>Clade C</b>	AY695370 AY695377	Rh10 Hu17 Hu6	AY243015 AY530582 AY530621
<b>Avian AAV strain DA-1</b>	NC_006263, AY629583	Hu9 Hu10	AY530629 AY530576	Rh25 Pi2	AY530557 AY530554
<b>Bovine AAV</b>	NC_005889, AY388617	Hu11 Hu53 Hu55	AY530577 AY530615 AY530617	Pi1 Pi3 Rh57	AY530553 AY530555 AY530569
<b>Clade A</b>		Hu54	AY530616	Rh50	AY530563
<b>AAV1</b>	NC_002077, AF063497	Hu7 Hu18	AY530628 AY530583	Rh49 Hu39	AY530562 AY530601
<b>AAV6</b>	NC_001862	Hu15	AY530580	Rh58	AY530570
Hu48	AY530611	Hu16	AY530581	Rh61	AY530572
Hu43	AY530606	Hu25	AY530591	Rh52	AY530565
Hu44	AY530607	Hu60	AY530622	Rh53	AY530566
Hu46	AY530609	Ch5 Hu3	AY243021 AY530595	Rh51 Rh64	AY530564 AY530574
		Hu1	AY530575	Rh43	AY530560
<b>Clade B</b>					
Hu19	AY530584	Hu4	AY530602	<b>AAV8</b>	AF513852
Hu20	AY530586	Hu2	AY530585	Rh8	AY242997
Hu23	AY530589	Hu61	AY530623	Rh1	AY530556
Hu22	AY530588				
Hu24	AY530590	Rh62	AY530573	<b>Clade F</b>	
Hu21	AY530587	Rh48	AY530561	Hu14 (AAV9)	AY530579
Hu27	AY530592	Rh54	AY530567	Hu31	AY530596
Hu28	AY530593	Rh55	AY530568	Hu32	AY530597
Hu29	AY530594	Cy2	AY243020	<b>Clonal isolate</b>	
Hu63	AY530624	<b>AAV7</b>	AF513851	<b>AAV5</b>	Y18065, AF085716
Hu64	AY530625	Rh35	AY243000		
Hu13	AY530578	Rh37	AY242998	<b>AAV 3</b>	
Hu56	AY530618	Rh36	AY242999		NC_001729
Hu57	AY530619	Cy6	AY243016	<b>AAV 3B</b>	
Hu49	AY530612	Cy4	AY243018		NC_001863
Hu58	AY530620	Cy3	AY243019	<b>AAV4</b>	
Hu34	AY530598	Cy5	AY243017		NC_001829
Hu35	AY530599	Rh13	AY243013		AY243001
<b>AAV2</b>	NC_001401			Rh33	AY243002
Hu45	AY530608	Rh38	AY530558	Rh32	AY243003

and appear to be dependent upon a helper virus for their replication ([www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm](http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm)). Two of these mammalian AAVs, one from avian (AAAV) and another from bovine (BAAV), have been cloned and sequenced, and have undergone initial characterization as rAAV vectors (Bossis and Chiorini, 2003; Schmidt *et al.*, 2004). Other members of the genus *Dependovirus*, although clearly not dependent upon helper virus, are the autonomous avian parvoviruses: goose parvovirus (GPV), Barbarie duck parvovirus (BDPV), and Muscovy duck parvovirus (MDPV) ([www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm](http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm); Lukashov and Goudsmit, 2001).

An extensive phylogenetic analysis has established that the primate AAVs and the autonomous avian parvoviruses share common ancestry (Lukashov and Goudsmit, 2001). Among the primate AAVs the sequences of only eight complete genomes (AAV1–7 including AAV3b) are known and exhibit an overall nucleotide identity of 45.1 percent. Elimination of the two most divergent isolates, AAV4 and AAV5, from these analyses indicate that the remaining five isolates are closely related exhibiting approximately 72.4 percent overall identity.

Bossis *et al.* have speculated that AAAV provides a ‘missing link’ between the autonomous avian parvoviruses and the primate AAVs (Bossis and Chiorini, 2003), primarily based on the observation that the *rep* and *cap* genes of AAAV are equally divergent from those of the autonomous avian parvoviruses and the primate AAVs. Interestingly, the AAV4 capsid protein sequence is 76.4 percent identical to the BAAV capsid sequence and the AAV5 Rep sequence is 89.8 percent identical to the BAAV Rep protein, suggesting that BAAV is a recombinant between AAV4 and AAV5 or represents some type of cross-species transmission (Schmidt *et al.*, 2004).

Although there are fewer identified examples of the Rep protein than there are of the cap protein (15 Rep sequences versus 124 capsid sequences deposited in Genbank), there does not appear to be much variability in the AAV Rep sequence information with the single exception of the AAV5 Rep (Rep5) which is only 58 percent identical to the other Rep proteins. The functional relatedness of the Rep proteins is suggested by studies that show that the Rep protein of AAV2 (termed Rep2) can support replication and packaging of vector genomes into the capsids of other AAV isolates (Chiorini *et al.*, 1999a; Rabinowitz *et al.*, 2002). Again, the only exception is the Rep protein of AAV5 (Rep5), which utilizes a novel terminal resolution site (TRS) present only in AAV5 terminal repeats (Chiorini *et al.*, 1999a). Accordingly, the known ITRs are greater than 95 percent identical, with the exception of the AAV5 ITRs, which are only 60 percent similar to the other known ITRs. In addition to the novel TRS, the AAV5 ITRs also contain a difference in the spacing between the rep-binding element (RBE) and the TRS. Together, the distinct differences in both the AAV5 Rep and ITR support the idea that the Rep protein and its respective ITR must co-evolve and that changes in

the ITR must be compensated by changes in the Rep protein and vice versa.

Another example of co-evolution concerns the ability of AAV to integrate into its host. Amiss *et al.* determined the sequence of the African green monkey chromosomal locus utilized by a monkey virus isolate, AAV4, for integration and found that this site is homologous to the human integration site with the exception of the RBE (Amiss *et al.*, 2003). The monkey RBE is expanded, containing five repeating GAGC tetramers as opposed to the three tetramers found in the human chromosome. Interestingly, the AAV4 ITRs contain four RBE tetramers, instead of the three found in AAV2. The expansion of similar elements on both the monkey integration site and the AAV4 ITR strongly suggest co-evolution between the virus and its host.

The diversity of primate AAV capsid isolates will be the major focus of this chapter. Much emphasis has been and will continue to be placed on understanding the features of the AAV capsid responsible for receptor binding and cellular trafficking. The receptors for three of the AAV isolates are known and are in concordance with the promiscuity of AAV. Heparan sulfate proteoglycan (HSPG), a molecule found on the surface of many cell types, is used as AAV2’s primary receptor (Summerford and Samulski, 1998). In addition, two different molecules, FGFR and  $\alpha v\beta 5$  integrin, serve as secondary receptors for AAV2 (Qing *et al.*, 1999; Summerford *et al.*, 1999). Both the AAV4 and AAV5 isolates bind to sialic acid (Kaludov *et al.*, 2001; Walters *et al.*, 2001; 2002). Further AAV5 uses PDGFR as a high affinity receptor, a molecule found on the surface of many cell types (Pasquale *et al.*, 2003).

The crystal structure of the AAV2 capsid has been elucidated (Xie *et al.*, 2002) and the regions of the capsid responsible for AAV2 binding to HSPG have recently been mapped to the 3-fold axis of symmetry (Kern *et al.*, 2003; Opie *et al.*, 2003). The structural information for two other AAV isolates, AAV4 and AAV5, is beginning to become available (Kaludov *et al.*, 2003; Walters *et al.*, 2004); however, the exact locations of the receptor binding epitopes on these capsids are unclear at present. Knowledge of the receptors and crystal structures of the different AAV isolates give us a framework to begin to assess the newer AAV isolates with regard to their potential usefulness in gene therapy applications. Further, the diversity of the newer isolates gives us so much more ‘ready-made’ information to choose from in the rational design of new and hopefully better gene therapy vectors. Deciphering this information is a primary focus of many laboratories.

## AAV CAPSID CLASSIFICATION

A classification system for all 124 AAV capsid isolates (Table 2.1) has been problematic but it is clearly necessary to shift through the redundant isolates in order to propel

the field forward. The adeno-associated virus isolates have been subdivided by three different classification systems based on differences in the capsid:

- serology;
- subgroups via capsid subunit mixing compatibility; and
- clades based on genetic relatedness.

The hope of any classification scheme, at least for those investigators using AAV isolates for gene therapy, is to predict the tropism of each isolate based on some unyielding physical or genetic property rather than rigorous testing of the transduction profiles of each isolate.

Serology, a method classically used to group viruses, is no longer in vogue in the AAV field as it has become obvious that there are not 124 distinct AAV serotypes. The AAV isolates can and do fall into serologically distinct 'groups'. For example, Li and Samulski (unpublished data) have determined that the AAV1 and AAV6 capsids that differ by only six amino acids are serologically similar. In addition, many of the newly identified capsids differ from each other only in a few amino acids (Gao *et al.*, 2002, 2003, 2004). One could not envision each of these as unique antigenic sites.

Because of the inherent problems with the use of serotypes for classification, the AAV field is now moving away from its use and examining alternative classification systems. 'Transcapsidation' is a method developed by Rabinowitz *et al.* (Rabinowitz *et al.*, 2004) that allows one to distinguish the degree of relatedness of the different capsids based on their ability to form mixed shells composed of capsid subunits from different isolates. This study assessed the mixing of AAV isolates 1, 2, 3b, 4, and 5 and proposed that three subgroups of AAV exist based on capsid structure. Similar mixing experiments performed with newly identified AAV isolates should allow assignment of these newer variants to an appropriate subgroup.

Although the structural relatedness elucidated from the transcapsidation studies can serve to predict the functional relatedness of the AAV capsid variants, the most straightforward approach to determine evolutionary changes is to examine the amino acid and the DNA sequences of each isolate. Gao *et al.* (Gao *et al.*, 2004) used the ClustalX1.81 program to align the amino acid sequences of many of the AAV capsid isolates. They then used several different computational approaches to determine the phylogenetic tree of these isolates. These analyses suggest that six AAV clades (a group of viruses defined by sequences originating from three or more sources) exist (Table 2.1). Interestingly, similar to what was seen in the transcapsidation approach, both AAV4 and AAV5 do not fit into any of the clades and were regarded as clonal isolates along with several other clonal isolates (Table 2.1). Although only a limited number of isolates were examined, the clade classification seemed to correlate with serological grouping with the exception of AAV1 and AAV5, which appear to be, somewhat unexpectedly, cross-reactive (Gao *et al.*, 2004).

In spite of our attempts to group the known AAV isolates into a unifying scheme, it is still not easy to predict the tropism of any particular isolate. For example although both AAV8 and rh20 are members of clade E, they exhibit remarkably distinct *in vitro* transduction profiles (Gao *et al.*, 2004). Perhaps as the tropisms of more isolates are evaluated, particular capsid 'motifs' will be used to predict which isolates are better suited for delivery to a particular tissue. Evaluation of the newer capsid variants from a structural perspective, using information gleaned from both crystallography and transcapsidation studies will give us the necessary tools to make these predictions and then to test them in model systems. Future determination of operationally distinct subgroups will have to include considerations of sequence identity, structural similarity, structural compatibility, tissue tropism, and immune profiles in order to group new isolates with science-driven understanding. At the moment, this is a tall order and only in its infancy.

## AAV CAPSID DIVERSITY

A clear understanding of AAVs' natural infection remains elusive because of their inability to cause a disease and their latent lifestyle. Until we understand where and how the AAVs exist in nature, we will not understand their evolution. Historically, the AAVs were brought to the attention of the scientific community when they were isolated as contaminants of adenoviral preparations (AAV1–4 and AAV6) (Atchison *et al.*, 1965; Hoggan *et al.*, 1966). Serological evidence indicates that AAV is prevalent among both the human and non-human primate population (Erles *et al.*, 1999; Xiao *et al.*, 1999; Moskalenko *et al.*, 2000) and infectious AAVs have been directly isolated from humans (Blacklow *et al.*, 1967; Bantel-Schaal and zur Hausen 1984).

Recent work by Gao *et al.* sheds new light on the diversity of AAV and indicates that a plethora of distinct AAV capsid genes exist in both human and non-human primates (Gao *et al.*, 2004). Some isolates are predominantly found in the human host (AAV1 [clade A], AAV2 [clade B], AAV2/3 hybrid [clade C], and AAV9 [clade F]), others predominate in non-human primates (AAV7 [clade D]), and some can possibly 'shuttle' back and forth between human and monkeys (AAV8 [clade E]). Approximately 18 percent of human tissues and 19 percent of monkey tissues examined were positive, indicating that these viruses are common in the population. (Gao *et al.*, 2002, 2003, 2004). For both human and non-human primates the spleen and liver were the organs in which most isolates were identified. Other tissues giving rise to a high frequency of isolates included bone marrow, brain, colon, lung, kidney (for humans), heart, and lymph nodes (for non-human primates).

Why, almost 40 years after its initial discovery, has there been such an explosion in the isolation of diverse AAV variants? Perhaps the simplest explanation is that investigators

began to look using very sensitive techniques such as polymerase chain reaction (PCR) and DNA sequencing. Although these molecular biology techniques can be excellent tools for the detection of viral DNA and give us a 'snapshot' of the viral DNA sequences present in the cell at a particular time, these DNA sequences may merely represent failed variants and not reflect the viruses that are successful in nature. Since AAV is not a lytic virus and does not destroy the cells that it infects, it is likely that this may be the case for many of the capsid variants being identified in this manner. Additionally, PCR is subject to an intrinsic error rate proportional to both the type of enzyme used for the analyses and the number of cycles used in the amplification scheme. Details concerning these parameters were not mentioned in the studies performed by Gao *et al.* but could have influenced the perceived diversity in capsid sequences. In parallel with these notes of caution, a number of labs are independently confirming these original observations.

If all of these capsid variant sequences do represent successful virions, it is still unclear whether the DNA analyzed in these studies represents a latent or a replicating viral genome or even a viral genome that was previously encapsidated prior to DNA isolation. It is well known that mucosal epithelial cells are the primary site of herpesvirus replication and that transcriptionally inactive viral genomes remain latent in neurons (Taylor *et al.*, 2002). A similar situation may exist for AAV; the virus may replicate initially in certain tissues but establish latency in another tissue. It would be of interest to ascertain whether the DNA analyzed in these reports could be amplified from genomic or episomal DNA, whether viral mRNA can be isolated and subjected to reverse transcription PCR to determine expression patterns, and/or whether the AAV viral proteins could be detected in these tissues in order to better understand the dynamics of AAV infection. A new chapter of AAV *in vivo* biology will clearly be written while investigating the new capsid variants currently being isolated from primary tissues.

Interestingly, diverse capsid sequences were observed both in the same animal and among members of the same animal species (Gao *et al.*, 2003). In particular nine distinct capsid sequences were characterized from the lymph node of one animal, the diversity of which was compared with the 'swarms of quasispecies' seen during an RNA virus infection. Two possible but not mutually exclusive mechanisms exist to explain the AAV capsid diversity observed in these studies:

- error-prone nucleic acid replication, or
- recombination between co-infecting AAV isolates.

Error-prone nucleic acid replication is frequently observed in RNA viruses, where it is based on the lack of proofreading activity among the RNA-dependent and DNA-dependent RNA polymerases. This is unlikely to explain the majority of variant AAV capsids as AAV DNA replication is carried out by host DNA polymerases. However, recent work on *Caenorhabditis elegans* indicates that the rate of

mutation is much higher than previously thought (Denver *et al.*, 2004) and may explain some of this diversity.

Recombination between co-infecting parental viruses is the mechanism most likely to explain the majority of the capsid variants. Gao *et al.* were able to map how recombination between two different co-infecting viruses could explain the different variants seen in the lymph node of one animal and remarked that these mostly differed from each other in the capsid hypervariable regions (Gao *et al.*, 2003). Thus, the observed AAV diversity may resemble the mosaicism that occurs during bacteriophage recombination (Hendrix, 2002). In that system, random non-homologous recombination first occurs between distinct viral genomes to generate a vast pool of mosaic genes, many of which do not encode functional proteins. This is followed by a period of re-assortment mediated by homologous recombination that serves to clean up the genomes. The single-stranded genome of AAV would be a perfect substrate for non-homologous recombination followed by re-assortment mediated by homologous recombination. In essence, this would be the driving force for the diversity observed in the numerous capsid sequence isolated and characterized to date. However, one would expect the non-homologous recombination to occur across the viral genome. The propensity to observe such changes within specific domains of the capsid coding sequences may be influenced by host selection (i.e. both intracellular selection for competitive growth and potentially extracellular selection to evade the host immune system (see 'Selection of variants' below). The fact that analogous observations have been extensively documented in bacterial phages, leading to the genetic Cuisinart model proposed by Dr Hendrix (Hendrix, 2004), lends compelling support to the idea that identical recombination pathways may also be found among eukaryotic viruses, and that AAV may be the poster child for this pathway.

In further support of recombination occurring between two parental isolates, many examples of AAV2–AAV3 hybrids were observed in human tissues (Gao *et al.*, 2004). Interestingly, this was recapitulated in a marker rescue approach (Bowles *et al.*, 2003) that examined the recombination between AAV2 and AAV3. Although the common breakpoint observed in the two reports differed, results were consistent with recombination occurring between blocks of homologous sequences.

Several concurrent events appear to be required for these recombination events to occur successfully. First, the same cell in the host must be infected with two different AAV variants. Second, if rapid AAV replication is required for increased recombination, then the appropriate helper virus should be present or the cells need to be in a state that is conducive for such rapid replication. As a further complication, it is likely that transcapsidation will also occur when the host is infected with two or more AAVs. Interestingly, when viruses were mixed with other members of the same subgroup, transduction properties were displayed that were

not observed with either parent (Rabinowitz *et al.*, 2004). This ability to transcapsidate may result in virus progeny, albeit dead-end, that are able to infect a greater variety of cell types. Thus, much of the DNA characterized by Gao *et al.* may not encode the AAV capsid that is best evolved to transduce these tissues, perhaps explaining why viral DNA identified in certain tissues may not correlate with the ability of that capsid to transduce those particular tissues. For example, both AAV7 and AAV8 DNA sequences were identified in cardiac muscle (Gao *et al.*, 2002) but may not turn out to be best suited for this tissue.

The actual events that occur during the infection process to promote this recombination are unknown. Mechanisms that increase the proximity of the variant DNAs are likely to be of importance. Gao *et al.* speculated that annealing of the positive and negative single-stranded DNA genomes from the different variants could be a mechanism. Alternatively, in a situation that closely mimics recombination between clades of HIV, heterodimers of AAV genomes could be packaged into the same capsid, serving to increase the proximity of different molecules and increasing recombination (Burke, 1997). Finally, a strand-switching event during viral DNA replication, similar to that observed with *Circovirus*, another single-stranded DNA virus (Cheung, 2004), could account for some of these events.

## SELECTION OF VARIANTS

The differences between many of the AAV capsid variants cluster in the 12 hypervariable regions located in the loops of the beta barrel formed by each capsid protein subunit. These loop regions are exposed on the surface of the capsid, and are responsible for binding to receptors, and creating the epitopes recognized by antibodies. What is clear is that minimal, even single amino acid changes to the capsids of parvoviruses can have profound implication on receptor usage, cell tropism, trafficking, and pathogenesis (Hueffer and Parrish, 2003). Even within the AAV clades, the capsid variants may exhibit distinct biological properties depending upon where the capsid changes are located. The examples discussed below involve only small amino acid differences in the capsid and concern the binding of AAV to the cellular receptor HSPG.

Examination of all isolates that fall into the AAV2 clade (clade B) indicate that only 22 percent of these isolates contain both of the amino acids (R585 and R588) critical for heparin binding in the context of the AAV2 capsid (Opie *et al.*, 2003). The majority of clade B isolates are thus predicted not to bind HSPG or to use alternate sites on the capsid for HSPG binding. One possibility to explain the lack of arginines at these key positions is that the AAV2 isolate that we know today may have been selected to bind heparin sulfate in cell culture from a diverse pool of AAVs. There are several examples of viruses evolving to use heparin sulfate

in cell culture (Klimstra *et al.*, 1998; Mandl *et al.*, 2001), although the others are all RNA viruses that can undergo rapid changes in response to the environment. Still it may be possible that AAV2 was adapted to cell culture prior to its cloning. If this is true, heparin binders should outgrow the non-heparin binders if a pool of the new clade B isolates were passaged in cell culture. Alternatively, the clade B heparin binders may have distinct infection profiles *in vivo* and potentially novel helper viruses.

An example of alternate heparin binding site usage concerns the AAV3 isolates AAV3a and AAV3b. AAV3b binds heparin columns (Rabinowitz *et al.*, 2002) but infection with AAV3b is not inhibited by heparin (Rabinowitz *et al.*, 2004). In contrast, Handa *et al.* determined that the ability of AAV3a to infect tissue culture cells is inhibited by heparin (Handa *et al.*, 2000). The heparin sensitivity phenotypes of both of these isolates is not due to the arginines at positions 585 and 588, as both AAV3a and AAV3b and all isolates in clade C (the AAV2/3 hybrid clade) lack arginines in these or nearby positions. There are only six amino acid differences in the capsid of these two isolates to account for this variation in heparin sensitivity (Muramatsu *et al.*, 1996; Rutledge *et al.*, 1998). Interestingly, all of the isolates in clade C resemble AAV3b in four out of six amino acid positions. In the remaining two positions, 52.6 percent of the AAV2-AAV3 hybrid clade resembles AAV3a while none resembles AAV3b. It will be quite a challenge to examine the known AAV3 variants to resolve the heparin-binding differences noted in cell culture and to determine whether these differences in the binding affinities correlate with novel properties *in vivo*.

Two isolates, AAV1 and AAV6, have both been reported to be superior vectors for the transduction of skeletal muscle (Xiao *et al.*, 1999; Gregorevic *et al.*, 2004). These two variants share greater than 99 percent amino acid identity, yet differ in their ability to bind to heparin columns. Gregorevic *et al.* state that AAV6 can be purified via heparin columns while AAV1 cannot (Rabinowitz *et al.*, 2002; Gregorevic *et al.*, 2004), although the exact column conditions for AAV6 binding were not mentioned and may not be identical to those used for AAV1. Does AAV6 represent a virus that has been adapted in cell culture to bind to heparin? AAV6 was originally isolated from a human adenovirus preparation and appears to be a hybrid that arose from recombination between AAV1 and AAV2 (Xiao *et al.*, 1999). When this recombinatorial event occurred and whether it happened *in vitro* or *in vivo* is unknown. Interestingly, all of the other isolates that fall into clade A resemble AAV1 rather than AAV6 at these six positions. It will be of interest to determine whether these additional isolates exhibit the same muscle enhanced transduction, what components of the capsids are responsible for this, and which of the six amino acid differences are responsible for the differences in heparin binding.

Because little information is available regarding the natural infection process of the newer AAV variants and because

they were characterized directly from DNA extracted from the cell, it is quite possible that many have had no selective pressures placed on them. Since AAV can coexist with the host in latent phase or undergo mobilization by a helper virus, it is likely that multiple helper virus infections would induce periodic blooms of AAV in the same host. Many groups have clearly demonstrated immune responses to AAV viral particles *in vivo* (reviewed in Monahan *et al.*, 2002), suggesting the important role the immune system may play in selecting new capsid variants when a subsequent bloom of AAV particles ensues in the same host. This scenario, which may explain the large number of capsid variants carrying changes only in the hypervariable regions, can be mimicked in animal models and should shed important insights into the role of immune selection on virus capsid variation.

## EVOLUTION BY THE HELPER VIRUS

A unique pressure that drives the evolution of adeno-associated viruses is their need for an unrelated helper virus to provide the proteins necessary for many aspects of their life cycle (Buller *et al.*, 1981; Janik *et al.*, 1981; Cockley and Rapp, 1987; Weindler and Heilbronn, 1991). One of the major contributions from these helper viruses is their influence on AAV DNA replication; in fact, some herpes viruses seem to carry their own Rep homolog (Thomson *et al.*, 1994). It seems probable that increased AAV DNA replication is a positive factor in AAV recombination. In this regard, the presence of the helper virus proteins may function to increase the rate of recombination and influence the evolution of the AAVs.

One possible example of AAV coevolving with its helper is AAV5, the most divergent of the AAV isolates. Recent studies by Qiu *et al.* suggest that AAV5 is less dependent on the adenovirus helper than AAV2 (Qiu *et al.*, 2002). Is this because AAV5 has evolved a dependence on another helper such as herpes virus? This seems possible since seroconversion for AAV5 coincides with seroconversion for HSV (Georg-Fries *et al.*, 1984).

Qiu *et al.* report that AAV5 efficiently utilizes a polyadenylation (pA) site in the middle of the transcript originating from the p7 and p19 promoters (Qiu *et al.*, 2002). Because this pA site is within the central intron, AAV5 does not express the Rep proteins generated from the use of the alternate splice site (Rep68 and Rep42). It is known that adenoviral transcripts undergo extensive splicing while only a few of the HSV transcripts are spliced (Julius Rajcani, 2004). This decreased reliance of AAV5 on adenoviral help may thus reflect AAV5's infrequent splicing pattern. Interestingly, the HSV ICP27 protein functions to transport intronless HSV transcripts (Chen *et al.*, 2002). The presence of HSV then may aid in the replication of AAV5 by actively transporting these AAV5 transcripts. Conway *et al.* (1997) determined that HSV devoid of the ICP27 protein

produces more rAAV2 than HSV containing ICP27. A likely prediction from these studies is that HSV containing ICP27 would be the better helper for rAAV5 production.

## EVOLUTION WITH THE HOST

It is generally agreed that the AAVs do not cause disease or any observable pathological effect. Only one AAV isolate, AAV5, was isolated *in situ* from a primary human infection, a flat condylomatous penile lesion (Bantel-Schaal and zur Hausen, 1984), and its role in this pathology was unclear. Other AAVs have been isolated from clinical situations with no apparent pathology other than an ongoing adenoviral infection (Blacklow *et al.*, 1967). Stilwell *et al.* using microarray analyses, demonstrated that while infection with a pathogenic virus such as adenovirus induced the expression of immune and stress response genes, infection with AAV2 resulted in minimal transcriptional activation (Stilwell and Samulski, 2004). In essence, these AAVs have reached the ultimate point of viral evolution. By specifically minimizing all negative effects on their host, AAV gets a 'free ride', allowing it to hide out in its host via integration and escape elimination by an immune response. Can the presence of an innocuous virus actually be beneficial to the host and aid in its evolution? A theory proposed by Fisher and Mayor in the 1980s speculated that the AAVs serve a role in cellular defense against disease-causing viruses (Fisher and Mayor, 1986). The ability of AAV to inhibit the replication of their helper virus was first commented on by Hoggan *et al.* in 1966. Further, AAV appears to interfere with oncogenic transformation induced by viruses. Limited serological evidence indicates a negative correlation of AAV with cervical carcinoma (Odunsi *et al.*, 2000; Smith *et al.*, 2001). It is postulated that AAV prevents the replication of human papillomavirus (HPV), an etiologic agent of this type of cancer.

A final way in which the AAVs may help the host evolve, at least indirectly, may come from the use of these viruses as gene delivery agents for the field of gene therapy. The fact that this group of viruses does not seem to cause a disease is clearly advantageous for gene therapy. Their ability to transduce tissues *in vivo* has been widely investigated in lower mammals, but is only beginning to be explored in humans. The only isolate approved for the approximately 25 ongoing clinical trials using rAAV vectors is AAV2 ([www.wiley.co.uk/wileychi/genmed/clinical](http://www.wiley.co.uk/wileychi/genmed/clinical)). However, as more information becomes available it is evident that isolates other than AAV2 may be the vectors of choice for different gene therapy applications. The non-human primate clades (AAV7- and AAV8-based clades) may be of particular interest to the gene therapy community, as human sera rarely contain neutralizing antibodies against either isolate (Gao *et al.*, 2002).

Direct comparison of the transduction profiles of several AAV isolates have been made in many tissue types using both marker and therapeutic genes. A general assessment is

that AAV1 and AAV5–9 exhibit the most favorable transduction profiles; AAV2 seems to transduce cells in culture better than tissues *in vivo*. The differential transduction profiles of the AAV isolates are not easily explained, but may reflect differences in binding receptors on the transduced cells and/or the ability to deliver their recombinant genomes to the nucleus more efficiently. Still to be determined is whether the enhanced transduction profiles seen in animal models extend to humans. This will only come from numerous phase I clinical trials. Therefore, we wait with anticipation for the new ‘evolved’ serotypes to make their way to the clinic.

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# The genus *Erythrovirus*

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KEVIN E. BROWN

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Erythroviruses are named for their tropism for red blood cell progenitor cells. The type member of the genus, parvovirus B19, is the only member of the *Parvoviridae* family known to be pathogenic in humans. Acute infection causes fifth disease in children, polyarthropathy syndrome in adults, transient aplastic crisis in patients with underlying chronic hemolytic anemia, and chronic anemia from persistent infection in immunocompromised patients (Brown *et al.*, 1994c). Infection in pregnancy can lead to hydrops fetalis with possible fetal loss or congenital infection (Brown *et al.*, 1994a).

The erythroid tropism is mediated in part by the cellular receptor for parvovirus B19, the neutral glycolipid globoside (blood group P antigen) (Brown *et al.*, 1993). Hematologic disease due to parvovirus B19 is the result of a direct viral cytotoxic effect on erythroid progenitor cells in bone marrow with interruption of erythrocyte production. The physiology of host hematopoiesis and the competence of the immune antiviral response both determine clinical manifestations of the infection (Brown and Young, 1997).

Cynomolgus monkeys are infected by a simian parvovirus (O'Sullivan *et al.*, 1994) that is remarkably similar to human B19 parvovirus in its predilection for host bone marrow *in vitro* and ability to cause serious anemia in some infected animals. In addition, two other parvoviruses have been identified in anemic macaques: a pig-tailed macaque parvovirus (PmPV) and a rhesus monkey virus (RmPV); at the molecular level all three viruses are as different from each other as they are from the human virus, but all are associated with profound anemia in immunosuppressed animals.

With the realization of the fundamental differences in the viral sequence, transcription map and cellular biology between parvovirus B19 and the other mammalian parvoviruses, in 1993 a new genus within the *Parvoviridae* was created, the *Erythrovirus* genus, initially with a single member, parvovirus B19. Subsequently, the three other

non-human primate erythroviruses have been identified with similarities to B19 in the clinical diseases they cause and in their genome sequences. In addition, viral sequences were obtained from Manchurian chipmunk, fetal calf serum and pigs, that have similarity to the erythroviruses. However, apart from the sequence data little else is known about these viruses, including their role in disease in the natural host.

## DISCOVERY OF THE VIRUSES

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### **Parvovirus B19**

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Parvovirus B19 was first discovered in England during 1974 when Yvonne Cossart was evaluating tests for hepatitis Bs antigen (HBsAg) using panels of serum samples (Cossart *et al.*, 1975). One serum sample (coded 19 in panel B) gave anomalous results: a positive result in the relatively insensitive CIE (counterimmune electrophoresis) assay, which used human sera as antibody, but negative in the more sensitive and specific radioimmunoassay and reverse passive hemagglutination tests, which employed hyperimmune animal sera as antibody. The CIE precipitin line was cut out and electron microscopy (EM) showed the presence of 23 nm particles resembling parvovirus. There was no reactivity with antisera to adeno-associated viruses or rat parvovirus, and the virus was originally labeled 'serum parvovirus-like particle' (SPLV). Approximately 30 percent of adults had antibody to the new virus detectable by CIE.

The virus was independently described by workers in Japan using similar techniques who gave it the name 'Nakatani' virus (Yamano *et al.*, 1980). However, subsequent testing of both 'Nakatani' antigen and antibody confirmed

that it was identical to the virus discovered in England (Okochi *et al.*, 1984).

Initially there was no disease associated with the new virus. The first report of symptomatic human parvovirus appeared a few years after its discovery. Two soldiers returning to the UK from Africa developed a brief febrile illness. Serum taken at the time of the illness contained parvovirus visualized by EM, and the soldiers subsequently cleared the virus and developed an antibody response (Shneerson *et al.*, 1980). The first clinically significant illness associated with B19 infection was hypoplastic crisis in patients with sickle-cell anemia (Pattison *et al.*, 1981), and development of specific serological assays showed that patients with fifth disease (erythema infectiosum) also had evidence of acute infection with the virus (Anderson *et al.*, 1983). Subsequent studies demonstrated the link between chronic B19 infection and anemia in immunosuppressed patients, and the development of fetal hydrops following maternal B19 infection in pregnancy.

Analysis of the genetic material of viral particles from sera showed that they contained complementary single strands of DNA, of ~5.5 kb length (Summers *et al.*, 1983), with terminal hairpin sequences, and that the particles had a buoyant density in cesium chloride of 1.43 g/ml (Clewley, 1984), all characteristics allowing classification of the virus as a member of the *Parvoviridae*. In 1985 the International Committee on Taxonomy of Viruses (ICTV) accepted the virus as a member of the *Parvoviridae* and recommended the name B19 to prevent confusion with other viruses (i.e. HPV, human papillomavirus) (Siegl *et al.*, 1985). Although originally classified as a member of the autonomous parvovirus genus, in 1993 it was reclassified as the type member of the newly created *Erythrovirus* genus.

## Simian parvoviruses

The first simian parvovirus, SPV, was initially identified in 1992 in a group of severely anemic cynomolgus monkeys at the Bowman Gray School of Medicine's Comparative Medicine Clinical Research Center (CMCRC). Clusters of anemic cynomolgus monkeys had been noted previously at CMCRC, most recently in 1991, and the absence of a specific diagnosis in those cases had raised the possibility of an underlying viral etiology. Serum samples were obtained from the anemic monkeys in 1992, and because of the clinical similarity to human patients with transient aplastic crisis who are viremic (normocytic normochromic anemia with reticulocytopenia), sera were tested for parvovirus DNA by dot blot hybridization using a B19 parvovirus probe (pYT103; Clewley 1985). The four anemic monkeys all tested strongly positive in this test while sera from healthy monkeys were negative, and parvovirus-like particles were identified in serum samples of two animals by immune electron microscopy using a hyperimmune human anti-B19 serum sample as a source of antibody. Viral DNA was obtained from the serum of viremic monkeys,

cloned, and sequenced to confirm the isolation of a novel simian parvovirus (SPV) (Brown *et al.*, 1995b).

The second simian parvovirus was obtained from a similar outbreak of anemia in pig-tailed macaques in Seattle, Washington. The animals were used in a study of SHIV-2 infection; animals receiving one source of inoculum reproducibly developed profound anemia in addition to becoming immunosuppressed. Although tests, including polymerase chain reaction (PCR), for B19 and SPV were negative, some SPV-specific PCR primer pairs gave a unique product on ethidium bromide staining. When this amplified DNA was cloned and sequenced, the PCR products were found to show limited sequence homology (~70 percent) with both SPV and B19, confirming that this infectious agent was a second novel simian parvovirus, named pig-tailed macaque parvovirus (PmPV) (Brown *et al.*, 1995a; Green *et al.*, 2000).

The third simian virus was obtained from an outbreak of anemia in rhesus monkeys at Kansas University Medical Center. As with the pig-tailed macaques, these monkeys were being used in infection studies with SHIV-2, when there was an outbreak of anemia among both the cynomolgus and rhesus animals. SPV infection was suspected, and sera were tested by dot-blot hybridization using an SPV probe. The cynomolgus monkeys were negative (subsequently their anemia was shown to be due to blood parasites), but two of the rhesus samples were positive by dot-blot hybridization. PCR with SPV primers from the capsid region gave a single product on ethidium bromide staining, and this amplified product was cloned and sequenced. Again, the sequence had only 80 percent homology to SPV, PmPV, and B19, confirming the existence of another novel simian parvovirus, named Rhesus monkey parvovirus (RmPV) (Green *et al.*, 2000).

The true prevalence or incidence of primate infection with erythroviruses is unknown. The original outbreak of SPV was not an isolated event. Similar outbreaks of infection have been documented in other cynomolgus colonies (O'Sullivan *et al.*, 1996; and unpublished observations) and we have identified RmPV and PmPV in monkey colonies (Foresman *et al.*, 1999; Joag *et al.*, 1997). Some of the earlier reports of the hematologic abnormalities seen with SAIDS (simian acquired immunodeficiency syndrome) may well represent unrecognized parvoviral infection (Henrickson *et al.*, 1984; MacKenzie *et al.*, 1986; Watanabe *et al.*, 1990). In our own preliminary studies approximately 50 percent of captive cynomolgus monkeys, and 35 percent of captive rhesus macaques have Western blot evidence of previous infection with SPV or related viruses. Similar seroprevalence studies suggest that ~40 percent of wild Indonesian macaques have antibodies to SPV (James *et al.*, 2003).

## Manchurian chipmunk parvovirus

In studies very reminiscent of those leading to the discovery of parvovirus B19, in 1999 Byung Chul Yoo and colleagues

were testing sera for HbsAg in an attempt to discover a new animal model of hepatitis B (Yoo *et al.*, 1988). Four of 62 animals tested for HbsAg were reactive, and EM showed the prevalence of 20–22 nm particles in the serum. DNA extraction of serum under denaturing conditions revealed the presence of a single-stranded DNA genome of approximately 5900 nucleotides (nt). As with other members of the *Erythrovirus* genus, the virus packaged approximately equimolar amounts of minus and positive strands that self-annealed if DNA was extracted under annealing conditions. Sequencing of ~5 kb confirmed homology to the *Parvoviridae* with the greatest similarity to parvovirus B19 and SPV (Yoo *et al.*, 1999). Nothing is known about the pathophysiology of this virus, and it is not known whether it is erythrotropic, or associated with any disease.

## Bovine and porcine viruses

In studies to develop a modified form of SISPA (sequence-independent single primer amplification) to detect novel viruses, two novel parvoviral genomes were detected in fetal calf serum used as a diluent. On analysis of almost the full length genome, neither sequence clustered with any of the known parvoviruses. However, one sequence, BPV3, had significant similarity to the chipmunk and primate erythroviruses (Allander *et al.*, 2001).

In a study to identify hepatitis E in sera in Myanmar, a parvoviral sequence was detected in 9 of 86 pig samples tested (Hijikata *et al.*, 2001). No information is given about the pigs. However, the sequence, which again differed from all known parvovirus sequences, was most similar in the capsid region to BPV3.

## PHYSICAL CHARACTERISTICS

Parvovirus B19 virions have similar physical characteristics to the other members of the *Parvoviridae* (Siegl *et al.*, 1985). On electron microscopy the particles are seen to be non-enveloped, 19–23 nm in diameter, with icosahedral symmetry and often both 'empty' and 'full' capsids visible (Summers *et al.*, 1983). Mature infectious parvovirus particles have a molecular weight of  $\sim 5.6 \times 10^6$ , whereas empty particles have a molecular weight of  $4.2 \times 10^6$ . Parvovirus B19 particles appear to be slightly denser than other members of the *Parvoviridae*, have a sedimentation coefficient of 110S (Cotmore *et al.*, 1986) and band at a density of 1.41–1.45 g/ml in CsCl gradients (Clewley, 1984).

The DNA in infectious particles makes up 19–37 percent of the total mass of the virion. The virus packages approximately equal amounts of plus and minus strands, which, if extracted under optimal annealing conditions, form duplex DNA. The 5' and 3' terminal sequences are identical, and are sufficiently long that they can be visualized

by electron microscopy (Mori *et al.*, 1987; Zuccheri *et al.*, 2001).

B19 virions comprise only two types of capsid protein, VP1 (84 kDa) and VP2 (58 kDa) Parvovirus B19 is stable in lipid solvents (ether, chloroform) but can be inactivated by formalin,  $\beta$ -propiolactone and oxidizing agents. Gamma irradiation will also inactivate B19, with 1.4 Mrad producing a  $10 \log_{10}$  reduction in infectivity (Cohen and Brown, 1992). The heat stability varies widely depending on the environment. Although parvovirus B19 is not quite so resistant to heat as other parvoviruses, transmission by heat-treated blood products has been well documented (Blumel *et al.*, 2002a), since it still survives 48°C for 30 minutes. In blood products, parvovirus B19 retained infectivity after heating to 56°C for 60 minutes, although in one report there was diminished B19 infectivity after 30 minutes at 56°C (Mortimer *et al.*, 1983). However, the heat sensitivity varies depending on the diluent used. Although B19 is inactivated by >4 logs at 60°C when diluted in 25 percent albumen (Blumel *et al.*, 2002b; Yunoki *et al.*, 2003), it is virtually resistant to 60°C heat for >1 hour when suspended in 60 percent sucrose (Yunoki *et al.*, 2003).

## MOLECULAR BIOLOGY

### DNA

#### CLONING OF THE B19 VIRAL GENOME

The cloning of the B19 (Wi isolate) genome was accomplished by exploiting the fact that B19, like AAV, packages both complementary strands separately, and that the hairpin sequences can be used to prime extension with *Escherichia coli* Klenow fragment. DNA was extracted, the second strand synthesized, BamHI linkers added to the termini, the product cut with BamHI (there is one internal BamHI site in the B19 genome) and the two subgenomic BamHI fragments cloned into the BamHI site of pAT153, a derivative of pBR322. Two plasmids, pYT101 and pYT102, containing the B19-specific 1.5 kb and 3.9 kb BamHI fragments were isolated. Although this strategy allowed some of the hairpin sequence to be obtained, subsequent analysis showed that the clones underwent a series of deletion events with loss of up to 200 nucleotides at the termini (Cotmore and Tattersall 1984).

A second molecular clone was obtained from the serum of a child with homozygous sickle cell anemia in the early phase of reticulocytopenic aplastic crisis (Cotmore *et al.*, 1986) by similar methods. The plasmid pYT103 from this isolate of B19 (Au isolate) was cloned by methods similar to those described above, but using EcoRI linkers, following which the two subgenomic fragments were joined together. Again, the sequence obtained was incomplete, missing the extreme left and right ends of the B19 termini (Shade *et al.*, 1986).

Intact hairpin sequences were first obtained by Veronika Deiss and colleagues by cloning the plasmids into JC8111 bacteria, which are deficient for exonuclease and recombinase genes and had been used to maintain other large palindromic sequences. The resultant plasmids permitted structural analysis of the B19 palindromic termini for the first time, and confirmed that the 5' and 3' ends were identical and existed in the flip and flop format seen in other *Parvoviridae* (Deiss *et al.*, 1990). However, they were unable to obtain a stable plasmid when the fragments were joined together. More recently, by using a similar approach with recombinase-negative bacteria and growing the plasmids at 30°C, we have produced a full-length clone, including intact termini, and have shown that after transfection into permissive cells it can produce infectious B19 particles (Zhi *et al.*, 2004).

### B19 DNA

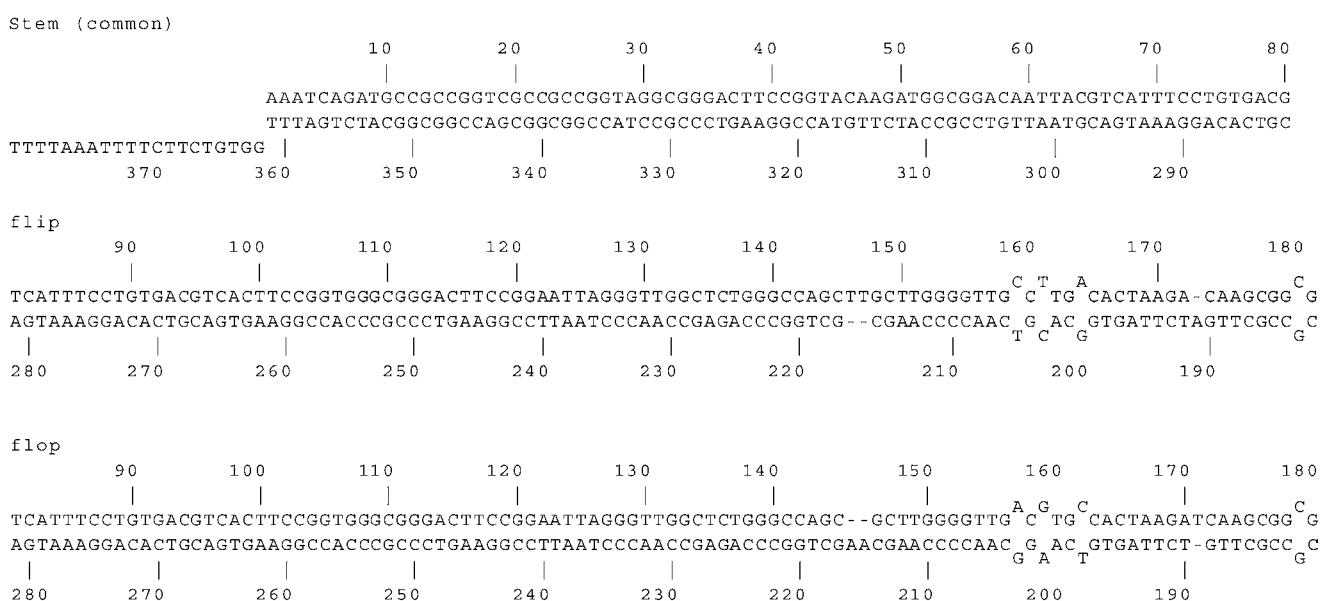
The B19 genome thus consists of a single linear DNA molecule of 5596 nucleotide; it is composed of an internal coding sequence of 4830 nucleotide, flanked by the terminal repeat sequences of 383 nucleotides each. The terminal 383 nucleotides at each end of the genome were found by sequence analysis to be identical inverted repeats. The distal 365 nucleotides of the repeat represent an imperfect palindrome, which folds over to form a hairpin structure. Notably, the sequence of the hairpin occurs in two distinct configurations, which are related in that one is the inverted complement of the other. Such alternative configurations of the terminal hairpins have been described for all parvoviruses analyzed to date and are referred to as > flip = and > flop = (Figure 3.1). Viral DNA replication is

thought to be analogous to that of the dependoviruses that also have identical terminal hairpin structures (although not as long) and are discussed in Chapter 2.

### B19 GENOMIC RELATEDNESS AND STRAIN VARIATION

Strain variation has been studied by several investigators, initially by restriction endonuclease analysis (Morinet *et al.*, 1986; Mori *et al.*, 1987), and more recently by sequence analysis (Erdman *et al.*, 1996; Hemauer *et al.*, 1996; Dorsch *et al.*, 2001). Initial studies showed that the viral genome is well conserved with approximately 5 percent sequence variation between isolates from different parts of the world and from different time periods. In a study of 12 strains isolated in Japan at two different times, 1981 and 1986–1987, the genome type differed at each time and B19 viruses with similar genome types disseminated widely in Japan during each prevalence (Umene and Nunoue, 1990). Although isolates can be divided into groups based on their sequenced differences, so far no correlation has been demonstrated between viral sequence and specific disease presentation (purpura, aplastic crisis, arthralgia).

In 1995 Quang Tri Nguyen, then working in Paris, identified a B19 variant in a G6PD-deficient child with transient aplastic crisis. The viral sequence was amplified by PCR using B19 primers and a low annealing temperature, but was only detected by the probe with low stringency washes (Nguyen *et al.*, 1998). Subsequent sequence analysis showed that the isolate, V9, had ~12 percent sequence variation from other isolates of B19 (Nguyen *et al.*, 1999). In a study to find similar variants in samples sent to NIH for testing, he found an additional variant, A6, which by sequence analysis showed ~11 percent sequence variation from B19 sequences, and



**Figure 3.1** Structure of the B19 terminal presented in its hairpin form. The flip and flop orientations at the 5' = end (+ strand) are shown. The numbers indicate the nucleotide positions in the viral genome of B19-J35 isolate (GenBank AY386330).

8 percent variation from V9 (Nguyen *et al.*, 2002). In independent studies in Finland, Kati Hokynar also identified a B19 variant in skin samples. They named this genotype K71, and sequenced the coding region of one isolate, LaLi (Hokynar *et al.*, 2002); LaLi and A6 sequences are virtually identical and are clearly members of the same genotype. Similar sequences can be identified in serum, bone marrow (Servant *et al.*, 2002) and liver samples (Wong *et al.*, 2003).

Although the nomenclature remains unclear, it appears that B19 sequences can be divided into three distinct genotypes (Figure 3.2). It has been proposed that the original B19 sequences comprise genotype 1, the A6-like, LaLi-like and Vx-like comprise genotype 2, and the V9-like sequences comprise a third group, genotype 3 (Servant *et al.*, 2002). Although there are distinct differences in the sequences between the genotypes, most of the nucleotide differences are at the third nucleotide position (i.e. silent mutations), and the viral proteins have 96–97 percent homology with other B19 isolates, with no reported difference in antigenicity (Heegaard *et al.*, 2002). It is not known at present if these different genotypes reflect different biological properties, pathophysiology or even tissue tropism.

## OTHER ERYTHROVIRUS DNA

The coding sequence of all three simian parvoviruses has been obtained, although the complete terminal sequences are not known. All three viruses have a single-stranded DNA genome of approximately 5600 nucleotide, and encapsidate both plus and minus strands in approximately

equal numbers. To date 4986 nucleotides of SPV, ~5049 nucleotides of PmPV, and 5342 nucleotides of RmPV have been sequenced, including the promoter, all coding regions and the polyadenylation sequences (Table 3.1). However, none of the hairpin regions of the simian viral genomes has been cloned, and the nature of the terminal structures remains unresolved. At least 300 nucleotide of the hairpins of RmPV have been obtained, and RmPV-like B19 has virtually identical sequences at the 5' and 3' ends.

Comparison of the simian parvovirus sequences with nucleic acid sequences in GenBank confirmed that they have little homology with the autonomous parvoviruses or the dependovirus AAV-2, but 50 percent overall homology with parvovirus B19 DNA.

It was predicted that the chipmunk parvovirus genome may be slightly larger than the other erythroviruses, with a genome of ~5900 nucleotides (Yoo *et al.*, 1999). However, only 5097 nucleotides have been cloned and sequenced, including the putative TATA box and 3' polyadenylation signal. The structure of the hairpins is unknown. Similarly the structure of the hairpins of the porcine H1 and bovine BPV-2 and BPV-3 viruses is unknown, with ~5000 nucleotides sequenced of each of the viruses (Table 3.1).

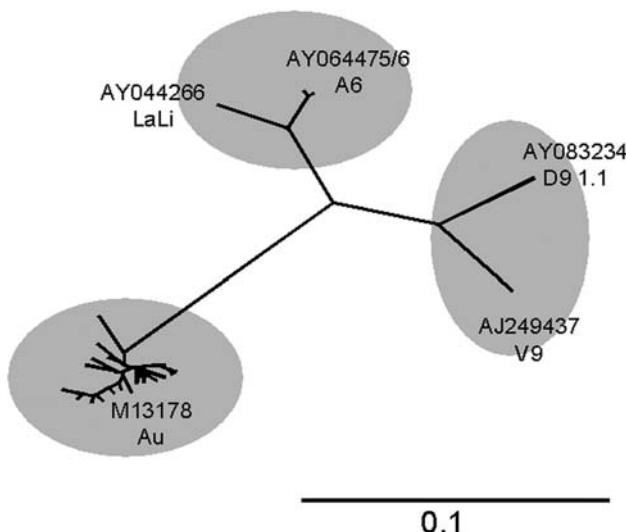
## GENOMIC ORGANIZATION

Analysis of the open reading frames of all known erythroviruses reveals two large open reading frames, with the capsid proteins encoded by genes on the right side of the genome and non-structural proteins by genes on the left side (Figure 3.3). There are no large open reading frames in the complementary DNA strand, suggesting that it does not encode proteins. However, in contrast to the other *Parvoviridae*, B19 appears to have a single strong promoter, p6, at the left-hand side of the genome and capsid proteins are produced by alternative splicing. Of the non-B19 erythroviruses, only SPV has been grown in tissue culture to date. Studies of the SPV transcription map have recently been published and also suggest the presence of a single strong promoter (Liu *et al.*, 2004, Vashisht *et al.*, 2004). Further studies are required to determine whether the other viruses also have only a single promoter and multiple termination signals (using putative internal polyadenylation sites, as in parvovirus B19), or if the viruses use multiple promoters (with a single polyadenylation signal, as in most other members of the *Parvoviridae*).

## B19 PROMOTER

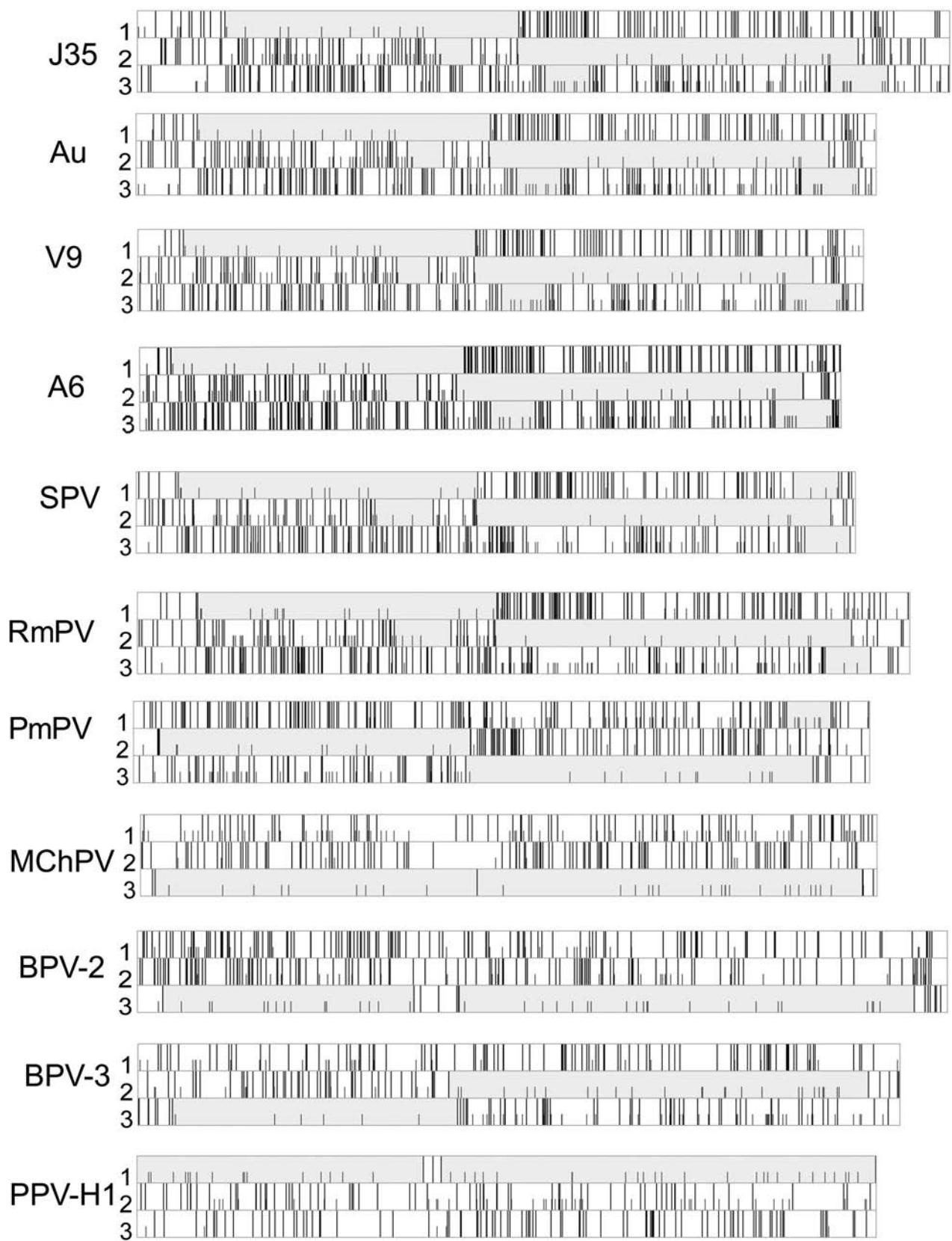
Shortly after the cloning of B19 parvovirus, two groups reported the identification of the left-sided non-structural gene promoter at map unit 6 (the P6 promoter). Using *in vitro* run-off transcription assays in HeLa cells it was shown that a region between nucleotides 258 and 321 (map unit 6) was necessary for *in vitro* transcriptional activity (Blundell *et al.*, 1987), and that the transcriptional start site was at nucleotide 350–351 (of the B19-Au sequence, GenBank

**Figure 3.2** Phylogenetic analysis of 31 B19 strains (sequences with >4000 nucleotides) showing division into three distinct genotypes. Multiple alignment was by Clustal W, and the phylogenetic tree was obtained with TreeView (Page, 1996). Sequences similar to A6, LaLi and D7.1.1 are genotype 2, and V9-like sequences are genotype 3.



**Table 3.1** Summary of the erythrovirus genomes, with the length of DNA sequenced (only B19-J35 is a full genome), and the position of the TATA box and main viral proteins.<sup>H</sup> For the 11 kDa protein, the B19-Au and J35 sequence starts at the in-frame ATG after the splice junction. For V9 and A6 the splice junction has not been mapped and the numbers refer to the first ATG in the small open reading frame. For SPV, RmPV, and RmPV there is no ATG start codon in the ORF and the size of the orf is given (see Figure 3.8, p. 39)

Virus	Strain	Gene bank accession number	Length	TATA	NS	aa	VP1	aa	11 kDa <sup>H</sup>	7.5 kDa	X kDa
B19	Au	M13178*	5112	319	436-2451	671	2444-4789	781	4710-4994	1904-2128	2694-2939
	J35	AY386330	5596 <sup>I</sup>	499	616-2631	671	2624-4969	781	4890-5174	2084-2308	2874-3119
	V9	NC_004295	5028	211	328-2343	671	2336-4681	781	4575-4886	1796-2020	2586-2831
	A6	AY064476	4844	118	235-2250	671	2243-4588	781	4509-4793	1709-1927	2493-2738
	LaLi	AY044266	4612	168	285-2300	671	2293-4612+	773+	nk	1753-1977	2543-2788
SPV	B20	SPU26342	4986	195	307-2370	687	2363-4819	818	4638 <sup>H</sup> -4949	1667-2062	2718-2963
RmPV		AF221122	5342	326	439-2490	683	2480-4942	820	4761 <sup>H</sup> -5075	1790-2173	2841-3080
PmPV		AF221123	5049	171	296-2314	672	2307-4664	785	4483 <sup>H</sup> -4788	?	2563-2802
MChPV		CPU86868	5097	20	198-2333	711	2511-5000	829			
BPV-2		AF406966	5610	121	306-1919	537	2268-5384	1038			
BPV-3		AF406967	5276	nk	261-2216	651	2219-5062	947			
PPV-H1		AB076669	5118	nk	1-1986	662	1987-2	981			



**Figure 3.3** Analysis of the Erythrovirus genomes, including tentative members, illustrating the open reading frames present in the + strand of the virus. For each reading frame, solid bars indicate a stop codon, and half bars an ATG site. Sequences are as in Table 3.1.

M13178), 31–32 nucleotides downstream from the TATA box at nucleotide 319 (Blundell *et al.*, 1987). A second group of investigators used transient expression of cloned DNA transfected into HeLa cells to detect and map a strong promoter at map unit 6 (Doerig *et al.*, 1987). Neither group was able to detect any other promoter of comparable strength to the P6 promoter in the B19 genome (despite numerous TATA boxes in the middle of the genome).

Initially it was assumed that parvovirus B19, like other members of the *Parvoviridae* would have a second internal promoter for transcription of the capsid proteins. Putative TATA boxes are also present in the middle of the genome and it was suggested that the TATA boxes at p44 and p55 (nucleotide 2247 and nucleotide 2308) may be functional. One group (Doerig *et al.*, 1990) was able to detect promoter activity by cloning fragments containing p44 B19 DNA upstream of the CAT gene, transfecting the DNA into HeLa cells, and measuring CAT expression. The strength of the p44 promoter was similar to that of the capsid gene promoter, p39, of MVM. In contrast to the p6 promoter there was no transactivation of promoter activity by expression of NS protein in (non-permissive) HeLa cells, and the authors speculated that this might determine tissue tropism and the selective replication of B19 in erythroid progenitors. However, similar studies using the same construct with the more sensitive luciferase gene were unable to detect any transcriptional activity in either HeLa cells or the semipermissive UT7/Epo cell line (Liu *et al.*, 1991a). In addition, no putative transcripts coming from a mid-genome promoter have been identified in transcription maps from permissive or non-permissive cells.

Initial studies all confirmed that p6 was a strong promoter whose activity was not restricted to permissive cells (Blundell *et al.*, 1987; Doerig *et al.*, 1987, 1990; Liu *et al.*, 1991a; Gareus *et al.*, 1998). A number of investigators therefore looked for evidence of enhancer elements in the upstream regions of the promoter. It is now recognized that the upstream region is very rich in sequences that bind transcription factors, and mapping of the region has confirmed that regions between nucleotide 100 and 190, and nucleotide 233 and 298 (of B19-Au) are particularly important for promoter activity (Gareus *et al.*, 1998), and that these regions contain binding sites for SP1 (Blundell and Astell, 1989; Raab *et al.*, 2001), YY1 (Liu *et al.*, 1991b; Momoeda *et al.*, 1994a), GA binding protein to the Ets motif (Vassias *et al.*, 1998) and Oct-1 proteins (Raab *et al.*, 2001), all of which have been shown to bind to the B19 promoter sequence (Figure 3.4). The p6 promoter has been shown to have increased activity in K562 cells, which, although not permissive for B19 infection, have many erythroid transcription factors, and motifs for the erythroid transcription factors **GATA** and **MZF-1** are found in the p6 promoter. Interestingly, although there are differences in the promoter regions of the three different B19 genotypes, most of the transcription motifs appear to be conserved (Figure 3.4).

Several studies have shown that the B19 NS upregulates p6 promoter activity (Doerig *et al.*, 1990; Carter and Flotte, 1996), and it has been shown that nucleotides 100–160 are essential for this transactivation (Gareus *et al.*, 1998). The NS protein appears to bind directly to the DNA in this region as oligonucleotides containing nucleotides 126–162 (region D) could be immunoprecipitated with anti-NS antibody (Raab *et al.*, 2002).

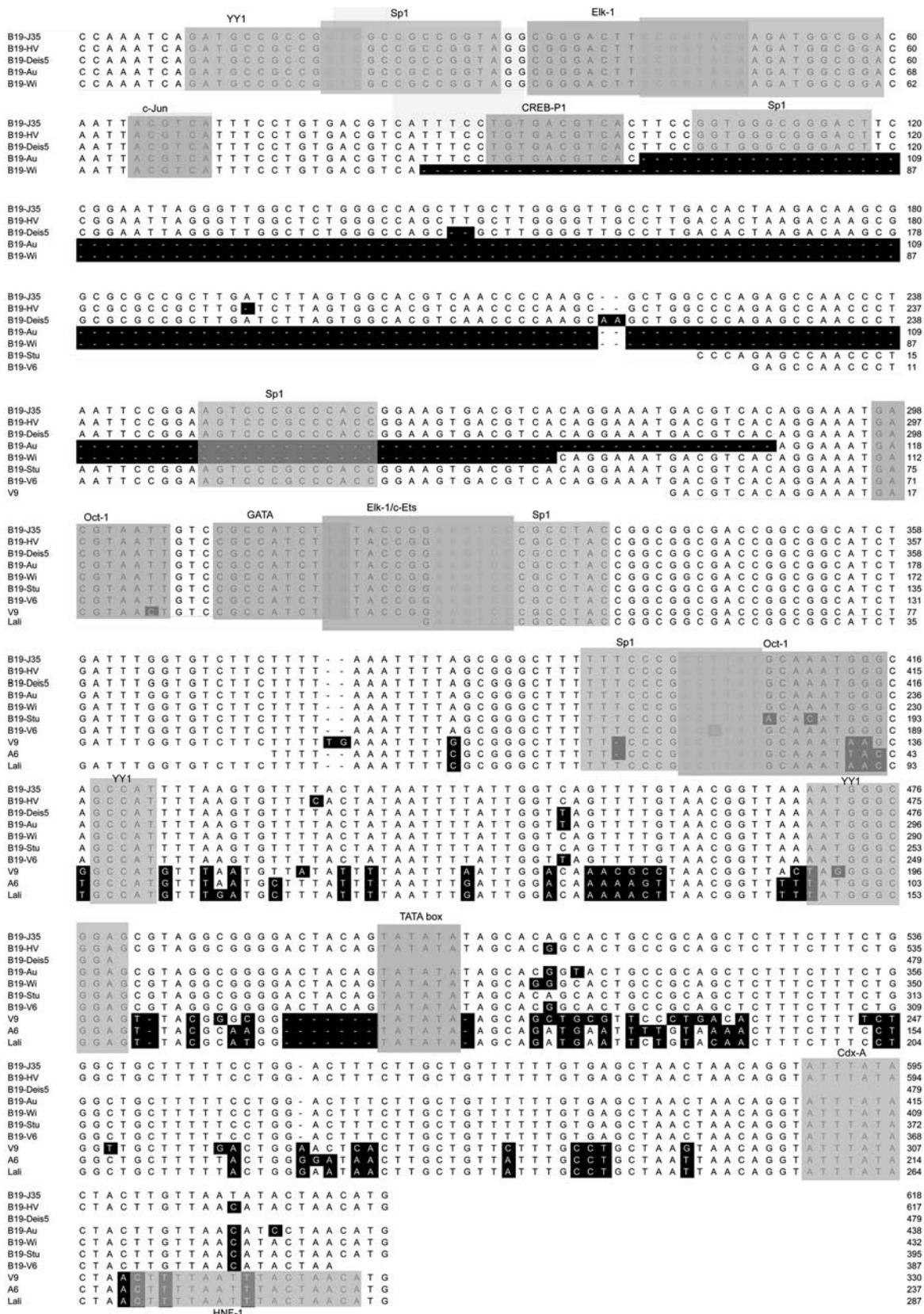
## RNA

### TRANSCRIPTION MAP

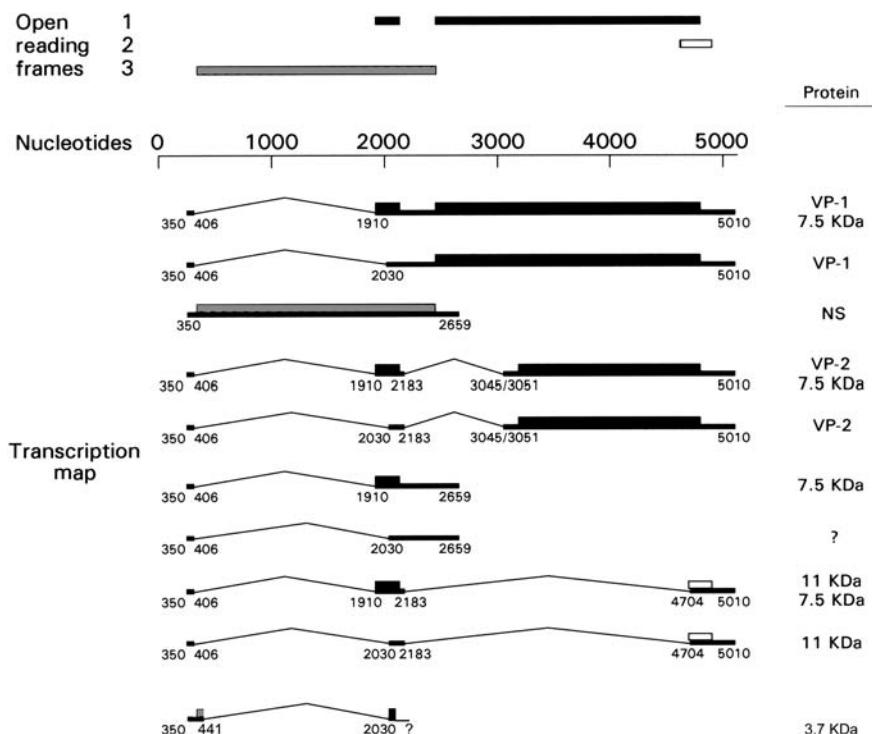
The transcription map of parvovirus B19 was initially determined by analysis of RNA from infected erythroid bone marrow cells and found to be remarkably different from those of other animal parvoviruses and adeno-associated viruses (Ozawa *et al.*, 1987a). There are at least nine overlapping polyadenylated transcripts, all but one (encoding the NS protein) containing introns (Figure 3.5). In contrast to other *Parvoviridae*, no separate, internal promoter for the capsid protein genes could be detected, but all transcription initiated from the single p6 promoter, with the capsid transcripts, encoded in the right side of the genome, driven through short leader sequences with subsequent splicing of the large introns. In addition, whereas other parvoviruses terminate all RNA transcription at a common polyadenylation site at the far right side of the genome, several B19 transcripts terminate in the middle of the genome and appear to use an unusual polyadenylation signal. In addition, short 700–800 nucleotides transcripts from the middle of the genome, not present in other parvovirus maps, were particularly abundant. These RNAs are derived from the left side of the genome and share an open reading frame with the NS gene. In addition a second group of abundant RNAs, the short 500–600 nucleotides transcripts are present.

The B19 transcription map was also analyzed following transient expression in COS-7 cells transfected with B19-SV40 hybrid vectors (Beard *et al.*, 1989). These hybrid vectors contain the SV40 origin of replication, allowing them to replicate to high copy number in COS cells. Transient expression from these hybrid vectors resulted in RNA species and polypeptides that were indistinguishable from those seen in B19-infected erythroid marrow cells. Interestingly the hybrid B19-SV40 vectors replicated at a low level (copy number about 1000/transfected cell) unless a frameshift mutation was introduced into the NS gene.

B19 cDNA libraries were constructed from B19-transfected COS-7 cells and B19-infected human erythroleukemic cells and used to confirm the splice junctions and the translational potential of the small polyadenylated RNAs (St Amand *et al.*, 1991). The 700–800 nucleotides RNA species were not represented in the COS cell library, suggesting that the variant polyadenylation signal (ATTAAA



**Figure 3.4** B19 sequence upstream of the TATA box with putative binding site for transcription factors indicated (shaded boxes). The B19-Au and B19-Wi sequences have deletions in the promoter region, shown as a black box. Nucleotides differing from the consensus sequences are on a black background. There is an MZF1 motif in all the viral promoters, but in different positions for the different genotypes (open box). Motifs were identified using >Match v1.0 at [www.gene-regulation.com](http://www.gene-regulation.com)



**Figure 3.5** Transcription map for parvovirus B19. Upper panel indicates open reading frames. Lower panel, RNA transcripts identified in infected cells. The bottom incomplete transcript has only been detected by RT-PCR, and it is not been verified whether it codes for a functional splice protein, or whether this splice junction is present in the capsid and 11 kDa transcripts.

or AATAAC) in the middle of the genome is not recognized well in COS cells. In contrast, in the library generated from B19-infected human erythroleukemic cells, the 700–800 nucleotides species were highly represented. The 500–600 nucleotides transcripts were shown to direct synthesis of an 11 kD protein, which could be detected in transfected COS cells (Guillot *et al.*, 1987) and in infected bone marrow cells (St Amand and Astell, 1993). Subsequently the 700–800 nucleotides species were shown to encode a 7.5 kDa protein (Luo and Astell, 1993).

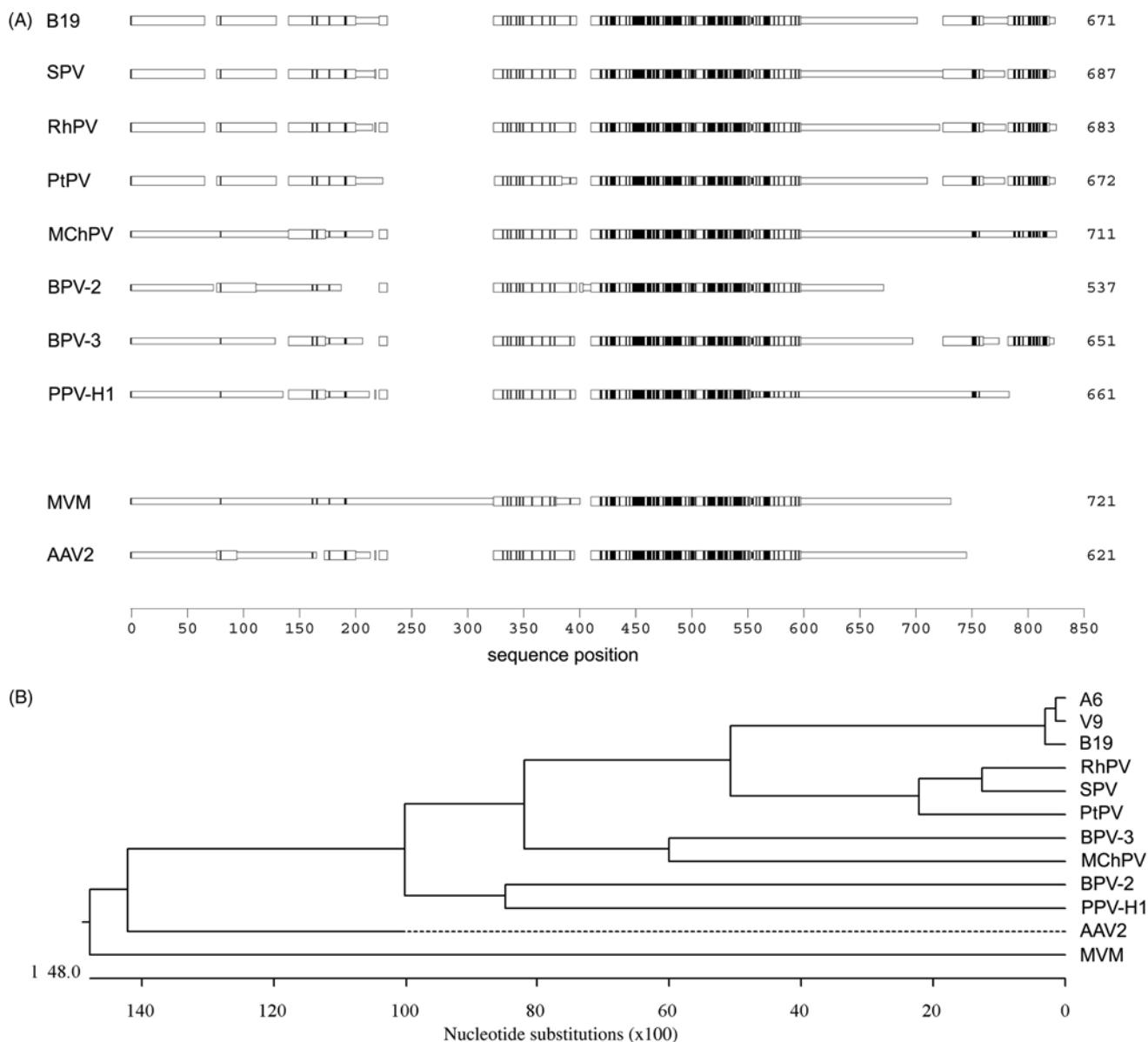
More recently, a reverse transcription-polymerase chain reaction (RT-PCR) based study suggested that in the semi-permissive cell line for B19 replication, MB-02, a second splice donor site at nucleotide 441, rather than the site at nucleotide 406 observed in erythroid progenitor cells and COS cells, is used (Brunstein *et al.*, 2000). This transcript was only detected by RT-PCR, and it was not determined if this alternative splice junction is seen in all the capsid and 11 kDa transcripts. The change of donor site would allow for a truncated protein (Figure 3.6), and it was suggested that this alternative splice donor or fusion protein may have a role in the restricted replication seen in these cells.

The transcription map for SPV has also been determined (Liu *et al.*, 2004, Vashisht *et al.*, 2004), and although SPV shows some similarities to B19, there are also marked differences. As noted above there is a single promoter at the 5' end of the genome, both spliced and un-spliced RNA transcripts, and polyadenylation signals in the middle of

the genome and the 3' end. However, SPV RNA shows three introns, compared with the two seen in B19, with the additional intron, in the capsid region of the genome. Transcripts with this third intron encode a 14 kDa protein analogous to the 11 kDa protein in B19.

## REGULATION OF RNA PROCESSING

Tissue culture analyses (see below) have demonstrated that there is a temporal order in the expression of the NS and capsid proteins in infected cells, and that this seems to be controlled at the level of the viral transcripts. In addition, differential transcription of NS and capsid transcripts has been demonstrated in non-permissive and permissive cells: in one study using RNase protection assays in permissive erythroid bone marrow cells the steady-state level of right-side structural-gene transcripts predominated over that of left-side NS transcripts; in non-permissive HeLa cells transfected with the B19 viral genome, NS-gene transcripts predominated. Similarly, in a subclone of UT7 cells found to be non-permissive for B19 infection, the NS transcripts predominated over the capsid transcripts (Leruez *et al.*, 1994). This differential transcription appears to be controlled by unique post initiation events (Liu *et al.*, 1992). Removal of 3' processing signals located in the middle of the viral genome increases transcription of the far right-side transcripts, and disruption of a polyadenylation signal in this region enables read through of the full-length right-side



**Figure 3.6** Comparison of the amino acid sequences of the non-structural proteins of different members of the Erythrovirus family. (A) Alignment performed by MACAW (Multiple Alignment Construction and Analysis Workbench, NCBI, NIH) v2.0.5 with BLOSUM 62 scoring matrix. Shading indicates the relative mean score for aligned residues; boxes indicate blocks of locally high homology; narrow boxes indicate areas lacking significant homology; gaps in the sequence lineups are not boxed. (B) ClustalW analysis using a gap penalty of 10, a gap length penalty of 0.2 and the Gonnet 250 protein weight matrix. MVM and AAV2 Rep proteins are included as representative of the Parvovirus and Dependovirus families respectively.

transcripts. These results suggest that the abundance of B19 RNAs is determined by active 3' processing and is coupled to DNA template replication.

## Erythrovirus proteins

### NON-STRUCTURAL PROTEIN (NS)

In contrast to the other parvoviruses, parvovirus B19 encodes a single large non-structural protein NS, of 671aa, with a predicted molecular weight of 74 kDa. However, in several

studies of either infection or transfection of B19 DNA into mammalian cells, investigators have detected up to three protein bands on Western blot or immunoprecipitation with MW of ~71 (77), ~68 (63), ~55 (52), and/or 34 kDa (Cotmore *et al.*, 1986; Ozawa and Young 1987; Beard *et al.*, 1989; Astell *et al.*, 1997; Leruez-Ville *et al.*, 1997). These proteins all appear to arise from the single non-spliced transcript, and it is unclear as to whether the different size proteins represent post-translational cleavage and/or modification, internal initiation, or non-specific degradation. Antibody mapping of the different proteins indicates that

the smallest protein (34 kDa) does not contain aa 655–671 (Astell *et al.*, 1997), arguing against this protein, at least, being a product of internal initiation. It is also unknown whether these different sized proteins have a functional role in replication.

Antibody studies have demonstrated that in infected erythroid cells, NS is present only in the nuclear fraction of infected cells, and is absent from cytoplasm and culture supernatants as well as from intact virions in sera (Ozawa and Young, 1987). It was suggested that this localization could be due to the possible nuclear transport signals present in the NS sequence at aa 177–180 (KKPR) and aa 316–321 (KKCGKK). These motifs are not present in the non-human primate erythroviruses.

### SEQUENCE SIMILARITIES IN THE ERYTHROVIRIDAE

For SPV, translation of the first open reading frame would produce a predicted NS protein of 687 aa, with a predicted molecular weight of 77 kDa, and with highest amino acid homology (50 percent) to B19 NS protein. Similar sized proteins are predicted for PmPV (672 aa, MW 75.5 kDa), RmPV (683 aa, MW 76.7 kDa), chipmunk parvovirus (711aa, MW 78.6 kDa), BPV-2 (537aa, MW 62.6 kDa), BPV-3 (651aa, MW 72.8 kDa) and porcine H1 virus (662aa, MW 74.8 kDa; Table 3.1, p. 30). Comparison of their putative amino acid sequences indicates that the proteins are distinctly different from the NS proteins of the other *Parvoviridae* (Figure 3.6), but with the greatest similarity in the mid third of the genome (see below).

*Function of NS protein.* Like other members of the *Parvoviridae* the NS of B19 has a central region of ~160 aa with high homology to Papovaviridae proteins (Astell *et al.*, 1987). This region contains an ATPase and nucleotide-binding motif (Astell *et al.*, 1987). Crucial to its role in viral replication, it is assumed that B19 NS has nickase and helicase activities for resolution of the hairpin structures, and that it enables encapsidation of the DNA into capsids, but these properties have not been formally shown. Similarly, although B19 NS is phosphorylated (Kajigaya, unpublished observations), the phosphorylation sites have not been determined.

Apart from viral replication, NS also affects other cellular functions. Transfection of NS into HeLa cells led to marked cytotoxicity and blocked cellular proliferation (Ozawa *et al.*, 1988a). The cytotoxicity was blocked if a frame shift mutation was introduced into the NS protein. Similar results were obtained using an AAV vector containing the B19 NS sequence (Srivastava *et al.*, 1990). This marked toxicity has limited studies of the function of NS and, combined with the inability to readily culture B19 *in vitro*, most studies have been performed using either transient transfection or NS under an inducible promoter.

Mutation analysis confirmed that the A domain of the NTP binding domain was critical for this cytotoxicity (Moffatt *et al.*, 1998; Momoeda *et al.*, 1994b). This cytotoxicity

is mediated by induction of apoptosis through activation of the caspase cascade, and could be blocked by caspase 3, 6 and 8 inhibitors or over expression of Bcl-2 (Moffatt *et al.*, 1998; Sol *et al.*, 1999). Fas–FasL interaction does not appear to be involved in this apoptosis, but cells expressing NS were sensitized to TNF- $\alpha$ -induced apoptosis (Sol *et al.*, 1999).

NS transactivates its own promoter, p6 (see above), but can also transactivate a number of other promoters, including proteins under the control of the HIV-1 LTR (Frickhofen and Young, 1989), the IL-6 promoter through interaction with the NF- $\kappa$ B binding site (Moffatt *et al.*, 1996), and TNF- $\alpha$  in U937 cells through activation of AP1 and AP2 (Fu *et al.*, 2002). Increased levels of NF- $\kappa$ B have been reported in B19-infected nuclei (Sol *et al.*, 1999), and may have a central role in promoter upregulation. Interestingly, mutations in the NTP-binding site critical for the induction of apoptosis, had no effect on the upregulation of IL-6. Conversely pentoxyfylline, an inhibitor of NF- $\kappa$ B, blocked IL-6 upregulation, but had no effect on apoptosis (Moffatt *et al.*, 1998).

The role of NS in cell cycle control is less clear. B19 infected cells clearly stop proliferating, and arrest at G<sub>2</sub> with the accumulation of mitotic cyclins (Morita *et al.*, 2001). Transfection of NS has been shown to block cell proliferation (Ozawa *et al.*, 1988a), and it has also been shown that expression of NS in erythroid cells leads to cell accumulation at the G<sub>2</sub> phase of the cell cycle (Sol *et al.*, 1999). However, more recent studies using mitotic inhibitors, have suggested that although B19 virus infection leads to G<sub>2</sub> arrest, NS expression primarily induces arrest at G<sub>1</sub> with only a smaller fraction arresting at G<sub>2</sub> (Morita *et al.*, 2003). Following NS expression there was a corresponding increase in p21/WAF1, a known negative regulator for G<sub>1</sub>-S transition, in both UT7/Epo cells and in cells in which p53 was inactive, indicating that this induction of p21/WAF1 is not through p53 signaling.

### B19 CAPSID PROTEINS

As with the other *Parvoviridae*, the capsid proteins of the erythroviruses are encoded by the large ORF on the right side of the genome. B19 and the non-human primate viruses have two structural proteins VP1 and VP2 produced by alternative splicing; the number of capsid proteins in the other viruses is not known. In B19, the major structural protein, VP2, is a 58 kDa protein. The minor capsid protein, VP1, is an 84 kDa protein, which is identical to VP2 with the addition of 226 more amino acids (known as the VP1-unique sequence) at the amino-terminus. In infected erythroid cells, the ratio of VP2 to VP1 produced was 24:1, with VP1 representing only 4 percent of the capsid protein (Ozawa and Young, 1987). Immunoblotting and immunofluorescence studies have shown that capsid proteins are localized to the cytoplasm and nuclei of infected cells in culture. A non-conventional nuclear localization signal

(KLGPRKATGRW) has been mapped to the carboxyl sequence of VP2 (Pillet *et al.*, 2003). This sequence is relatively conserved in all the erythroviruses.

The capsid proteins have been expressed in a wide range of different systems, including bacterial (Sisk and Berman 1987; Morinet *et al.*, 1989; Rayment *et al.*, 1990), mammalian (Beard *et al.*, 1989; Kajigaya *et al.*, 1989) and insect cells (Brown *et al.*, 1991a; Kajigaya *et al.*, 1991). In mammalian and insect cells the capsid proteins can self assemble in the absence of viral DNA and these proteins have been used as a source of antigen for diagnostic assays and for functional analysis (see below).

The role of the VP1 unique sequence is not known. It is the main site of neutralizing antibodies (Saikawa *et al.*, 1993; Uemura *et al.*, 1995), and recombinant capsids that contain increased amounts of VP1 have increased immunogenicity (Bansal *et al.*, 1993). The VP1 unique region is, at least in part, exposed on the virion surface (Rosenfeld *et al.*, 1992). Recently it has been shown that the erythroviruses, like the other members of the *Parvoviridae* have a phospholipase motif in the VP1 unique region (Zadori *et al.*, 2001). This phospholipase motif is functional in B19 peptides and VP1-containing recombinant capsids (Dorsch *et al.*, 2002), but its role in B19 replication, infectivity or pathogenicity is not known.

## SPV CAPSID PROTEINS

SPV particles appear to be morphologically similar to other parvoviruses by electron microscopy, appearing as icosahedral virions of 18–26 nm diameter. Their buoyant density has not been estimated. Antigenically, there is some cross-reactivity with parvovirus B19, and SPV particles can be immunoprecipitated with human anti-B19 sera. SPV particles have also been analyzed by SDS-PAGE and Western blotting (O'Sullivan *et al.*, 1994). Two bands were observed to react with rabbit polyclonal anti-B19 capsid serum, corresponding to the SPV capsid proteins. The smaller capsid protein had a molecular weight of approximately 60 kDa and the larger capsid protein a molecular weight of 90 kDa (compared with 58 kDa and 84 kDa for B19), with similar ratios of VP1 and VP2 as in B19. These protein sizes are in keeping with the predicted size of the non-spliced capsid protein of 818 aa, and by analogy to B19 an identical possible splice acceptor site for a second spliced capsid protein (VP2) 81 nucleotides downstream, with predicted VP2 size of 556 aa. Similar sized capsid proteins are predicted from the sequences of PmPV and RmPV (Green *et al.*, 2000) (Table 3.1, p. 30).

Capsid proteins have been expressed in insect cells for the B19 variant V9 (Heegaard *et al.*, 2002) and SPV (Mills *et al.*, 2002). As with B19, the expressed V9 and SPV VP2 proteins self-assembled to form parvovirus capsids, of identical appearance to native particles on electron microscopy. Although no reactivity on Western blotting has been observed with B19 monoclonal antibodies, in ELISA assays

several B19 antibodies bind to SPV capsids (unpublished data), suggesting that the viruses share conformational epitopes.

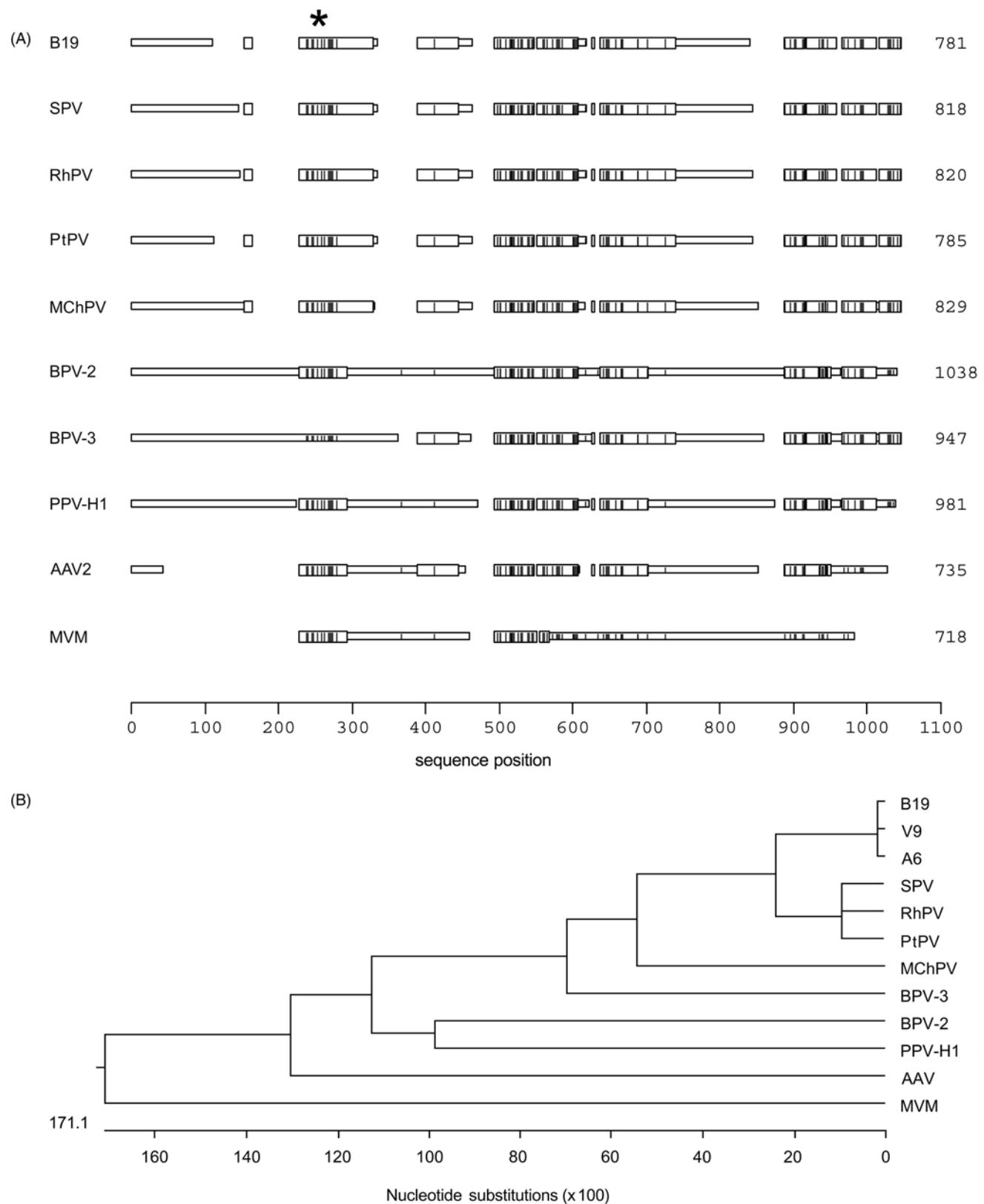
Similar expression studies have not been performed for the other erythroviruses. However, as with the NS proteins, the amino acid sequences cluster separately from the other Parvoviridae, with the primate erythroviruses showing the greatest similarity (Figure 3.7). All the viruses, apart from BPV-3, have the conserved phospholipase motif in the predicted region of the VP1 unique region (Figure 3.7).

## TRANSLATIONAL REGULATION

In infected erythroid progenitor cells B19 parvovirus capsid proteins are produced in strikingly different quantities (VP2 > 96 percent and VP1 < 4 percent). When an *in vitro* translation system was used with RNA transcripts produced by T7 RNA polymerase, translation of VP1 RNA was very inefficient compared with VP2 RNA, indicating that capsid production was regulated at the level of translation (Ozawa *et al.*, 1988b). Immediately upstream from the VP1 translation initiation site is an unusual sequence containing multiple AUG triplets, and during RNA processing this sequence is spliced out of VP2 RNA. Removal of these upstream AUG sequences from VP1 RNA greatly improved the efficiency of translation (Ozawa *et al.*, 1988b), suggesting that the upstream AUG-rich region acts as a negative regulatory element in the translational control of B19 capsid protein production. Similar multiple ATG sequences are also present upstream of the capsid ORF in the simian erythroviruses, but not in the chipmunk, bovine, or porcine viral genomes.

## CAPSID STRUCTURE

The B19 virion has an icosahedral structure consisting of 60 copies of the capsid proteins. Most of the capsid protein is VP2, with only ~5 percent of the larger VP1 protein (Kajigaya *et al.*, 1989). As noted above, capsid proteins can be expressed in a variety of both mammalian and insect cells, and have been shown to self assemble in the absence of B19 DNA (recombinant empty capsids). VP1 is not required for capsid formation (Brown *et al.*, 1991a; Kajigaya *et al.*, 1991), and VP1 alone will not produce an icosahedral capsid structure (Wong *et al.*, 1994). The atomic structure of B19 VP2 empty capsids has been resolved to 8 Å at present (Agbandje *et al.*, 1991), with many of the observations confirmed by cryo-electron microscopy (cryo-EM) studies (Chipman *et al.*, 1996). Although some features are similar to the other parvoviruses (Tsao *et al.*, 1991; Agbandje *et al.*, 1993; McKenna *et al.*, 1999) and AAV structures (Kaludov *et al.*, 2003; Xie *et al.*, 2003), which have now been resolved, there are also clear differences. In B19, although the central structural motif of eight GKB>-pleated sheets that is found in many other icosahedral viruses is maintained, the surface features differ greatly, with depressions at the 2-fold and 3-fold axes and canyon-like regions around the 5-fold



**Figure 3.7** Comparison of the amino acid sequences of the capsid proteins of different members of the Erythrovirus family. (A) by MACAW and (B) ClustalW analysis. Settings are as in Figure 3.6. MVM and AAV2 Rep proteins are included as representative of the Parvovirus and Dependovirus families respectively.

	*	
B19	-----	-MKS LKN CG QPKAV CTHCKHSPPCPQPG 27
J35	-----	-MQNNT T DMDM KSL KN CG QPKAV CTHCKHSPPCPQPG 36
A6	-----	-MQTNT T NTDM KSL KN CG CLPKAV CTHCKHSPPCPQPG 36
V9	-----	-MYCMT PQLQM QMSNT T DTD M KSL KN CG CLPKAV CTHCKHSPPCPQPG 45
SPV	vPENIQEGCTPNQHAYPPTLQDIYPTYCTIPRSPKTPRTIDTGTRSRKNAGVPKSEYTLFKHYVCPLQNP	70
Pt PV	vLENTLADGIHNQQYTLPMQRDTCPMSSMTLSQKPKHTDTGMKNRRSCGQQRKEYTPFKYYMCPLTSP	70
RhPV	aLEN TREGGT PNP HVHLPTLQD TY PTY CTT QRL PKTPRTIDTGMRNQRNA G ALRNES TFFKHYVCPLGNP	70
 B19	 CVTKRPPVPPRLYLPPPVEIRQPNKTDIDNVEFKYLTRYEQHIVRMLRLCNMYQNLEK-----	85
J35	CVTKRPPVPPRLYLPPPPIPIRQPNKTDIDNVEFKYLTRYEQHIVRMLRLCNMYQNLEK-----	94
A6	TVTNRPPVPPRLYVPPPIPRREPLVKDTNAVEYKLLTRYEQHIVRMLRLCNMYTNLEK-----	94
V9	TVTHRPPVPPRLYVPPPIPRRQPSIKDTDAVEYKLLTRYEQHIVRMLRLCNMYTSLEK-----	103
SPV	PPSM-----YGALSPQE KHLIQCFLCSAYPNLHGLvkk	104
Pt PV	PPNM-----YGMLTPGEQHHLIQCFLCAKYLEINNV---	101
RhPV	PPSM-----YGALSPQE KHLIQCFLCSAYPDLHGLlk	104

**Figure 3.8** Alignment of the amino acid sequences of the small 11 kDa protein encoded by B19 and the primate Erythroviruses using MACAW (Multiple Alignment Construction and Analysis Workbench) v2.0.5 with BLOSUM 62 scoring matrix. \*The B19-Au sequence starts at the in-frame ATG after the splice junction. For B19-J35, V9 and A6 the sequence begins at the first ATG in the small open reading frame. For SPV, RmPV and PmPV there is no ATG start codon in the ORF and the amino-acid sequence for the whole ORF is given.

axis. The receptor globoside (see below) appears to bind to depressions on the 3-fold axis. The structural distribution of VP1 in the B19 capsid structure is not known.

## SMALL PROTEINS

Parvovirus B19 contains three small open reading frames (ORFs) in the genome with coding potential. The largest of these small ORFs is on the far right side of the genome, and is conserved in the non-human primate erythroviruses (Figure 3.3, p. 31). In B19 there is an in-frame ATG in this ORF in the small 500–600 nucleotides transcripts (Ozawa *et al.*, 1987a). When antibodies to these putative proteins were tested, a family of three 11 kDa proteins were detected in human cells and in COS cells transfected with the B19 genome (St Amand *et al.*, 1991). Although these localized in the cytoplasm of transfected COS cells, in infected erythroid cells, the 11 kDa protein was detected in both nucleus and cytoplasm (St Amand and Astell, 1993). The protein contains three proline-rich regions, and binds *in vitro* to growth factor receptor-binding protein 2 (Grb2) (Fan *et al.*, 2001). However, its role in infection is unknown. SPV, RmPV and PmPV have a similar ORF in this region (Figure 3.8), although there is no in-frame ATG start codon. Recently it was shown that, for SPV, the protein is generated from a spliced transcript, and has a putative size of 14 kDa (Liu *et al.*, 2004).

The second small ORF in B19 that has an in-frame ATG is found in the 700–800 nucleotides transcripts. In B19 it appears to be functional, and produces a protein product of about 7.5 kDa in infected erythroid cells (Luo and Astell 1993). In contrast to the 11 kDa protein, although the

7.5 kDa protein was detected in both the nucleus and cytoplasm of transfected cells, it was found only in the cytoplasm of infected erythroid cells. The function of this protein is unknown. In SPV and RmPV there is a similar, but larger ORF, but the sequence has limited similarity to the B19 sequence. There is no similar ORF in PmPV.

The third potential minor ORF is in the middle of the genome, in the region of the VP1-unique region. It has not been shown to produce a functional protein, the putative protein X, but the predicted amino acid sequence appears to be conserved in the human erythroviruses.

## CELLULAR BIOLOGY

### Virus receptor

Parvovirus B19 capsids hemagglutinate primate red cells (Brown and Cohen, 1992), and it was this assay that was used to determine the nature of the B19 receptor on the host cell (Brown *et al.*, 1993). In addition capsids containing VP2 alone agglutinated cells as efficiently as capsids containing VP1, suggesting that the epitopes for binding were part of the major capsid protein. Purified globoside, also known as blood group P antigen, a tetrahexose ceramide, inhibited hemagglutination, and erythrocytes lacking globoside or P antigen on their surface could not be agglutinated. The biological relevance of this binding was shown by competing infection with globoside or monoclonal antibody to P antigen, or using cells that do not express P antigen. In tissue culture, using the CFU-E assay (Mortimer *et al.*, 1983),

globoside blocked the B19-induced cytotoxicity of erythroid progenitors. In addition, preincubation of target cells with globoside-specific monoclonal antibody completely blocked infection. There was no protection with similar antibodies to other blood group P antigens. Finally, bone marrow from individuals that genetically lack P antigen on their bone marrow cells, was unable to be infected with parvovirus B19, even at high doses (Brown *et al.*, 1994b), and in serological studies it appeared that individuals with this blood group p phenotype were naturally resistant to B19 infection.

A direct interaction of globoside with the B19 virion was demonstrated by cryo-EM studies (Chipman *et al.*, 1996). Globoside bound to depressions on the 3-fold axes of the virion, in the same region as two neutralizing monoclonal antibodies known to block B19 hemagglutination (Caillet-Fauquet *et al.*, 1990; Chipman *et al.*, 1996).

The tissue distribution of P antigen is consistent with the known tropism of B19. The antigen is found on erythroblasts and megakaryoblasts (von dem Borne *et al.*, 1986). It is also found on endothelial cells, which may be viral targets involved in the pathogenesis of transplacental transmission and the rash of fifth disease, on fetal myocardial cells, in the kidney (Rouger *et al.*, 1987), in the placenta (Jordan and DeLoia 1999), on myeloblasts (Cooling *et al.*, 2003) and on some B cells (Wiels *et al.*, 1991).

Other studies have confirmed that P antigen is required for B19 infectivity (Weigel-Kelley *et al.*, 2001). However, the P antigen receptor may not be the only mediator of permissivity. Initial experiments suggest that pig erythroid cells (which also bear globoside on their surface) and a B cell line expressing globoside on the surface cannot be infected with B19 (unpublished observations). For another autonomous parvovirus, MVM (minute virus of mice), target cell specificity is due to intracellular factors (Spalholz and Tattersall, 1983), and a similar mechanism has been proposed for B19 (Liu *et al.*, 1992), as discussed above. In addition, it was recently proposed that  $\alpha 5\beta 1$  integrins may facilitate viral entry in some cells (Weigel-Kelley *et al.*, 2003), although it was not shown whether they are involved in viral entry into permissive cells.

Following the expression of VP2 capsids of the B19 variant, V9, and SPV in insect cells, particles were also tested for their ability to bind to globoside. Both capsids bind to globoside in a similar manner to B19 capsids (unpublished observations; Mills *et al.*, 2002), suggesting that both these viruses also use globoside as the receptor for viral infection.

## *In vitro* infection

The discovery that infection with B19 in sickle cell patients was associated with an arrest of erythropoiesis suggested that the target cell for B19 was an erythroid precursor. This was confirmed by *in vitro* studies: B19 virus inhibited colony formation of late erythroid progenitors (CFU-E) and the more primitive burst forming erythroid progenitors

(BFU-E), but had minimal effect on myelopoiesis (Mortimer *et al.*, 1983). Southern analysis of suspension cultures of human mononuclear cells infected *in vitro* showed that the virus replicated in the erythroid and not the leukocyte fraction of bone marrow (Ozawa *et al.*, 1986; Ozawa *et al.*, 1987b). Parvovirus B19 could be demonstrated, by *in situ* hybridization for DNA or by immunofluorescence for viral capsids, in infected cell nuclei.

Erythroid progenitor cells from a number of different sources have been shown to support B19 replication. Examples of this include human bone marrow (Ozawa *et al.*, 1986; Srivastava and Lu, 1988), fetal liver (Yaegashi *et al.*, 1989; Brown *et al.*, 1991b), erythroid cells from a patient with erythroleukemia (Takahashi *et al.*, 1989), and peripheral blood (Serke *et al.*, 1991). All systems show an absolute requirement for erythropoietin, probably to maintain rapidly dividing erythroid cells. With cells taken from bone marrow it was shown that the susceptibility of erythroid progenitors to parvovirus B19 increased with differentiation. The pluripotent stem cell appeared to be spared and the main target cells were CFU-E and erythroblasts (Takahashi *et al.*, 1990). Immunophenotyping of susceptible cells from fetal liver showed that these cells were also of pronormoblast phenotype, expressing CD36 and glycophorin A (Morey and Fleming, 1992).

In erythroid progenitors, infection with B19 parvovirus causes a cytopathic effect, with characteristic light (Ozawa *et al.*, 1987b) and electron microscopic (Young *et al.*, 1984; Brown *et al.*, 1991b) changes. Infected cultures are characterized by the presence of giant pronormoblasts or 'lantern cells' (Van Horn *et al.*, 1986). These are early erythroid cells, 25–32  $\mu\text{m}$  in diameter, with cytoplasmic vacuolization, immature chromatin and large eosinophilic nuclear inclusion bodies. By electron microscopy the cells show marginated chromatin, pseudopod formation and virus particles in the nucleus and lining cytoplasmic membranes. Immunostaining studies showed that these cells express Ki-67 antigen and p53 in the nucleus (Sadahira *et al.*, 2001).

## Cell lines for B19 culture

All these culture systems are primary culture explants only, and not suitable for long-term culture. Unsuccessful attempts have been made to culture B19 in erythroleukemic cell lines K562, HEL, KMOE-2, KG1, KG1a, probably because the cells need to be at a more differentiated stage to support B19 replication. B19 can be propagated in a few megakaryoblastoid or myelod cell lines adapted to grow in erythropoietin, UT-7/Epo (Shimomura *et al.*, 1992), MB-02 (Munshi *et al.*, 1993), KU812Ep6 (Miyagawa *et al.*, 1999) and in a human erythroleukemia cell line, JK-1. However in all these cell lines the level of B19 expression is low, and none are suitable as a practical source of virus. Even in infected erythroid cells, although the genome copy number in infected bone marrow cells was approximately 30 000/cell,

at low multiplicity of infection (moi) there was only a 200-fold increase in B19 DNA (Ozawa *et al.*, 1987b). However, they do allow further investigation of the molecular pathogenesis of the virus, and infectivity assays to look for infectious virus (Miyagawa *et al.*, 1999; Blumel *et al.*, 2002b; Yunoki *et al.*, 2003), or neutralizing antibodies have been established (Bostic *et al.*, 1999; Saito *et al.*, 2003).

Parvoviruses have a propensity to produce high ratios of defective particles (Tattersall and Cotmore, 1988) and these infectivity assays have allowed calculation of the ratio of infectious to non-infectious particles for parvovirus B19. In our infection assay using UT7/Epo-S1 cells, we can detect infectious virus with inoculation of  $<10^4$  genome copies (Wong and Brown, 2003). A similar figure of  $\sim 10^3$  genome copies has been obtained with KU812Ep6 cells (Miyagawa *et al.*, 1999). These numbers are similar to those obtained for autonomous parvoviruses (1:520 for porcine parvoviruses (Choi *et al.*, 1987) and between 1:500 and 1:1000 for parvovirus H-1 (Paradiso 1981).

## Cellular aspects of SPV

Like parvovirus B19, SPV appears to be highly tropic for erythroid progenitor cells, as demonstrated by *in vitro* infection of cynomolgus monkey marrow (Brown *et al.*, 2004). In colony-inhibition assays, parvovirus SPV is erythrotropic and inhibits simian erythroid colony formation, without inhibition of myeloid colonies, and SPV DNA replication can be detected by increased total SPV DNA with time and demonstration of replicative DNA by Southern hybridization. SPV infection can be confirmed by *in situ* hybridization of infected cells, and detection of SPV capsid proteins within cells by immunofluorescence. Interestingly, in similar studies, B19 has also been studied for its ability to infect cynomolgus marrow (Gallinella *et al.*, 1995), and DNA and capsid proteins of B19 and SPV showed similar localization within infected cells.

## Life cycle and time course studies

The B19 life cycle must include binding to the cell surface and viral entry, translocation of the genome to the nucleus and uncoating, DNA replication, RNA transcription, empty capsid assembly, insertion of DNA into the capsid, virion maturation, cell lysis, and release of virions. Most of the various stages of the life cycle have not been studied for B19 or other erythroviruses. DNA replication and RNA transcription patterns have been studied in synchronized cells from the human megakaryocytoblastoid line, UT-7/Epo (Shimomura *et al.*, 1993). In freshly inoculated UT-7/Epo cells, RNA transcription appeared as an early event following infection, with viral RNA detected about 6 hours after infection. In contrast, dimer replicative intermediate forms of parvovirus DNA did not appear until more than

16 hours after infection. In UT-7/Epo cells synchronized by hydroxyurea treatment, northern analysis of specific transcripts showed an earlier appearance of NS protein RNA (6 hours), compared with capsid protein RNA (24 hours). Addition of an inhibitor of protein synthesis to block synthesis of NS protein abolished capsid protein RNA transcription as well as DNA replication. The study concluded that RNA transcription preceded detectable DNA replication.

## Infectious clone

Many studies into the biology of parvovirus B19 or the related erythroviruses have been limited by difficulty in culturing the virus, and by the absence of an infectious molecular clone. Recently, in collaboration with Peter Tijssen's group in Canada we have cloned the entire B19 genome, including the intact terminal hairpin structures, into a bacterial plasmid, and can show that transfection of permissive cells leads to the production of infectious B19 virus (Zhi *et al.*, 2004). This plasmid is stable and can be manipulated *in vitro* to introduce mutations in different parts of the B19 genome. Such studies are in progress, and should enable us to determine the functional roles of the different transcripts and proteins in B19 replication.

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# Autonomous parvovirus variation and evolution

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This review examines the variation and evolution of the autonomous parvoviruses including various rodent parvoviruses, the parvoviruses of carnivores including canine parvovirus (CPV), feline panleukopenia virus (FPV), and Aleutian mink disease virus (AMDV), and the erythroviruses (human B19 virus and related viruses of humans and other primates). As is reviewed in detail elsewhere in this text, the parvoviruses are genetically simple and have between one and three transcriptional promoters depending on the particular virus. Through a variety of strategies those give rise to messages for between one and four non-structural proteins, and between two and four capsid proteins.

## EPIDEMIOLOGY AND ANTIVIRAL IMMUNITY

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Understanding the epidemiology of the parvoviruses is necessary for understanding the variation and evolution of the virus over historical time periods. Many autonomous parvoviruses, including CPV, FPV, porcine parvovirus (PPV), and B19 human parvovirus, normally cause acute infections of their host animals, which last for <10 days, by which time the virus is cleared by the host immunity, infectious virus cannot be readily demonstrated, and the hosts are no longer infectious for other animals (Parrish, 1995). However, prolonged replication and persistence is a feature of infection by some other parvoviruses, including rodent parvovirus infections where prolonged viral persistence in the kidneys and shedding in the urine can be demonstrated, and in AMDV-infected mink the virus persists and continues to replicate in a number of tissues for the life of the animal (Bloom *et al.*, 1994). For B19 in humans a small number of chronic and persistent infections occur in

individuals who are immunosuppressed, or who for other reasons do not develop effective immunity (Frickhofen and Young, 1989; Brown *et al.*, 1994; Young, 1995). The epidemiology of the bovine parvoviruses is less well understood, but some viruses are recovered from fetal bovine sera, suggesting that they may also cause longer term infections (Allander *et al.*, 2001).

The mechanisms of transmission among the parvoviruses vary. For the carnivore parvoviruses related to CPV the virus uses fecal–oral transmission, replicating in the intestine and shedding in the feces (Macartney *et al.*, 1984a; Macartney *et al.*, 1984b; Parrish, 1995). Some of the rodent parvoviruses also replicate in the intestine, and they may also be transmitted through urine after replication in the kidney (Jacoby *et al.*, 1996; Ball-Goodrich *et al.*, 1998). Although the human B19 virus replicates primarily in the bone marrow, it is thought to be transmitted by respiratory routes (Brown and Young, 1995). There is undoubtedly an important role for cell-mediated immunity in recovery from infection, but for most of the mammalian viruses humoral immunity, including maternal antibody or passively administered IgG, protects animals against infection. Antibodies also appear to be important in recovery from infection, as antibody treatments can arrest CPV replication in dogs, and can terminate chronic human infections by the B19 parvovirus (Brown *et al.*, 1994; Brown and Young, 1995).

However, in the case of AMDV, the antibodies produced in most virus-infected mink reduce viral titers, but in many animals that antibody does not efficiently neutralize the virus and a persistent chronic infection is established (Alexandersen *et al.*, 1994; Bloom *et al.*, 1994). The level of virus replication and intracellular gene expression is reduced by the presence of circulating antibodies, although the

mechanism by which that occurs is not understood (Alexandersen, 1986; Alexandersen *et al.*, 1994).

The antigenic structure of the capsid has been examined for several parvoviruses, and there appear to be significant differences between the various viruses. Conformation-dependent neutralizing epitopes are important targets of neutralizing antibodies on the exposed surface of CPV, minute virus of mice (MVM) and B19 capsids (Yoshimoto *et al.*, 1991; Saikawa *et al.*, 1993; Strassheim *et al.*, 1994; Lopez-Bueno *et al.*, 2003). Linear epitopes have also been defined on the capsid proteins, including the N-terminus of VP2, which is exposed to the exterior of CPV full (DNA-containing) capsids, and the N-terminus of VP1 for the B19 parvoviruses (Saikawa *et al.*, 1993; Langeveld *et al.*, 1994a,b; Musiani *et al.*, 2000; Zuffi *et al.*, 2001). There appear to be differences in the topologies of the capsid proteins of the different parvoviruses since, although the VP1 unique sequence is inside the CPV and MVM capsids, that sequence is exposed on the outside of virus-like particles assembled after co-expression of VP1 and VP2 of B19 in insect cells, and anti-VP1 antibodies neutralize infectious B19 (Weichert *et al.*, 1998; Cotmore *et al.*, 1999; Musiani *et al.*, 2000; Dorsch *et al.*, 2001).

The role of antigenic variation and immune selection in the epidemiology of the parvoviruses is not well understood, and the analysis is complicated by the fact that, where it has been examined for CPV, many of the changes that result in antigenic variation also alter host range or other properties of the virus (Chang *et al.*, 1992; Parker and Parrish, 1997). Although genetically widely separated viruses can be distinguished by neutralization or hemagglutination inhibition with polyclonal sera, within a particular virus group less antigenic variation is detected even among viruses that have been separated by decades. Variation of capsid epitopes of natural CPV or mink enteritis virus (MEV) isolates has been detected using monoclonal antibodies, but the epidemiological significance of those antigenic differences is not well understood (Parrish *et al.*, 1991; Strassheim *et al.*, 1994; Martella *et al.*, 2004; Nakamura *et al.*, 2004). MVM antigenic variants were readily selected in tissue culture after selection with neutralizing monoclonal antibodies, but it is not known whether these arise readily in nature (Lopez-Bueno *et al.*, 2003). Widely separated viruses (such as the different strains of human erythroviruses) are antigenically distinct, but those viruses are quite genetically distinct – as described below.

For many parvoviruses, neutralizing antibodies from homologous or slightly variant strains are protective, and most animals that have recovered from infection resist reinfection by antigenically related viruses, even if those are variant at one or more epitope. However, it is also likely that in many natural transmission cycles the immune selection on the virus is controlled primarily by the antibody found in the animals that acquire maternal immunity from their mother. In those animals the low levels of waning maternal antibody that are present when the animals become infected may allow selection for variation

in the virus compared with the antigenic type that infected the mother.

## GENETIC VARIATION AND REPLICATION ERROR RATE

The level of variation of the parvoviruses can be high when measured over defined periods of replication when the virus is subject to selection. This has been seen in studies of the emergence of CPV (Badgett *et al.*, 2002; Shackelton *et al.*, 2005), in the growth of CPV through serial passage in tissue culture (Badgett *et al.*, 2002), and in the analysis of MVM during replication under various selective conditions such as alternative hosts (Lopez-Bueno *et al.*, 2004) or monoclonal antibody selection (Lopez-Bueno *et al.*, 2003). However, it is not known how much variation occurs during the replication of the parvovirus genome. Although the DNA is replicated using host cell DNA polymerases, the fidelity of the viral replication may be lower than is seen for the host cell replication with the full polymerase and associated replication complex. This may particularly be seen when filling in or replicating the ssDNA forms, leading to significant levels of DNA sequence variation, and allowing more variation and potential evolution of these viruses.

## Genetic relationships and variation among the members of the family parvovirus

A comparison of the sequences of conserved regions of the genome, in particular of the NS1 gene, shows that all of the parvoviruses have certain sequences in common, and they are most likely related through a distant common ancestor. However, the viruses are readily subdivided into several distinct clades that show some correlations to the hosts of origin (Lukashov and Goudsmit, 2001), although there are many variants. As more viruses are collected and analyzed, a variety of more or less related viruses have been seen to infect many species. For example, within the bovines there are three parvoviruses described as bovine parvoviruses, types 1, 2, and 3 (Allander *et al.*, 2001), and there are two distantly related parvoviruses infecting dogs (Schwartz *et al.*, 2002). The several erythroviruses from primates (B19 and related human and simian parvoviruses) were most closely related to each other and to the chipmunk parvovirus. Most viruses from rats, mice, and hamsters were found to be within the same clade with CPV and the related viruses of carnivores and PPV, while AMDV was found to be quite distantly related to all the other vertebrate viruses. The LuIII virus proved to be a recombinant between two different rodent viruses, most likely between MPV and hamster PV (Lukashov and Goudsmit, 2001), although whether that recombination arose in nature or during passage of the virus in tissue culture is not clear. The times of divergence

of the various viruses are not known as no molecular clock of the parvovirus sequences can be estimated. Whether the viruses have co-evolved along with their hosts or have crossed between different hosts in more recent times is also not known, although both origins are possible.

## Recent evolution and temporal variation

More variation of several parvoviruses has been studied in detail. Sequence variation of the B19 viruses in humans has been examined in several studies, where viruses from different regions of the world or from chronic and acute infections have been compared. CPV and the closely related viruses of other carnivores appear to cause exclusively acute infections and those have also been examined closely, particularly for the period since CPV emerged in dogs in 1978. AMDV in mink and some other carnivores causes mostly chronic and persistent infections and the variation in sequences has been examined in a small number of studies.

## Human B19 and related erythroviruses

There appear to be a number of human erythroviruses that are related to B19, including the V9-like viruses and the Lali-related viruses that are between 5 and 20 percent different at the DNA sequence level (Hokynar *et al.*, 2002; Servant *et al.*, 2002; Gallinella *et al.*, 2003; Candotti *et al.*, 2004). When the B19-related viruses are examined, there is between 1 and 4 percent sequence variation found between isolates within each clade. There appears to be global spread of the viruses, as there is often close similarity both between isolates collected from various regions of the world, and those collected at various times over the past two decades, although viruses collected from one geographic area are generally more similar to each other. There is a tendency for viruses from patients with persistent infections to have a higher variation compared with viruses from patients with acute infections.

Seven isolates collected in Italy between 1989 and 1994 from one geographic area have been compared, where there was no obvious connection between the isolates, and these showed a maximum variation of 0.61 percent in the 2400–3400 nucleotides sequence within the amino terminal end of the VP genes (Gallinella *et al.*, 1995). Those viruses were 0.7 percent and 0.77 percent different on average from the prototype Wi and Au sequences of viruses collected in the UK and USA, respectively. Of the 22 nucleotide sequence differences detected, nine resulted in amino acid sequences differences, and of those seven were between residues 4 and 114 in VP1 sequence, while two were within the VP1/VP2 common region (Gallinella *et al.*, 1995).

Fifty B19 isolates collected throughout the world were examined by the single-stranded conformational polymorphism (SSCP) assay with sequences from a 283 nucleotides region of the non-structural gene (1399–1682),

and that showed five different SSCP types among the isolates (Kerr *et al.*, 1995). All the variation was due to single or double silent nucleotide sequence differences within the region analyzed. There was found to be variation in the distribution of the different types both geographically and temporally, as strains designated type 3 predominated in Japan and the UK, while type 4 predominated in the USA. In Japan a number of SSCP types were detected among the strains collected between 1981 and 1987, while viruses collected between 1990 and 1994 were mostly type 3 (Kerr *et al.*, 1995).

The sequences of the complete VP1 and VP2 gene region (2343 nucleotide) of 29 isolates from 25 infected patients in various regions of the world were compared with each other, and with the two published Wi and Au sequences. Those viruses included 10 from an outbreak in Ohio, USA, one of which was a mother-child pair, and other isolates from throughout the USA, UK, Brazil, Ireland, Venezuela, Korea, Japan, and China (Erdman *et al.*, 1996). The sequences differed by as few as two nucleotides and up to as many as 99 (4.2 percent), and by between 0 and 13 (1.7 percent) amino acid sequence differences. No insertions or deletions were observed. Variation of nucleotides was found throughout the VP1 and VP2 genes, but the amino acid sequences clustered into three regions of the gene – in the VP1-unique region, around the junction of the VP1 and VP2 coding regions, and within the VP1 and VP2 overlapping region. In that study isolates from the outbreak in Ohio could be divided into two classes, represented by seven and two samples each (Erdman *et al.*, 1996). Within each group the sequences differed by only a few nucleotides and, where multiple isolates were collected from four individuals, only a single difference was found in the sequences from each person. Again, it appeared that the virus strains had a worldwide distribution, as genetically closely related viruses were also obtained from various regions of the world. However, there was some geographic clustering of the strains – viruses from China formed a distinct clade, while those from the USA were generally clustered, as were the isolates from Korea.

In other hospital outbreaks viral variants were collected that showed some variation in the genotype of the viruses, although it is unclear whether those were due to different virus introduction and co-circulation or to variation in the viruses during their replication and spread (Takahashi *et al.*, 1999; Miyamoto *et al.*, 2000).

Viruses recovered from chronic and acute infections were examined in several studies, and in most cases no specific correlation was seen between any particular disease syndrome and any virus type (Morinet *et al.*, 1986; Mori *et al.*, 1987; Gallinella *et al.*, 1995; Kerr *et al.*, 1995; Takahashi *et al.*, 1999). In one study a 2-fold higher genomic variability was seen in the NS1, VP1 and VP2 genomic regions of B19 sequences recovered from cases of persistent infection (arthritis or other chronic diseases) compared with those from acute infections. A distinct genome type was detected by restriction enzyme analysis in a case of B19-induced

encephalopathy, although any relationship of that variation to the disease seen has not been established (Umene and Nunoue, 1995).

## CPV and related viruses

DNA sequences of viruses from dogs, cats, raccoons, mink, and arctic foxes have been examined in a number of studies, with comparisons of the variation of the NS1 and VP2 genes (Truyen *et al.*, 1995; Horiuchi *et al.*, 1998; Ikeda *et al.*, 2002; Shackelton *et al.*, 2005). The studies show that the canine isolates all formed a single clade, and were therefore derived from a single common ancestor, which most likely arose during the late 1960s or early 1970s (Truyen *et al.*, 1995; Shackelton *et al.*, 2005). The sequences of viruses isolated from cats, mink, raccoons, or foxes could not be clearly distinguished from each other, suggesting that interspecies transmission of those viruses is common.

Although early studies using restriction enzyme analysis of CPV and related virus sequences suggested a relationship between CPV and vaccine strains of FPV (Tratschin *et al.*, 1982), more detailed analysis of the virus DNA sequences indicated that the CPV isolates were not more closely related to those vaccine strains, and in fact may have been more closely related to a virus of red foxes (Truyen *et al.*, 1998).

During the emergence of CPV the viruses showed rapid variation of the sequences, particularly during the emergence of the CPV lineage (Shackelton *et al.*, 2005). In the 26 years since CPV emerged there has been continuing accumulation of sequence changes compared with the ancestral CPV sequence, with a rate of change of around  $10^{-5}$ /site per year. Other studies have shown that many of the capsid protein gene differences between CPV and the FPV-like viruses are associated with phenotypic properties of the virus – including changes in host range, antigenicity, and sialic acid binding (Chang *et al.*, 1992; Horiuchi *et al.*, 1994; Hueffer *et al.*, 2003). The CPV lineage split into two major variants during the mid-1970s, giving rise to the CPV type-2 strains that were the first to emerge in 1978 and circulate worldwide, and the CPV type-2a variant, which replaced the CPV type-2 viruses during 1979 and 1980. For the FPV-related viruses the temporal rate of variation calculated was only slightly slower than that seen for CPV (Horiuchi *et al.*, 1998; Shackelton *et al.*, 2005).

The NS1 sequences showed similar overall phylogenetic relationships to those of VP2, although there were less variation compared with the capsid protein gene of the same viruses, and there was a lower proportion of non-synonymous sequence differences (Shackelton *et al.*, 2005).

## Antigenic variation of CPV-like viruses

Monoclonal antibody (MAb) analysis showed that the virus capsids from dogs all differed from those from cats, mink,

or racoons in at least two specific neutralizing epitopes – one present only on CPV, and the other present on FPV isolates (Parrish and Carmichael, 1983; Mochizuki *et al.*, 1989; Parrish *et al.*, 1991; Strassheim *et al.*, 1994). CPV isolates collected during 1978 and early 1979 were all antigenically identical worldwide, but antigenically variant strains were seen as the CPV type-2a strain that differed in the VP1/VP2 gene by four to five amino acids compared with CPV type-2 isolates, but which differed from CPV type-2 in having lost two epitopes and gained two different neutralizing epitopes on the capsid (Strassheim *et al.*, 1994). Other antigenic variants emerged around 1984, and during the later 1990s and early 2000s, and each of those contained a single amino acid sequence differences within neutralizing epitopes in the capsid (Ikeda *et al.*, 2000; Buonavoglia *et al.*, 2001; Ikeda *et al.*, 2002; Nakamura *et al.*, 2004). Despite the small numbers of differences between those viruses, they each became globally distributed within a year or two of first being detected. Natural antigenic variation of FPV and MEV isolates has also been described (Parrish and Carmichael, 1983; Parrish *et al.*, 1984; Mochizuki *et al.*, 1989).

## Host range

As reviewed in detail elsewhere, the various FPV and CPV isolates differ in their abilities to infect cells in culture and animals. The evolution of the viruses also involved changes in their natural host ranges, since the CPV type-2 isolates did not replicate in cats, but later viruses (CPV type-2a and its derivatives) replicated efficiently in cats after experimental inoculation (Truyen *et al.*, 1996). That this was a natural host range for the viruses was confirmed by the finding that CPV type-2a and related viruses were also isolated from between 10 and 20 percent of cats who had natural parvovirus disease in Asia, Germany, and the USA (Mochizuki *et al.*, 1993; Truyen *et al.*, 1996; Ikeda *et al.*, 2000).

## ALEUTIAN DISEASE VIRUS

A number of studies have shown that there is significant variation in the genomes of AMDV isolates, most likely due to the cocirculation and coinfection with variant virus strains. The genetic variability of the viruses from a single farm or region was seen to be up to 16 percent at the nucleotide sequence level (Olofsson *et al.*, 1999). AMDV isolates normally grow only in animals, but certain strains have been adapted to grow in feline cells in tissue culture. The genomic sequences showed a 2.5 percent sequence difference between a wild type pathogenic strain of AMDV and a tissue culture-adapted virus, and there was a hyper-variable sequence within the capsid protein gene at a position that is most likely on the surface of the VP2 protein structure (Bloom *et al.*, 1988; McKenna *et al.*, 1999; Oie

*et al.*, 1996). AMDV has also been recovered from ferrets, skunks, and raccoons. The racoon and skunk viruses appear similar to viruses isolated from farmed mink, although it is not known whether there is natural transmission between those hosts (Oie *et al.*, 1996).

The spectrum of AMDV sequences within the non-structural and structural protein genes of the genome was examined for experimentally infected Danish mink. Many different sequences were found in mink inoculated with a single inoculum. The sequences differed by up to 5 percent and they also differed markedly in the highly variable region (Gottschalck *et al.*, 1991). Since AMDV can establish long-term persistent infections with continuously circulating virus, it is likely that mixed infections occur, and that the experimental inocula that were prepared by mink infection can contain more than one virus strain.

Comparing virus non-structural gene sequences (nt 123–2208) from different AMDV isolates or virus stocks prepared from infected mink showed that there was extensive variation between the viruses in the different preparations. Some of those viruses had been originally prepared from pools of tissues from infected mink, and most had been repeatedly passaged through mink to prepare the working stocks (Gottschalck *et al.*, 1994). Variation of up to 11.4 percent was seen between the nucleotide sequences, and up to 16 percent between the amino acid sequences, and that variation was found distributed throughout the sequences compared.

## RODENT PARVOVIRUSES

Although the rodent parvoviruses MVM, LuIII and H1 have been intensively studied for their genetic and biochemical properties, information about the variation and evolution of the rodent viruses in nature is still being obtained. Many of the earliest viral isolates studied appear to be rodent viruses that were isolated from tissue cultures or transplantable tumors, and in some cases their true origin and the degree of tissue culture or host adaptation are not fully understood. Those viruses include the minute virus of mice prototype strain (MVMp) isolated from a murine adenovirus stock, H1 virus isolated from the HEP-1 human tumor transplanted into rats and most likely a rat virus, LuIII isolated from human cells, and a tissue culture isolate of MVM named MVM-Cutter (MVM-[c]). A number of viruses have been recently detected in mice (mouse parvovirus [MPV]), hamsters (hamster parvovirus [HaPV]), and rats (rat parvovirus [RPV]), which appear to be widespread in rodent colonies (Ball-Goodrich and Johnson, 1994; Besselsen *et al.*, 1996; Jacoby *et al.*, 1996; Ball-Goodrich *et al.*, 1998; Wan *et al.*, 2002). Many of those viruses are difficult to grow in tissue culture. The known and suspected rodent parvoviruses are >70 percent identical in DNA sequence, while viruses that are considered to be of the same type and given

the same name are generally >95 percent identical. Apart from the relationships between the viruses at the sequence level, little is known about the details of the evolutionary history or any host range differences between these viruses, nor about their temporal variation.

As reviewed elsewhere in this volume, the host range or tissue tropisms of some of the rodent viruses are controlled through changes in the capsid protein gene, and in the structures of the capsids. Many of those changes are likely to be due to passage in different tissue cultures, or to their different hosts of origin.

## CONCLUSIONS

Our understanding of the evolution of the parvoviruses is still being developed, but there are some interesting conclusions that can be drawn. The viruses are relatively stable in nature, but there may be less than 1 percent up to 6 percent sequence variation between related viruses from the same hosts. However, as more viruses are discovered, in many hosts these are showing variant forms that represent distinct lineages that have been co-circulating for many years. Higher rates of variation can be seen among many parvoviruses when put under selection or subjected to extended passage in alternative hosts or host cells, suggesting that their replication fidelity may be significantly lower than that of the host genomes.

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# Evolution of densoviruses

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Densoviruses (DNVs) are thought to be widely distributed among arthropods, from insects to crabs and shrimp, although only relatively few have been isolated thus far from the million-plus arthropod species (for reviews see Tijssen and Bergoin, 1995; Fédière, 2000; Bergoin and Tijssen, 2000). Most of the insect densovirus currently recognized were isolated from Lepidoptera (butterflies and moths) and from a few other orders, including Diptera (mosquitoes), Orthoptera (crickets, grasshoppers, etc.), Dictyoptera (mantids, cockroaches), and Odonata (dragonflies), but not from, for example, Coleoptera (beetles). Some hosts, such as the mosquito *Aedes albopictus* (or its C6/36 cell line), have yielded several isolates whereas other well-investigated insects, such as *Drosophila*, have yet to yield a single parvovirus. DNVs have also been isolated from Crustacea, notably from shrimp and crabs.

At first sight the distribution of densoviruses within the arthropods seems odd. Although the phylogenetic relationships of arthropods are currently much debated, evidence is emerging that some groups, such as Collembola, branched from the evolutionary tree much earlier than most insects and crustaceans and adapted to life on land independently (Nardi *et al.*, 2003). This view is supported by comparison of mitochondrial genomes (Boore *et al.*, 1995; Boore, 1999; Garcia-Machado *et al.*, 1999; Nardi *et al.*, 2003), molecular embryology markers such as the Hox genes (Averof and Akam, 1995; Akam, 2000; Cook *et al.*, 2001), nuclear ribosomal gene sequences (Friedrich and Tautz, 1995) and conserved neural characteristics (Strausfeld, 1998). Insecta (Hexapoda) are thus closely related to the Crustacea (shrimps, crabs), and much less closely related to the

Myriapoda (e.g. millipedes, centipedes) and the Chelicerata (e.g. spiders, mites ticks, scorpions, horseshoe crabs).

Not surprisingly, the isolation and characterization of DNVs from a rapidly increasing diversity of hosts within the arthropod (super)phylum and the early radiation of the arthropod orders during evolution, with whom these viruses have co-evolved, complicates their classification. Criteria that are suitable for the classification of vertebrate parvoviruses thus cannot be used for the densoviruses, since this would require many genera, often with a single representative.

## DEFINITION AND CLASSIFICATION

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Originally all viruses of arthropods that contained a single copy of a linear single-stranded DNA genome were defined as densoviruses (DNVs) and classified into the Densovirinae subfamily. However, all currently recognized DNVs package complementary linear single-strands of their 4–6 kb DNA genome into separate virions, as do several parvoviruses of vertebrates (e.g. B19, AAV), and replicate autonomously by self-priming and hairpin transfer mechanisms. This definition thus excludes some viruses that were initially, tentatively, included in the group, such as *BmDNV-2*, which has a bipartite single-stranded DNA genome with a total length of about 12.5 kb but lacks terminal hairpins. *BmDNV-2* is further differentiated from the Densovirinae by the fact that each of its DNA segments encode a structural protein of about 120 kDa, one of which carries a DNA polymerase motif involved in protein-primed

replication (Hayakawa *et al.*, 2000). Together with the Parvovirinae subfamily of vertebrates, with which they share little sequence identity, the Densovirinae make up the family of *Parvoviridae* (Berns *et al.*, 2000). Molecular characterization of the densoviruses allowed a further classification of the subfamily into at least three genera: the densoviruses, iteraviruses and brevidensoviruses. This classification was based on a limited number of densoviruses

and became strained as the number of characterized viruses increased. In the upcoming ICTV reclassification (Tattersall *et al.*, 2005) a single new genus, the *Pefudenviruses*, will be introduced, as discussed later, but the taxon will probably need to undergo continuous revision as our knowledge expands. Currently many densoviruses remain unclassified since only half of the ~30 reported DNV isolates have been sufficiently characterized.

### Box 5.1 Isolates and classification of densoviruses

#### Ambisense densoviruses

Subgroup A densoviruses (genus *Densovirus*, 6 kb genome with 0.55 kb ITRs, single ORF for VP)

<i>JcDNV</i>	<i>Junonia coenia</i>	Lepidoptera	NC_004284
<i>GmDNV</i>	<i>Galleria mellonella</i>	Lepidoptera	NC_004286
<i>DsDNV</i>	<i>Diatraea saccharalis</i>	Lepidoptera	NC_001899
<i>HaDNV</i>	<i>Helicoverpa armigera</i>	Lepidoptera	(sequenced, not submitted)
<i>MlDNV</i>	<i>Mythimna loreyi</i>	Lepidoptera	NC_005341
<i>PiDNV</i>	<i>Pseudoplusia includens</i>	Lepidoptera	(sequenced, not submitted)

Subgroup B densoviruses (5.5 kb genome with 0.2 kb ITRs, split ORFs for VP; *Pf* DNV will be classified in a new genus, *Pefudenvirus*, while others remain to be classified)

<i>AdDNV</i>	<i>Acheta domesticus</i>	Orthoptera	(sequenced, not submitted)
<i>BgDNV</i>	<i>Blattella germanica</i>	Orthoptera	NC_005041
<i>MpDNV</i>	<i>Myzus persicae</i>	Hemiptera	NC_005040
<i>PcDNV</i>	<i>Planococcus citri</i>	Hemiptera	NC_004289
<i>PfDNV</i>	<i>Periplaneta fuliginosa</i>	Dictyoptera	AB028936

Subgroup C densoviruses (6 kb genome with 0.3 kb ITRs, split ORFs for VP and NS)

<i>CpDNV</i>	<i>Culex pipiens</i>	Diptera	(sequenced, not submitted)
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#### Monosense densoviruses

Genus *Iteravirus* (5 kb genome, 0.25-kb ITRs)

<i>CeDNV</i>	<i>Caspalia extranea</i>	Lepidoptera	AF375296
<i>BmDNV-1</i>	<i>Bombyx mori</i>	Lepidoptera	AY033435
<i>DpDNV</i>	<i>Dendrolimus punctatus</i>	Lepidoptera	NC_006555

Genus *Brevidensovirus* (4 kb genome, no ITRs but terminal hairpins, no PLA<sub>2</sub> motif in VP)

<i>AaeDNV</i>	<i>Aedes aegypti</i>	Diptera	M37899
<i>AalDNV-1</i>	<i>Aedes albopictus</i> (cell line)	Diptera	NC_004285
<i>AalDNV-2</i>	<i>Aedes albopictus</i> (cell line)	Diptera	AY095351
<i>PmoDNV</i>	<i>Penaeus monodon</i>	Decapoda	AF499102
<i>PstDNV</i>	<i>Penaeus stylostris</i>	Decapoda	AF273215

#### Non-characterized densoviruses

<i>AvDNV</i>	<i>Agraulis vanillae</i>	Lepidoptera	Kelly <i>et al.</i> , 1980
<i>EaDNV</i>	<i>Euxoa auxilliari</i>	Lepidoptera	Sutter 1973
<i>LdiDNV</i>	<i>Lymantria dispar</i> (cell line)	Lepidoptera	Grignon 1982
<i>PrDNV</i>	<i>Pieris rapae</i>	Lepidoptera	Sun <i>et al.</i> , 1981
<i>SfDNV</i>	<i>Sibine fusca</i>	Lepidoptera	Meynardier <i>et al.</i> , 1977
<i>HeDNV</i>	<i>Haemagogus equinus</i> (cells)	Diptera	O'Neill <i>et al.</i> , 1995
<i>TaDNV</i>	<i>Toxorhynchites amboinensis</i> (cells)	Diptera	O'Neill <i>et al.</i> , 1995
<i>SvDNV</i>	<i>Simulium vittatum</i>	Diptera	Federici 1976
<i>LduDNV</i>	<i>Leucorrhinia dubia</i>	Odonata	Charpentier 1979

Of the three distinct DNV genera that were recognized in the last ICTV report, the densoviruses are exemplified by the *Jc*DNV (*Junonia coenia* DNV; Jousset *et al.*, 1990; Dumas *et al.*, 1992) and *Gm*DNV (*Galleria mellonella* DNV; Tijssen *et al.*, 2003), the brevidensoviruses by the *Aedes* (Afanasiev *et al.*, 1991; Boublík *et al.*, 1994) and shrimp (Shike *et al.*, 2000) viruses, and the iteraviruses by the *Casphalia extranea* DNV (Fédière *et al.*, 2002) and *Bombyx mori* densovirus type 1 (Li *et al.*, 2001). Viruses in the *Densovirus* genus have an ambisense genome organization, with the VP (structural proteins) and NS (non-structural or Rep proteins) on the 5' halves of the complementary strands, and with inverted terminal repeats (ITRs). Members of the *Iteravirus* genus have a monosense genome organization, also with ITRs (hence the name of the genus), and have so far only been found in the order Lepidoptera. The brevidensoviruses have a short genome (hence their name) with a monosense organization and terminal hairpins, but lack ITRs. The members of this group are found in *Diptera*, particularly in mosquitoes, and at least some densoviruses from shrimps are close relatives. Initially all ambisense densoviruses isolated from different insect orders were tentatively grouped into the *Densovirus* genus, which made this genus quite heterogeneous. Here, we divide this genus into three more uniform subgroups (A, B and C, see Box 5.1). Members of subgroup A make up a reasonably coherent group, and include all those viruses currently recognized as belonging to the genus *Densovirus*. Although subgroup B members share a common genome organization, that is distinct from subgroup A, they have very low sequence identities (10–30 percent), either with each other or with members of the subgroup A viruses. One of them, *Pf*DNV, will be transferred into the new *Pefudensovirus* genus in the next ICTV report (Tattersall *et al.*, 2005), but not enough is known about their expression strategies at the molecular level for their co- or separate classification.

## BIOPHYSICAL AND STRUCTURAL CHARACTERISTICS OF VIRIONS

A virus (*Gm*DNV) from the greater waxmoth, *Galleria mellonella*, was the first DNV to be isolated (Meynardier *et al.*, 1964). Its particles yield four clearly distinct polypeptide bands upon electrophoresis through polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE), with molecular masses that range from about 90 kDa (VP1) to 45 kDa (VP4) and vary in concentration, with VP1 being the least, and VP4 the most, abundant species (Tijssen *et al.*, 1976). These structural proteins have a strong identity upon peptide mapping (Tijssen and Kurstak, 1981), and were shown to be N-extended isoforms (Tijssen *et al.*, 2003). Restriction mapping confirmed a close relationship between the 6 kb genomes of *Gm*DNV and *Jc*DNV (Jousset *et al.*, 1990), and the sequence of *Jc*DNV demonstrated that these viruses carried a single gene encoding the structural proteins

(Dumas *et al.*, 1992). These viruses are the prototype strains for the genus *Densovirus*.

Shortly after the isolation of *Gm*DNV, *Bm*DNV-1 was discovered in sericultural farms in Ina City, Japan (Kawase, 1985). Although the virions resembled those of *Gm*DNV, the protein composition was different (Nakagaki and Kawase, 1980; Li *et al.*, 2001). Both *Bm*DNV-1 and the related *Ce*DNV particles contain five structural proteins, which are N-extended isoforms, with molecular masses of 76–48 kDa (VP1, 2, 3, 3' and 4; Li *et al.*, 2001; Fédière *et al.*, 2002). So far, these are the only three densoviruses classified in the *Iteravirus* genus. Capsids of both the iteraviruses and densoviruses possess phospholipase A<sub>2</sub> activity (Zádori *et al.*, 2001; Li *et al.*, 2001; Fédière *et al.*, 2002; Tijssen *et al.*, 2003).

In contrast, the viruses of the third genus, the brevidensoviruses, do not display phospholipase A<sub>2</sub> activity and have only two structural proteins (N-extended isoforms) of about 40 kDa. These viruses were all isolated from mosquitoes and shrimps.

The virions of the various DNVs resemble those of the vertebrate parvoviruses in that they have a buoyant density in CsCl of ~1.40–1.44 g/cm<sup>3</sup>, owing to their high nucleic acid content (~20–30 percent), and sedimentation coefficients of about 110 S. *Gm*DNV, *Jc*DNV, and *Bm*DNV-1 particles were shown to contain polyamines (spermine, spermidine, and putrescine) that could partially neutralize the viral DNA (Kelly and Elliott, 1977; Bando *et al.*, 1983), whereas so far no reports of polyamines in vertebrate parvoviruses have been published.

The near-atomic structure of *Gm*DNV was solved by X-ray crystallography (Simpson *et al.*, 1998). Some striking differences with the structure of vertebrate parvoviruses were observed. The  $\beta$ -barrel of the capsid proteins is highly conserved among parvovirus structures but, when rotational symmetry axes are superimposed, the *Gm*DNV capsid protein  $\beta$ -barrel must be rotated by 7.4° and translated radially inwards by 9.7 Å to superimpose it on the  $\beta$ -barrel of the capsid protein of canine parvovirus (CPV). Another difference with the vertebrate parvoviruses is the absence of loop 4 in *Gm*DNV capsid protein, resulting in a  $\beta$ -annulus type structure (channel) instead of a spike around the 3-fold axis. The  $\beta$ A strand folds back to its own subunit in vertebrate parvovirus structures thus far solved. In contrast, in *Gm*DNV, the  $\beta$ A strand is linearly extended from the subunit across the 2-fold axis allowing it to hydrogen bond with the  $\beta$ B strand of the neighboring subunit ('domain swapping'). Interestingly, cricket paralysis picornavirus displays similar domain swapping when compared to vertebrate picornaviruses (Rossmann and Tao, 1999). Although this may be a coincidence, it may also indicate a different assembly pathway in insect versus vertebrate cells. Overall, the outside of the *Gm*DNV capsid is much smoother than that of vertebrate parvoviruses (Simpson *et al.*, 1998), perhaps as a result of a different evolutionary pressure.

The 3D-structure of *Aal*DNV-2, from the *Brevi*densovirus genus, has been solved to a resolution of about 16 Å by

cryo-electron microscopy (Chen *et al.*, 2004). The structural proteins of this virus show less than 20 percent sequence identity with those of *GmDNV*, which is translated into some distinctive structural features. For example, *AalDNV-2* particles display density ridges around the 3-fold axis and prominent protrusions at the 5-fold axis, which are both absent in the *GmDNV* particle. Nevertheless, the 2-fold proximal depression as well as the inner surfaces and large cavities under the 5-fold vertices are conserved. Similarly, inner surfaces were most conserved among vertebrate parvoviruses (Simpson *et al.*, 2002).

Finally, recently collected diffraction data should allow the atomic structure of *BmDNV-1* to be solved in the near future (a collaboration between the laboratories of Michael Rossmann and Peter Tijssen).

## GENOME ORGANIZATION AND EXPRESSION STRATEGIES

### Alternative splicing and leaky scanning expression strategies

Vertebrate parvoviruses usually employ alternative splicing mechanisms to regulate the expression of different overlapping genes. Alternative splicing is controlled either by the initial binding of the general binding factors (Smith and Valcárel, 2000; Graveley, 2002) or by inhibition after intron removal has begun (Lallena *et al.*, 2002). Different small nuclear riboproteins (snRNPs) and accessory proteins bind to the 5' splice site, the branchpoint sequence and the pyrimidine tract near the 3' splice site (Graveley, 2002). The AG dinucleotide just upstream of the 3' splice site is required twice during splicing, during spliceosome assembly and prior to exon ligation. A splicing factor, SPF45, was shown to play a key role in the choice among alternative 3' splice sites (Lallena *et al.*, 2002). A well-known exception to alternative splicing in parvovirus gene expression is VP2 (B protein) of the *Dependovirus* genus members that is translated from an ACG initiation codon (Muralidhar *et al.*, 1994). This start codon, even though it is in a favorable context, is still inefficiently recognized and most of the scanning 40 S small ribosome units will recognize the next AUG initiation codon to start synthesis of VP3 (C protein).

In contrast, a leaky scanning expression strategy dictates to a significant degree the pattern by which densoviruses generate N-terminal extended isoforms of the structural proteins from the same reading frame or alternative proteins from different reading frames. For detailed reviews on leaky scanning mechanisms in translation, the reader is referred to Hellen and Sarnow (2001), Dever (2002) and Kozak (2002). Leaky scanning is promoted by very short 5' regions before the first AUG (Sedman *et al.*, 1990; Kaneda *et al.*, 2000), non-AUG start codons ACG or CUG in favorable contexts, often in conjunction with downstream highly

structured GC-rich leader sequences that slow scanning and allow Met-tRNA<sub>i</sub> mispairing (Kozak, 2002) or initiation codons in poor contexts, such as YNNAUGY (Kozak, 2002). Nevertheless, leaky scanning may also occur in good contexts, e.g. if the facilitating G<sup>+4</sup> is cancelled by U<sup>+5</sup> (Kozak, 2002). There is no evidence in parvoviruses for ribosomal shunting, in which the scanning ribosomal subunit shunts intramolecularly to a downstream landing pad to bypass secondary structures that may contain AUGs (Yueh and Schneider, 2000), or internal initiation. Context effects on initiation have not been studied in detail in insects.

Translation of several mRNAs is known to initiate from three alternate start codons, by leaky scanning and/or reinitiation. Both murine c-myc and rat C/EBP $\alpha$  mRNAs code for three isoforms that have different roles as regulators of transcription. In both cases the first initiation codon is CUG (Spotts *et al.*, 1997; Calkhoven *et al.*, 2000), which allows the majority of 43 S ribosomal subunits to continue scanning for the next AUG(s). Small out-of-frame upstream open reading frames (ORFs), between the initiation codons, may contribute to the leakiness of scanning (Calkhoven *et al.*, 2000). The translation mechanism of densoviruses is remarkable among the known leaky scanning examples. For example, from the single VP mRNA of *GmDNV*, *CeDNV* and *BmDNV-1*, four to five in-frame methionines stretching over a length of about 1100 nucleotides in the 5'-end of the transcripts initiate the translation of four to five isoforms of the capsid protein (Li *et al.*, 2001; Fédière *et al.*, 2002; Tijssen *et al.*, 2003). Moreover, six out-of-frame AUGs can be found between the first two in-frame methionines of the *GmDNV* VP transcript. The positions and number of out-of-frame AUGs are not conserved, however. The exact mechanism of how the translational mechanism copes with this large array of AUGs is unknown. VP4 can generate virus-like particles when expressed in baculovirus expression vectors (Croizier *et al.*, 2000). VP1 of parvoviruses are too bulky and cannot generate capsids. Therefore, these viruses have evolved expression strategies so that N-terminal extensions are present in only a minority of the capsid proteins of the virus and capsids can still be generated. These N-terminal extensions are located within or on the outside of the capsid.

Another common expression strategy observed in densoviruses is the use of two overlapping reading frames with initiation codons separated by a few nucleotides, usually four, which is less than the ~13–15 nucleotide footprint of the small ribosomal subunit. Of these two initiation codons, the 5'-proximal AUG is usually in a less favorable context. Inspecting the coding potential of vertebrate parvoviruses suggested that they might also employ a similar strategy (Zádori and Tijssen, unpublished results).

These three strategies, alternative splicing, leaky scanning translation mechanisms, and alternative initiation codon usage, are arranged to regulate protein expression in a temporally and quantitatively coordinated fashion. In addition, there is preliminary evidence that some densoviruses use frameshift translation.

## AMBISENSE DENSOVIRUSES

### Overview

As discussed previously, until recently all ambisense densovirus were classified into the *Densovirus* genus. However, an increasing number of new densovirus are being characterized that have ambisense genomes but share relatively few other characteristics with this, originally well-defined, genus. This probably reflects the fact that the first group of densovirus were all isolated from butterflies, with which they may have co-evolved over long periods of time, whereas the newer viruses are being isolated from other orders of insects. Nevertheless, they all encode their NS proteins on the 5'-half of one DNA strand and their structural proteins on the 5'-half of its complement.

The subgroup A densovirus members, *GmDNV* and *JcDNV*, have 6 kb genomes with ITRs of about 550 nucleotides. Their structural proteins are N-terminally extended isoforms of 45, 53, 58, and 89 kDa, whereas their nonstructural proteins have molecular masses of 20, 30, and 60 kDa. Several other ambisense densovirus have been isolated from butterflies, and these all resemble *GmDNV* and *JcDNV* (Box 5.1), showing about 80–90 percent sequence identity and with strong serological cross-reactivity. Their structural proteins are synthesized by a leaky scanning mechanism, NS1 and NS2 by alternative initiation after the transcript is spliced (see below), while NS3 is translated from the 5'-proximal AUG in the unspliced NS transcript (Tijssen *et al.*, 2003).

So far all ambisense densovirus from other insect orders have shorter genomes, of about 5.5 kb, and shorter ITRs, and use splicing and leaky scanning to generate the structural proteins. In contrast to the butterfly densovirus group, the genome organization of this group is not homogeneous (Figure 5.1), and neither are the relative amounts and molecular masses of their structural proteins. This heterogeneity probably reflects the fact that their insect hosts, such as mosquitoes and cockroaches, belong to different orders. Not surprisingly, the sequence identity among viruses of this group is also low.

Although densovirus from other genera have two NS proteins, most ambisense densovirus have a third NS protein. Sequence correlations between these NS3 polypeptides and non-parvoviral proteins suggest that this gene was obtained by horizontal transmission early in the evolution of the ambisense densovirus (see below).

The ORFs of the NS and VP genes in subgroup A ambisense densovirus are only separated by about 30 nucleotides. Since these genes are on opposite strands, their close proximity may commonly lead to the production of antisense RNA from this region. Indeed, mapping the transcripts of *GmDNV* and *MIDNV* (Tijssen *et al.*, 2003; Fédière *et al.*, 2004) demonstrated that the NS and VP transcripts have a terminal antisense sequence of about 60 nucleotides

that overlaps the 3'-end of the NS1 gene. Similar overlaps have been observed for transcripts of other subgroup A ambisense densovirus, such as *JcDNV* (Bergoin, unpublished observations), *PiDNV* and *HaDNV* (Tijssen, unpublished observations). The extent of antisense RNA at the end of the NS and VP transcripts in non-subgroup A ambisense densovirus is mostly unknown. However, in *BgDNV* the VP and NS ORFs are separated by three nucleotides, in *PcDNV* by 15 nucleotides and in *PfDNV* by 30 nucleotides, suggesting strongly that they may also generate antisense RNAs. Recently, we have mapped the transcripts of *AdDNV*, which were also found to have a 29 nucleotide antisense overlap. Interestingly, in the ambisense SV40 virus the ends of the antisense early and late transcripts also overlap by about 90 nucleotides (review: Cole and Conzen, 2001).

### Terminal hairpins and promoter sequences

The genomes of subgroup A ambisense densovirus, all isolated from butterflies and moths (*Lepidoptera*), have very long ITRs, of about 550 nucleotides, which comprise about 18 percent of the total genome. Only the terminal ~130 nucleotides can be folded into typical Y-shaped hairpins, while transcript mapping (Tijssen *et al.*, 2003) revealed that the rest of the ITRs contain the TATA-boxes and upstream promoter elements for the NS and VP gene cassettes. Dumas *et al.* (1992) reported that there are many short repeat sequences in the ITRs, but not all ITRs are the same length. The ITRs of *GmDNV* end one nucleotide downstream of the TATA-box (Tijssen *et al.*, 2003), the ITRs of *MIDNV* in the middle of the TATA-box (Fédière *et al.*, 2004) and the ITRs of *JcDNV* have a few, scattered mismatches before the TATA-box (Dumas *et al.*, 1992).

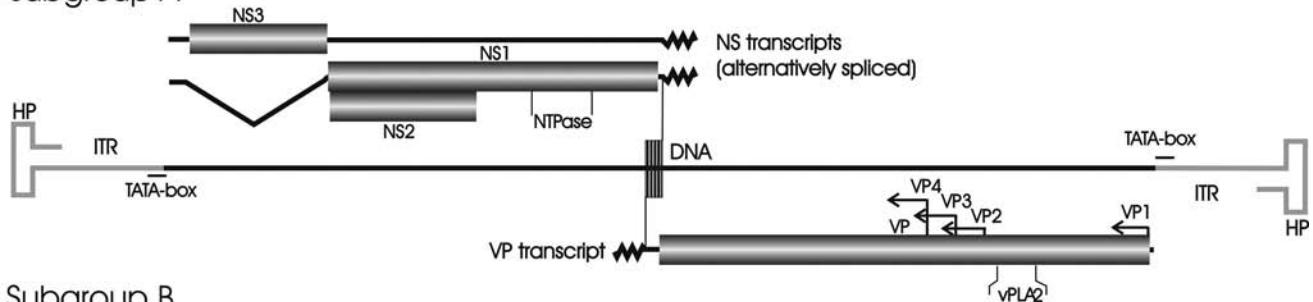
There are usually about 100 nucleotides missing from one end of the genome when it is cloned directly into bacterial plasmids (Dumas *et al.*, 1992). This is most probably an artifact as the number of nucleotides that are missing varies considerably, and the hairpins are notoriously difficult to clone, so that double-stranded DNA with one terminus missing is preferentially obtained. Moreover, full ITRs from both ends of the genome can be obtained when terminal restriction fragments instead of full-length genomes are cloned (Tijssen *et al.*, 2003; Fédière *et al.*, 2004).

Fédière *et al.* (2004) observed structural differences at the same position in the arms of the terminal hairpin (Figure 5.2). Whether this difference is merely coincidental or has some significance remains to be established. Flip/flop orientations were observed in the hairpins at both ends of the genome (Tijssen *et al.*, 2003).

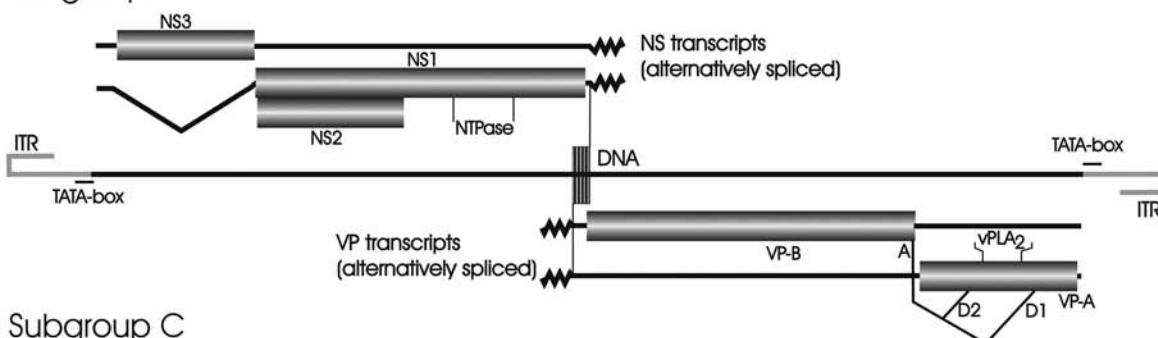
Only a few ITRs from the genomes of subgroup B densovirus have been completely sequenced. These are much shorter, only about 200–220 nucleotides, and make up about 7 percent of the total genome. The terminal hairpins of this group of viruses do not fold into the typical T- or Y-shaped structures observed with the subgroup A densovirus, but

## Ambisense densoviruses

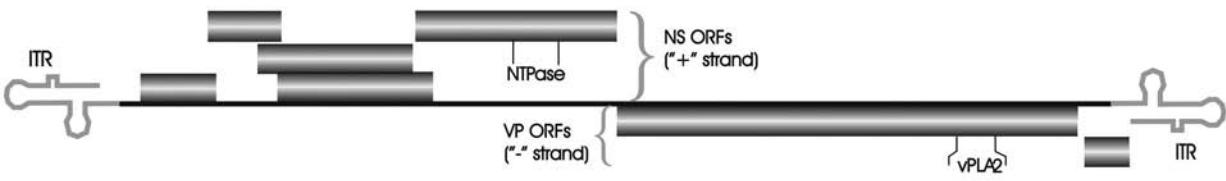
### Subgroup A



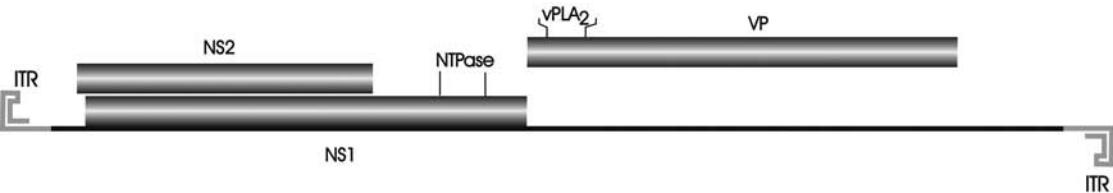
### Subgroup B



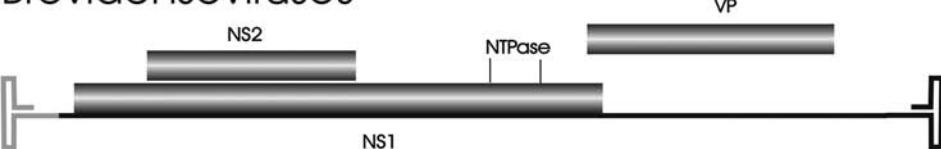
### Subgroup C



## Iteraviruses

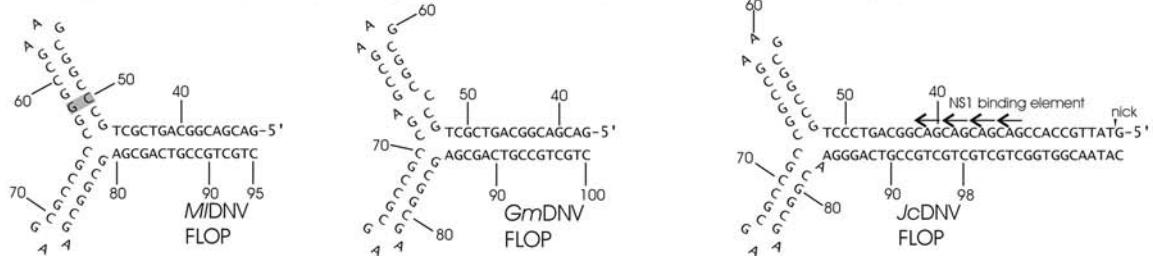


## Brevidensoviruses

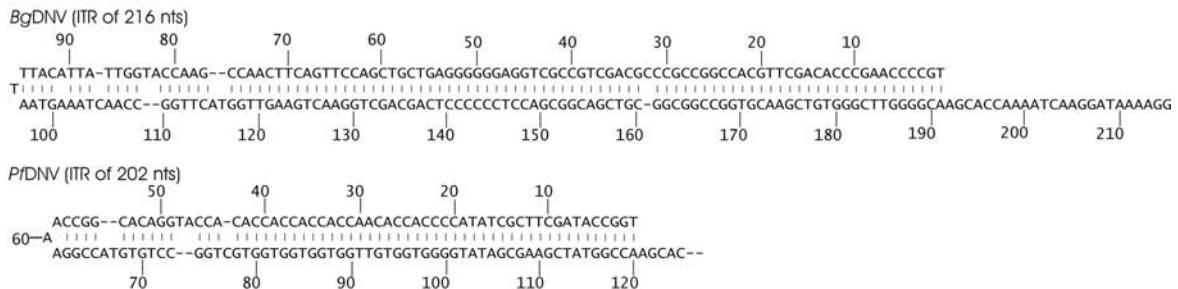


**Figure 5.1** Genome organization of densoviruses. The ambisense densoviruses can be divided into three subgroups (A-C) depending on size, terminal hairpin structure and organization of ORFs. All densoviruses, except brevidensoviruses, have ITRs and carry a PLA<sub>2</sub> motif in the structural proteins. The large NS proteins carry an NTPase (ATPase) motif and a rolling circle replication (RCR) motif.

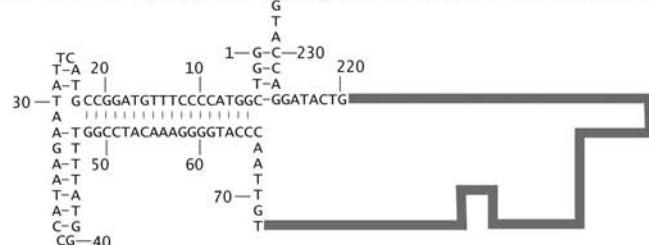
## A1. T-type terminal hairpin (HP) of subgroup A densoviruses (ITR is still about 450 nts longer)



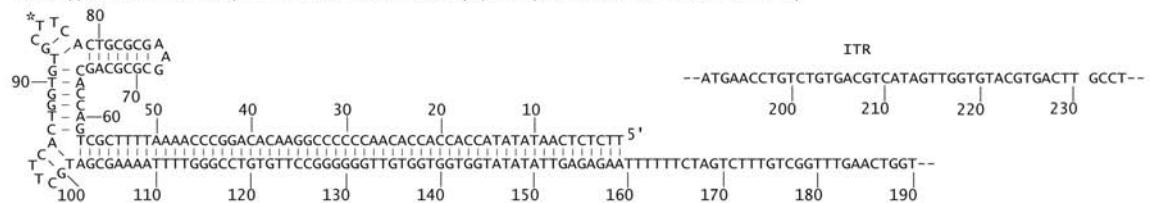
## A2. Terminal hairpins (HP) of subgroup B densoviruses (ITR of about 200 nts, BgDNV and PfDNV shown)



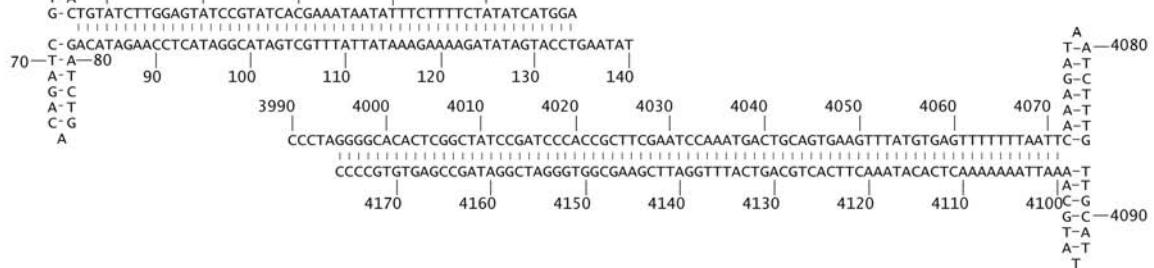
## A3. Terminal hairpins (HP) of subgroup C densovirus (ITR of about 300 nts, CpDNV)



## B. J-type terminal hairpin in ITR of Iteraviruses (flip sequence of BmDNV-1 shown)



## C. Terminal hairpins Brevidensovirus (− strand of Aa/DNV genome shown, no ITRs)



**Figure 5.2** Terminal hairpins of densoviruses from different genera and their subgroups. Only the Brevidensovirus members lack ITRs. Subgroup A of the Densovirus genus is relatively homogeneous and conserves the NS1 binding element. One distinguishing feature in their hairpins is indicated by shading in M/MDNV (position 50). The B and C subgroups are very heterogeneous often with less than 20 percent sequence identity. In the J-type terminal hairpin of the genomes of Iteravirus densoviruses flip/flop sequence there is a single nucleotide difference between BmDNV-1 and CeDNV but significantly more with DpDNV. Brevidensoviruses have T-type hairpins.

rather into simple foldback hairpins like those of the human B19 and waterfowl parvoviruses. Yamagishi *et al.* (1999) and Guo *et al.* (2000) determined the sequence of *Pf*DNV, isolated from the cockroach (*Periplaneta fuliginosa*), which has ITRs of 202 nucleotides and hairpins of 120 nucleotides (Figure 5.2). In contrast, *Bg*DNV has relatively long hairpins of 192 nucleotides in its 216 nucleotide ITRs (Figure 5.2). The *Pc*DNV genome has ITRs of 122 nucleotides and a total length of 5380 nucleotides (Thao *et al.*, 2001). These ITRs lack foldback structures and seem to be incomplete, maybe because truncated molecules were cloned preferentially, as mentioned above. Similarly, both *Mp*DNV ITRs lack terminal sequences (van Munster *et al.*, 2003). We are currently determining the sequence of the *Ad*DNV ITR, which seems to resemble that of *Pf*DNV.

So far, no transcript maps have been reported for the subgroup B densoviruses, and the location of their transcriptional promoters relative to the ITRs has yet to be established. However, we have mapped the transcripts and splicing patterns of *Ad*DNV, which also pointed to the use of two promoters, one for the NS gene cassette and one for the VP gene cassette (see below; unpublished results). The VP cassette can be recognized by the PLA<sub>2</sub> motif. The VP proteins of these viruses are most probably generated by a leaky scanning mechanism, although splicing is also involved (see below), and the proximity of the coding sequence to the ITRs could indicate that the promoter sequences are contained within the ITRs. The VP genes of this group of densoviruses are split into two ORFs, VP-A and VP-B, with VP-A located upstream. The most probable start codon in VP-A in *Pf*DNV is directly downstream of the ITR, at position 5253–5255, with the TATA-box positioned in the ITR, at nucleotides 5283–5289. In the case of *Bg*DNV, the putative ATG of the coding sequence in VP-A is located 26 nucleotides downstream of the ITR, whereas the TATA box

is located within the ITR (209–215). Finally, the putative ATG start codon of VP-A in *Pc*DNV is located 37 nucleotides downstream of the ITR, whereas the TATA-box is just inboard of the ITR. Similarly, the candidate ATG start codon of VP-A in *Mp*DNV is located 36 nucleotides downstream of its ITR. The presence of the TATA-box and all the upstream promoter elements for the VP transcripts in the ITR implies that the same elements are also present in the ITR upstream of the NS genes.

Thus, despite large size differences in the ITRs of the subgroup A and subgroup B densoviruses, these all seem to contain the TATA-boxes and upstream promoter elements for both the NS and VP genes.

### Non-structural proteins of ambisense densoviruses

The non-structural proteins of densoviruses have been studied in far less detail than those of the vertebrate parvoviruses. Nevertheless, motifs such as the RCR (rolling-circle replication) nuclease and Walker A- and B-motifs of the ATPase, identified in the NS1 molecules of vertebrate parvoviruses (Astell *et al.*, 1987; Campos-Olivas *et al.*, 2002), are also readily recognized in densoviruses (Figure 5.3). Ambisense densoviruses are unusual in having three distinct non-structural proteins (NS1–3). The roles of NS1 and NS3 have recently been studied in more detail (Ding *et al.*, 2002; Abd-Alla *et al.*, 2004).

The non-structural genes of the ambisense densoviruses are organized in quite similar ways, with the exception of *Mp*DNV, which lacks NS3 although it has some small ORFs (van Munster *et al.*, 2003). Invariably, in both subgroup A and B densoviruses, the NS2 ORF overlaps the 5' half of the NS1 ORF, with the proteins encoded in different reading

	RCR motif		ATPase motif	
	motif-2	motif 3	Walker A-site	B-site
JcDNV	132	GDHIHVIHD-41-DVFIYFFVRKR		
GmDNV	131	GDHIHVIHD-41-DVFIYFFVRKR		
BmDNV-1	289	QGHFHILHA-36-NIMFYNTKWPR		
CeDNV	289	EGHFHILHA-36-NIMFYNTKWPR		
Aa1DNV-1	316	GDHIHILFS-35-NYILYCIRYGI		
AaeDNV	372	GDHIHILFS-35-NYILYCIRYGI		
MVM	125	GWHCHVLIG-72-MIAYYFLTKKK		
PPV	126	GYHCHVLIG-69-MIAYYFLNKKR		
AAV2	88	YFHMHVLLM-55-YIPNYLLPKTQ		
SAAV	77	GYHMHVLLN-54-YLKNYFFRKTL		
B19	79	GYHIHVVIG-50-FIENYLMKKIP		
SiPV	79	GFHIHVVIG-49-FVTYYLMPKLY		
consensus	.	.uHuHuuu.	.u..Yu..K..	R
			S	S
			uu..GP...GKTu.....	EE

**Figure 5.3** Comparison of rolling circle replication and Walker ATP-ase motifs in NS1 of different densoviruses and parvoviruses (two viruses per genus). The Walker ATPase motif of the Densovirus and Iteravirus genus viruses do have GKN rather than the consensus GKS/T motifs but are, nevertheless, enzymatically active (Ding *et al.*, 2002). In the B-site we find in the Densovirus genus a single acidic residue rather than the conserved double acidic residues.

frames and using initiation codons just a few nucleotides apart. The NS3 ORF is located between the NS1/2 cassette and the upstream promoter. From the little information available it cannot be implied that all NS2 proteins, or all NS3 proteins, share the same functions. The strategy used by these viruses to express these non-structural proteins has only recently been elucidated, by transcript mapping (Tijssen *et al.*, 2003; Fédière *et al.*, 2004). The 5' proximal initiation codon in a 2.5 kb transcript generated from the ITR-overlapping promoter at the left end of the viral genome directs translation of NS3, a protein of about 20 kDa. However, some of these transcripts are spliced from a position just upstream of the NS3 initiation codon to a site just upstream of the tandem initiation codons of the NS1/2 cassette, so that the NS3 coding sequence is removed from the 2.5 kb transcripts, generating a 1.8 kb transcript. Although these mapping data have only been published for *Gm*DNV and *Ml*DNV, similar results were obtained for *Jc*DNV and *Ad*DNV. The organization and splicing motifs required for this strategy are conserved among all ambisense densovirus that encode an NS3 protein. The initiation codon of NS1 always seems to precede that of NS2 and, unlike that of NS2, is in a poor context (YnnAUG), which promotes leaky scanning. The proximity of the two initiation codons probably also promotes alternative initiation, as the footprint of the small ribosomal subunit is about 13–15 nucleotides.

*Mp*DNV probably employs the same strategy for expressing NS1 and NS2. The NS1 initiation codon is in a poor context, suggesting that a downstream initiation codon is used for NS2, even though that ORF is larger and starts upstream of NS1. In contrast to the other ambisense densovirus, the initiation codon of NS1 is not preceded by a splice acceptor motif, suggesting that the small ORFs upstream of NS1 are not expressed. The absence of NS3 in *Mp*DNV (if the sequence is correct, see Figure 5.1 and the discussion of phylogeny below) may be compensated by its NS1 protein, which is about 150 amino acids longer than the NS1s of other ambisense densovirus. Interestingly, the NS1s of densovirus that encode an NS3 are significantly shorter than the NS1s of all other parvoviruses. The absence of

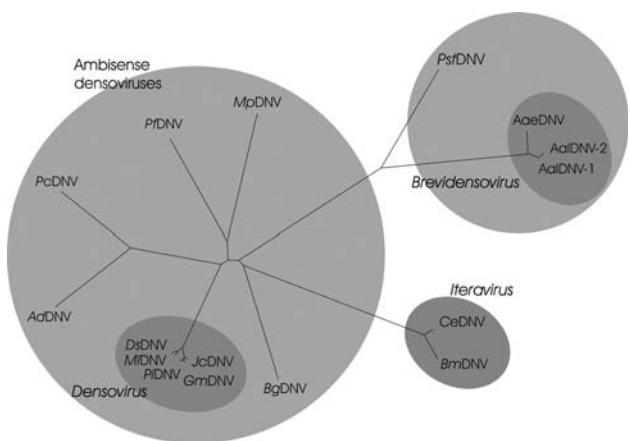
NS3 in *Mp*DNV and in densovirus of the other genera, as well as in vertebrate parvoviruses, suggests that this gene was acquired relatively late in evolution, probably with the concomitant loss of parallel function(s) in NS1. Blast searches of the database with the NS3 sequence detected related domains in the non-structural proteins of viruses from other families, suggesting horizontal transmission of this gene from such viruses to the ambisense densovirus (Figure 5.4).

NS1 of *Jc*DNV, expressed as a maltose-binding fusion protein, recognizes and binds the (GAC)<sub>4</sub> sequence in the stem of the hairpin (Figure 5.1) and nicks single-stranded forms of the hairpin preferentially at two sites (G\*TAT\*TG; Figure 5.1) (Ding *et al.*, 2002). Although the Walker A-motif (SxxxxGKNff) of the ambisense densovirus is not highly conserved when compared to the classic Walker motif (GxxxxGK[T/S]), Ding *et al.* (2002) nevertheless were able to demonstrate that the NS1 of *Jc*DNV had helicase activity. Abd-Alla *et al.* (2004) demonstrated that the NS3 of *Jc*DNV, which has two zinc-finger motifs (at amino acid sequences 144–151 and 184–190), is also critical for viral DNA replication. Thus, replication of NS3-minus mutants could be restored when vectors expressing NS3 were supplied in *trans* (Abd-Alla *et al.*, 2004).

The non-structural proteins of the ambisense densovirus are surprisingly divergent (Figure 5.5). The NS1 proteins are the most conserved. Using ClustalW (version 1.82) analysis, the scores (number of identities divided by the number of residues, excluding gap positions; Thompson *et al.*, 1994) for the NS1 proteins of subgroup A densovirus were about 90–95 percent, but comparisons with NS1s of the other groups gave very low scores (<28 percent). Among the subgroup B densovirus the highest score was obtained between *Ad*DNV and *Pc*DNV (38 percent) and between *Bg*DNV and *Pf*DNV (29 percent). *Mp*DNV NS1 gave scores of about 20 percent with all ambisense densovirus. Similarly, the scores for the NS2 polypeptides of subgroup A densovirus were about 90–95 percent, but homology with NS2s from other groups was very low (<25 percent), while among the subgroup B densovirus the highest scores

MLDNV:	AMEELVHWWDWTQNRPLPQLFLAVMH-----LNEI---PEWLDETLIESVYYFK-ELINYRDPYDSDEFNSW	130
	W+W K L +++ + +N P W D+ Y K +F +	
C1GV:	WESRFETWNWKPKTTLHTEMYNILFKYKTKYLINGA---PYWCDQFKWFNYKRYIKFNQQTGILKNLTEKFDEYY	86
	W+S F++WNW K L +Y +L Y + ++ P W + F RYI+ + ++NL+ ++F	
BmBDY:	WDSVFDWSNWSKGYKLPKPLYELLEIYMKLWKLDNRTTYPDWGESSMIFKRYRYIRNKRKMEDIENLSTEEFNDR	139
	w s f wnw K Lp Ly l y ln P w d m f rYik nl eFn	
MlDNV:	NLNGKPFKTMWK-ICKFCYTN---CEDPDEYRF---I-YNRTIFVEDAEDIIINRFQDGSSWCQMCHTCPLFTV	182
	++NG + ++ K +C CY + +Y I NR + E+ + + D +WC++C PLFT+	
C1GV:	DING--YGSVGKTVCKCYRE---NKFDHDYLIQDDI-ENRKLLYEN--EYFKKMMIDIKNWCEICKRTPLFTL	148
	+ NG + +C C+ N +DY D +N LL+ +YF +++ WC CK TPLFTL	
BmBDY:	NFNG-WPKTMWY-ICRNCFDNGIVINNNNSNDYYTCDFSDNPPLLHYT--DYFYIILNDYWCYDCKYTPLFTL	209
	n NG ktmwk iC Cy n n dY di Nrtll e dyf d WC CK tPLFTL	

**Figure 5.4** Horizontal transmission of the NS3 gene. The NS3 gene, *Ml*DNV in example, was not found in *Mp*DNV but was present in the C1GV granulosis virus (AY293731) and the BmBDY (Yamanashi strain, GenBank #S78547).



**Figure 5.5** Phylogenetic tree of the NS1 protein of densoviruses (replicator and helicase motifs; part of these motifs of MpDNV was outside the published ORFs). The lengths of the branches indicate the phylogenetic distance between the different viruses. The scale bar represents 10 mutations per 100 sequence positions. The tree was generated by distance matrix analysis (PHYLIP program package-PROTDIST, using the Dayhoff PAM 001 scoring matrix, followed by FITCH, applying the global search option).

were again obtained between *AdDNV* and *PcDNV* (28 percent), *AdDNV* and *PfdNV* (25 percent), and between *BgDNV* and *PfdNV* (20 percent). *MpDNV* NS2 scored <10 percent with all ambisense densoviruses. The scores were lower for NS3, about 80–88 percent for the subgroup A densoviruses, but only 10 percent between *AdDNV* and *PcDNV*, and even lower between other pairs. An exception was *PfdNV*, which gave relatively higher scores for NS3 than for NS1 and NS2 when compared with densoviruses from both subgroups A and B. No highly conserved domains were detected in either NS2 or NS3 from the ambisense densoviruses. The *CpDNV*, isolated from *Culex pipiens* (a mosquito species) (Jousset *et al.*, 2000), differs from the other ambisense densoviruses in having NS1 and NS2 coding sequences split into four ORFs (instead of two; subgroup C, Figure 5.5). This organization implies the splicing of a small intron to put in frame the N- and C-terminal sequences of the two polypeptides. Furthermore, the transcription of NS3 and NS1-NS2 are under the control of two different promoters (Jousset *et al.* unpublished).

## Structural proteins of ambisense densoviruses

Subgroup A densoviruses have a single ORF encoding the structural protein (VP) gene whereas subgroup B densoviruses use two ORFs, a smaller ORF (ORF-A) upstream of the main ORF (ORF-B), to code for VP. In subgroup A densoviruses ORF-A encodes about 280 amino acids from the N-terminal of VP. ORF-A is connected to ORF-B by splicing as shown for *PfdNV* (Yamagishi *et al.*, 1999), but after

splicing it may still require translational frameshifting, as demonstrated for *AdDNV* (unpublished results). The identity scores for the VP proteins, determined as described above using ClustalW alignment, between subgroup A and B ambisense densoviruses is <25 percent, whereas it is about 80 percent among subgroup A viruses and 20–25 percent among subgroup B viruses. Although these subgroup B viruses have mostly been lumped together so far because of similarities in the distribution of their ORFs and their split VP ORF, they have very divergent sequences.

Conserved motifs are more prominent in the structural proteins than in NS2 and NS3. The most important are the  $\text{Ca}^{2+}$ -binding loop (GPGN) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>) (DxxAxxHDxxY) motifs (Figure 5.1, p. 60), recognized in all ambisense densoviruses, and also found in the *Iteravirus* genus and the vertebrate parvoviruses (Zádori *et al.*, 2001). Although proline is conserved in the  $\text{Ca}^{2+}$ -binding loops of all parvoviruses, it is not present in secretory (non-viral) PLA<sub>2</sub>s. The distance between the  $\text{Ca}^{2+}$ -binding loop and the active site (HD) varies in the densoviruses, as it does among other PLA<sub>2</sub>s. The specific activity of densovirus PLA<sub>2</sub>s was significantly lower than that of iteraviruses or vertebrate parvoviruses (Zádori *et al.*, 2001; Li *et al.*, 2001; Fédière *et al.*, 2002; Tijssen *et al.*, 2003; Fédière *et al.*, 2004). All the PLA<sub>2</sub> motifs detected to date have been located on the least abundant capsid protein (VP1). A third motif that is prominent in vertebrate parvoviruses, a glycine-rich stretch, is clearly recognized as a (S/T)G sequence in *PfdNV*, but is not found in other densoviruses. Other motifs that are specifically conserved in those viruses with a split VP ORF are NEGQR-RYAVEQWQLARVRRG (sequence for *AdDNV*), positioned at the beginning of ORF-B, and QPSLHIGVKPVHALTT, positioned almost at the C-terminus of ORF-B.

The four forms of the structural proteins encoded by subgroup A densoviruses are produced by leaky scanning (Tijssen *et al.*, 2003; Fédière *et al.*, 2004) as discussed previously. In contrast, subgroup B densoviruses combine an alternative splicing strategy with leaky scanning to generate five isoforms of the structural proteins. In the case of expression *AdDNV* VP in the baculovirus system, alternative donor sites and a single acceptor site are found near the beginning of ORF-B, so that introns of either 131 or 356 nucleotides are spliced out. However, only the small intron is observed in the VP mRNA from its infected host, the cricket. The PLA<sub>2</sub> motif is located within the unique part (225 nucleotides) of the large intron, so that it would be removed when ORF-A is connected to ORF-B via the large intron observed in the baculovirus system instead of the small intron (unpublished results). Moreover, VP2 starts at the first methionine of ORF-B and there seems to be no need for alternative splicing to generate VP1. This alternative splicing strategy for expressing *AdDNV* VP thus seems to be an artifact owing to the expression in a different insect order. Nevertheless, ORF-A and ORF-B do not result in a combined ORF after splicing, so that translation would need to frameshift.

## MONOSENSE DENSOVIRUSES WITH ITRs: THE ITERAVIRUSES

So far, *BmDNV-1*, *CeDNV* and *DpDNV* are the only three densoviruses in the *Iteravirus* genus. Because of its economic importance to the silkworm industry, *BmDNV-1* was discovered early and has been studied in detail, particularly in Japan. SDS-PAGE analyses (Nakagaki and Kawase, 1980) revealed structural proteins with masses and stoichiometry unlike the ambisense densoviruses. Two protein doublets of 50/57 and 70/77 kDa were resolved, with the 50-kDa protein being the most dominant. However, Bando *et al.* (1984) showed by peptide mapping that these proteins were isoforms, while Li *et al.* (2001) later demonstrated that the 57-kDa band contained two isoforms of the VP3 protein of almost identical length. *CeDNV* particles were shown to contain five structural proteins, which were N-extended isoforms that were very similar to those of *BmDNV*, with molecular masses of 76, 65, 56, 55 and 48 kDa (Li *et al.*, 2001; Fédière *et al.*, 2002).

The genome organization of *Iteravirus* members was also significantly different from that of the ambisense densoviruses (Li *et al.*, 2001; Fédière *et al.*, 2002). Their genomes were only 5 kb in length, with J-shaped ITRs of 0.23 kb. Moreover, the NS and VP genes were located on the same strand, as in the vertebrate parvoviruses. Nevertheless, NS1 and NS2 were organized as an overlapping cassette, as in the ambisense densoviruses, while NS3 was absent. VP was located downstream of the NS ORF, with a short intergenic sequence of 65 nucleotides in the case of *CeDNV* but of 110 nucleotides in *BmDNV-1*, since it has a 45 nucleotide direct repeat. *DpDNV* also has an intergenic direct repeat (23 nucleotides).

J-shaped terminal hairpins in 230-nucleotide ITRs are typical for members of this genus (Figure 5.2, p. 61). The hairpin is 159 nucleotides long, with nucleotides 59–101 occurring in two orientations, flip and its reverse complement flop, which are highly conserved between *CeDNV* and *BmDNV-1* (showing 98 percent identity, versus 67 percent identity for the stem sequences between nucleotides 1–58 and 159–102) but less with *DpDNV*. These stems contain ACCA motifs, similar to those found in the centre of the right-end hairpin of minute virus of mice (MVM) where they are known to bind NS1 in order to induce hairpin-primed viral DNA replication (Willwand *et al.*, 2002). The high conservation of the flip/flop sequences may indicate that the sequence, as well as the structure, of these hairpins is important in the viral life cycle. The hairpins act as primers for DNA synthesis, allowing the conversion of single-stranded genomic DNA into duplex replicative forms, a prerequisite for transcription that is, in turn, important for the production of the non-structural proteins required to continue the replication cycle. This intermediate DNA molecule is converted into a closed circle by host cell ligases, which join the newly-extended complementary strand

to the 5' end of the ITR at the other end of the genome (Hayakawa *et al.*, 1997). When the NS proteins are produced, replication can proceed via a hairpin transfer mechanism. Strikingly, in both *BmDNV-1* and *CeDNV* the flip orientation of the hairpin dominates in actual virus populations and in cloned forms of the genome (Bando *et al.*, 1990; Fédière *et al.*, 2002). This would indicate that alternate orientations are asymmetrically generated or that the flip orientation is preferentially encapsidated.

The expression of iteravirus viruses has yet to be studied by transcript mapping. However, the ORFs in *BmDNV-1*, *CeDNV* and *DpDNV* are in identical positions, suggesting that they represent actual genes. Although the larger NS gene (NS1) contains the rolling-circle replication and Walker box ATPase motifs, NS2 is better conserved, in contrast to ambisense densoviruses. NS1 and NS2 are also substantially larger than those found in the members of the *Densovirus* genus. The putative initiation codons for both NS1 and NS2 (354/481 and 344/477 in *CeDNV* and *BmDNV*, respectively) are all in a favorable context. The VP proteins are generated by a strategy similar to that found in the subgroup A densoviruses, namely by leaky scanning without splicing (Fédière *et al.*, 2002). The conserved Inr site suggests that VP transcription in *CeDNV* starts at position 2661, 13 nucleotides prior to the start codon for its VP1. Not only is this start codon near the 5' end of the predicted transcript, it is also in a poor context, both features that are conducive to leaky scanning. Expression from different 5' proximal ATGs in a baculovirus system indicated that the five first ATGs are used to generate five N-terminally extended isoforms of the VP polypeptide. The AATAAA polyadenylation motif was found to overlap with the stop codon of the VPs.

## MONOSENSE DENSOVIRUSES WITHOUT ITRs: THE BREVIDENSOVIRUSES

The monosense densoviruses with the shortest genomes are classified into the *Brevidensovirus* genus. These are about 4 kb in length and have disparate hairpins at their two termini. Hosts include the mosquito (*Diptera*), or its cell lines, and shrimp species. Examples are *AaeDNV* from *Aedes aegypti* (Lebedeva *et al.*, 1973; Afanasiev *et al.*, 1991), *AalDNV-1* and *AalDNV-2* from the *A. albopictus* cell line C6/36 (Jousset *et al.*, 1993; Boublík *et al.*, 1994; Chen *et al.*, 2004). Other mosquito cell lines have also yielded virus isolates (O'Neill *et al.*, 1995), whereas Kittayapong *et al.* (1999) and Rwegoshora *et al.* (2000) obtained isolates from *A. aegypti*, *A. albopictus* and *Anopheles minimus*. Their sequences are very divergent from other densoviruses, and they do not contain the PLA<sub>2</sub> motif that is found in all other densoviruses and vertebrate parvoviruses except Aleutian mink disease virus (Zádori *et al.*, 2001). This may be one of the reasons why the structural proteins VP1 and

VP2 are much smaller than those of other parvoviruses, each being only about 40 kDa. Interestingly, *Aal*DNV-2 shares 90 percent identity with *Aae*DNV at the amino acid level, but only about 75 percent with *Aal*DNV-1, although the latter was isolated from the same cell line (Chen *et al.*, 2004). However, the genome organizations of *Aal*DNV-1 and 2 are closely related, which led these authors to suggest *Aal*DNV-2 has a common ancestor with *Aae*DNV but underwent convergent evolution with *Aal*DNV-1.

The terminal hairpins of these genomes can be folded into perfect T-shaped structures of about 60 nucleotides. Like porcine parvovirus strain NADL-2, a large perfect tandem repeat is found near the hairpin at the 3' end of the plus strand (37, 92, and 160 nucleotides for *Aae*DNV, *Aal*DNV-1, and *Aal*DNV-2, respectively). These T + A-rich tandem repeats contain a TTTCCAC motif, which is the inverted complement of the GTGGAAA SV40 transcription enhancer motif. Predicted promoters at map units 7 and 61 are conserved among these viruses. However, mutation of the TATA sequence at map unit 61 had little effect on expression. Instead, the CAGT Inr sequence 60 nucleotides upstream of this TATA sequence was found to be critical (Ward *et al.*, 2001).

The rolling-circle replication nuclease motifs are present in NS1, but these motifs are not specific for parvoviruses as they are found in many prokaryotic, eukaryotic and viral systems such as geminivirus and circovirus Rep proteins, bacteriophage  $\phi$ X174 gene A protein, and transposon IS91 TnpA (Campos-Olivas *et al.*, 2002). The ATPase (helicase) motif is also readily recognized (Figure 5.3).

Infectious hypodermal and hematopoietic necrosis virus (IHHNV or *Pst*DNV) infects shrimp (*Penaeus stylostris*) (Lightner *et al.*, 1983). Its sequence (Shike *et al.*, 2000) revealed an organization of ORFs that is similar to the mosquito brevidensoviruses, and its NS1 amino acid sequence has about 20–30 percent identity with *Aal*DNV-1 and *Aae*DNV NS1 sequences (but 10–15 percent with the subgroup A densoviruses). Again, direct repeats are found at the 3' end of the plus strand. NS2 and VP sequences failed to show significant sequence identities with the mosquito *Brevi*densovirus members, and may be closer to *Bm*DNV-1 (Roekring *et al.*, 2002). Analyzing different shrimp isolates, these authors found that the viruses do not form a single cluster but rather fall into two different insect parvovirus clades, suggesting that horizontal transmission occurred independently, and may still occur, between these distantly related arthropods.

## CONCLUSIONS

Molecular analysis of the genomes of densoviruses has revealed a striking diversity, which parallels the divergence of their host species and suggests that they may have undergone considerable co-evolution with their host. With the

exception of the *Iteravirus* genus, which so far has only three recognized members, *Densovirus* genera are far more heterogeneous at the molecular level than the vertebrate parvovirus genera. To assign each individual virus to a separate genus, based on their very limited molecular relatedness, would not serve a useful purpose. Therefore, to date, densoviruses are mostly classified according to their gross genome organization, which may reflect common ancestors but could also simply represent different transfers of viral entities that subsequently co-evolved in a particular host.

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# SECTION B

## The Rugged Virion

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# Introduction

SUSAN F. COTMORE AND PETER TATTERSALL

Parvovirus virions are amongst the smallest, most dense, and most rugged viral particles known to man. They are unique in containing a linear, single-stranded DNA genome, which is translocated vectorially into the capsid by a viral helicase, in a way that appears to allow the maximum possible genetic information to be accommodated in the smallest conceivable space. This modus operandum appears highly successful. Parvoviruses have long been known to infect an exceptionally broad range of animal species, both vertebrate and invertebrate, and as our ability to detect, sequence, and catalogue low level genetic contaminants in animal tissue expands, new viruses and new host phyla emerge at a surprising rate. Among these viruses, many are so well adapted to their host that their presence is essentially asymptomatic, while a few others remain highly virulent, causing lytic disease of dividing cell populations that can prove fatal, most commonly to fetuses and newborn animals. In this section of the book we explore the compact physical and genetic structure of the virion, which is the hallmark of these viruses, and reflects a life style where to be small, i.e. 'parvo', is of supreme importance.

Virions are 18–28 nm in diameter, non-enveloped, and exhibit  $T = 1$  icosahedral symmetry. They are generally made up from 2–4 virion protein species, called VP1–4, where VP1 is the largest with a molecular mass ( $M_r$ ) of around  $80\text{--}96 \times 10^3$ , while the others make up a nested set of polypeptides derived from the carboxy-terminus of VP1, with masses ranging from  $85\text{--}49 \times 10^3$ . Particles are constructed from 60 copies of this carboxy-terminal core sequence, with the amino-terminal extensions carried by some of the polypeptides serving as 'luggage tags' and entry tools that can be exposed or concealed from specific host cell compartments, as required to mediate cell exit and re-entry. Virions have a molecular mass of around  $5.5\text{--}6.2 \times 10^6$ , their buoyant density in aqueous isopycnic cesium chloride gradients is  $1.39\text{--}1.43 \text{ g/cm}^3$ , and they have sedimentation coefficients ( $S_{20w}$ ) of around 110–120 S. Packaging, uncoating and maturation intermediates, as well as particles with subgenomic defective DNAs are commonly present in viral stocks and all have slightly different

sedimentation coefficients and buoyant densities. In particular, empty capsids, which appear to serve as virion precursors and make up a variable, but often high (>90 percent), proportion of many stocks produced in cell culture, band at around 1.3–1.32 g/cm<sup>3</sup> in CsCl<sub>2</sub> and have sedimentation coefficients of around 70 S. Unlike most other viruses, parvovirus genomes account for approximately 25 percent of the particle mass, with the capsid proteins making up the residual 75 percent, generating virions of unusual density and with high  $E_{260}$  extinction coefficients (for example, the  $E_{260}$  for MVM virions is 7.12, with an OD<sub>260/280</sub> ratio of 1.38, while empty MVM particles have an  $E_{260}$  of 1.78, and a OD<sub>260/280</sub> ratio of 0.67). There is no evidence of cellular enzymes or chromatin constituents such as histones in the viral particle, and although in one study spermidine, spermine, and putrescine were identified as components of some insect viruses, their presence in other virions remains uncertain.

Mature virions are stable in the presence of lipid solvents, which may even potentiate the infectivity of species such as Aleutian mink disease virus (AMDV) by removing masking cell lipids. They also survive exposure to pH 3–9, and many will tolerate heating to 56°C for 60 minutes. This latter characteristic often applies only to concentrated suspensions of particles, or in situations where they are protected by animal tissue, since in dilute solution many species are metastable, undergoing an inactivating conformational transition in response to heat or denaturants. Viruses that undergo this type of heat-induced transition are thought to undergo similar structural changes upon entry into the cytoplasm, where the transition is required to expose essential motifs in the amino-termini of VP1. A well-documented, but surprising, exception to this pattern is seen in the human virus B19, where the vulnerable but essential VP1 amino-termini are thought to be exposed at the surface of both full and empty capsids. However, in general parvoviral particles are rugged shells, constructed to protect the delicate genome during transit through its host cell and the external environment, while able to shift through a series of minor structural changes that expose

trafficking information and enzymes needed to navigate host cell compartments. Concomitant with this stability is extreme longevity, so that many parvoviruses routinely survive for months or years at room temperature with little loss of infectivity. Such characteristics greatly facilitate rapid widespread transmission. Canine parvovirus (CPV) illustrates this fact since, as a newly-emerged virus in the late 1970s, it appears to have been distributed around the world within a few months, most probably disseminated in fomite traces on the shoes of airline passengers.

Within the viral particle lies a single, linear, non-permuted DNA molecule 4–6 kb in length, with a Mr of  $1.5\text{--}2 \times 10^6$ . The 3' and 5' ends of this chromosome contain small palindromic sequences of between approximately 120 and 420 nucleotides, which fold back on themselves to form duplex hairpin telomeres that are essential for viral DNA replication. These viruses are unusual in that they can encapsidate strands that are either positive or negative sense with regard to transcription, some viruses packaging equimolar copies of both strands while others package minus-sense DNA predominantly or exclusively. Because of this disparity a unifying convention has arisen by which the 5' end of the strand that encodes the non-structural

proteins is described as the ‘left end’ of the genome, and its 3' end the ‘right end’, with the whole sequence divided into 100 map units, starting from the left end. All parvoviruses possess two gene cassettes: a non-structural gene in the left end of the genome, encoding proteins that are essential for viral replication, and a structural gene located in the right half, encoding the proteins of the capsid. As might be expected for such a diminutive virus, genome usage is remarkably efficient, with protein sequences encoded in multiple overlapping reading frames that also contain regulatory elements for transcription or mRNA splicing. Nevertheless, the limited genetic capacity of the parvoviruses ultimately means they must rely primarily on the synthetic machinery of their host cell, augmented and diverted by a small battery of multifunctional viral proteins. To introduce the virion we first examine the viral blueprint, its genome, comparing the structure and compact coding strategies employed in different virus genera, and considering how the constraints required to construct a minimal icosahedron, that is small enough to enter the nucleus, severely limit genome diversification, while the single-stranded nature of this chromosome and its mode of replication promote its rapid genetic evolution.

# Structure and organization of the viral genome

SUSAN F. COTMORE AND PETER TATTERSALL

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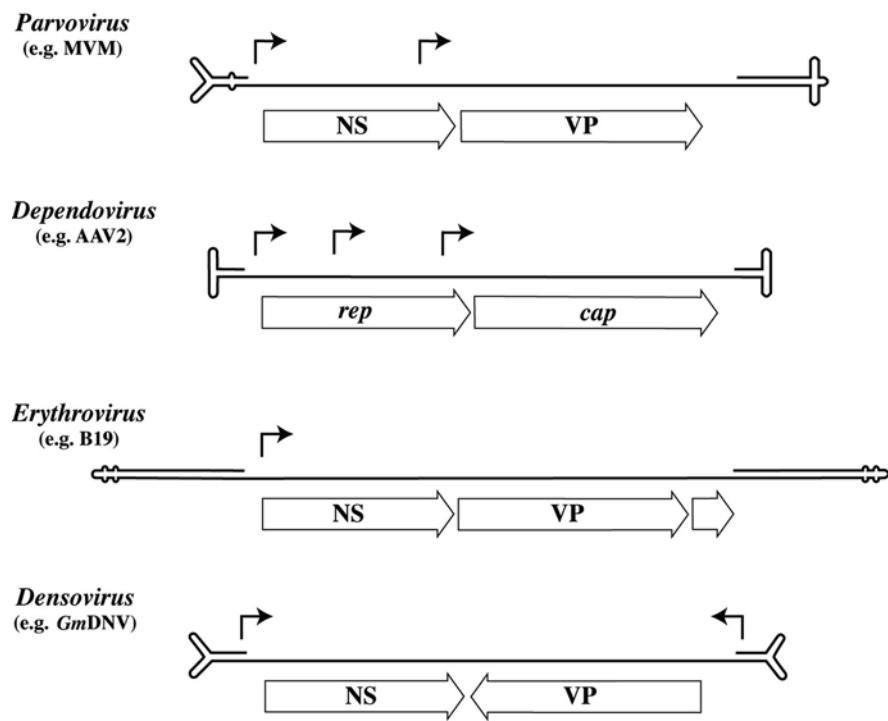
Viral genomes are small, non-permuted, linear single-stranded DNA molecules, ranging from 4–6 kb in length. They consist of a relatively long (<5.8 kb) single-stranded coding sequence bracketed by short imperfect terminal palindromes, ranging in size from approximately 120–420 bases, which fold back on themselves to form duplex telomeres. These termini vary dramatically in size, sequence and shape between viral genera, and even between species within the genus *Dependovirus*. In some viruses they are part of a terminal repeat element, so that the hairpins at both ends of the genome are closely related or identical in sequence, while in other genera they are disparate, with the two telomeres differing both in structure and in their activation mechanisms. These hairpins play a critical role in the unique ‘rolling-hairpin replication’ (RHR) strategy employed by parvoviruses, since they contain the sequences that create viral origins of DNA replication, and because they serve as essential ‘toggle switches’ that repeatedly unfold and refold to reverse the direction of DNA synthesis, thus adapting an ancient ‘rolling-circle’ replication strategy seen predominantly in prokaryotes, for the amplification of a linear chromosome. The hallmark of a parvovirus, which thus dictates and is dictated by their replication strategy, is a small single-stranded linear DNA genome, flanked by palindromic, self-priming telomeres.

As illustrated in Figure 7.1, parvoviruses encode two major genes, a non-structural (NS or *rep*) gene that encodes a small number of replication proteins, by convention said to occupy the ‘left’ half of the coding sequence, and a structural (VP or *cap*) gene, which occupies the ‘right’ half. Almost all other structural characteristics, such as the sense of the packaged strand, the number of transcriptional promoters or polyadenylation signals, or even the direction of

transcription, can vary within and between genera. Figure 7.1 illustrates that while the great majority of viruses studied to date are ‘monosense’, meaning that all their gene products are encoded in tandem from a single DNA strand, two genera of arthropod viruses, the densoviruses and pefudenviruses, are ‘ambisense’, encoding their non-structural and capsid genes in opposite directions from each ITR.

Although each particle contains a single DNA molecule, these can be exclusively negative sense or of mixed polarity depending upon the individual virus species. This unusual characteristic is controlled by the relative efficiency of the replication origins associated with each terminus (Cotmore and Tattersall, 2005a), so that viruses with terminal repeats, which thus have equivalent origins at both ends, encapsidate plus and minus strands with equal efficiency, while viruses with different 3' and 5' origins package a variable proportion of the two strands, and frequently package only minus-sense strands, depending upon the precise efficiency of each terminus. In newly released virions, the 5' end of the genome remains covalently attached to a copy of the viral replication initiator protein (called NS1 in most genera, but Rep1 or Rep68/78 in the dependoviruses), which is located on the outside of the particle, attached to the protected bulk of the genome via a short ‘tether’ nucleotide sequence that projects through the capsid wall (Cotmore and Tattersall, 1989). This sequence, with its attached NS1, can be removed without impairing particle infectivity, and is generally cleaved during natural infection as the virus transits the extracellular environment or is trafficked through the endosomes of its prospective host cell.

In this section we explore the structure of these compact viral genomes, their DNA sequences, cis-acting replication signals and expression strategies.



**Figure 7.1** Comparison of genome strategies across the Parvoviridae. Diagrams of representative genomes from four of the nine genera of Parvoviridae, showing their terminal hairpin structures magnified approximately 10-fold with respect to the intervening single-stranded region. The positions of promoters are indicated by arrows, and their major gene blocks by open arrows indicating the N- to C-terminal direction.

Abbreviations: MVM, minute virus of mice; AAV2, adeno-associated virus type 2; GmDNV, *Galleria mellonella* densovirus.

## THE VIRAL CHROMOSOME

Genome size is strictly limited in the parvoviruses, possibly because viral particles that are small enough to enter the nucleus can only accommodate DNA strands of a certain size, with the largest genomes, of around 6 kb, being found in the *Densovirus* genus and the smallest, at 4 kb, in the *Brevipedensovirus*es. Genomes are introduced vectorially in a 3' to 5' direction into these particles, translocated via a reaction which, for AAV2, has been shown to require the helicase activity of the Rep40/52 proteins for completion (King *et al.*, 2001). Specifically, the first 1–2 kilobases from the 3' end of each strand are internalized relatively easily, perhaps even facilitated by interactions between the DNA and the amino acids lining the capsid as discussed below, but packaging the rest of the strand depends upon the availability of Rep40/52, which is a member of the viral SF3 helicase family that is structurally related to the AAA+ family of cellular ATPases (James *et al.*, 2003, Yoon-Robarts *et al.*, 2004). Rep40/52, possibly in association with full-length Rep68/78 molecules and possibly functioning as a hexameric ring, forms a helicase motor. This translates ATP hydrolysis into conformational changes that effectively translocate the single-stranded DNA into the particle shell, presumably via an, as yet unidentified, entry portal at one of the capsid

vertices. In MVM, sequences with conspicuous secondary structure, such as internal stem-loops or poly-G tracks, interfere with the packaging mechanism, frequently causing the residual 5' sequences from these strands to remain outside the particle, where they are susceptible to nuclease degradation (Cotmore and Tattersall, 2005b). One such stem-loop structure occurs near the left end of MVM, between nucleotides 218 and 350, where it induces folding in both genomic DNA and messenger RNA (mRNA). This results in transcriptional attenuation *in vivo*, leading to the accumulation of prematurely-truncated 150 nucleotide RNAs of unknown function (Ben-Asher and Aloni, 1984; Resnekov *et al.*, 1989; Spegelaere *et al.*, 1991; Perros *et al.*, 1994). Since MVM packages predominantly negative sense DNA, this sequence element occurs, and is tolerated, in the 3' half of the packaged negative strand, but it is highly deleterious to the packaging mechanism, and generally leads to incomplete encapsidation, when experimental manipulation causes it to be packaged last, at the 5' end of positive-sense strands (Cotmore and Tattersall, 2005b).

Difference electron-density maps, derived by comparing X-ray crystallographic data from MVMi virions and empty particles, show that just over a third of the viral genome exhibits some level of icosahedral symmetry within the particle (Agbandje-McKenna *et al.*, 1998). This symmetry,

which in MVM involves 29 nucleotides per icosahedral asymmetric unit, reflects the interaction of bases in the genomic single-strands with side chains of amino acids lining the inner surface of the capsid. The outer layer of DNA, at least, has an unusual loop conformation, with its bases hydrogen bonded to side chains from the capsid amino acids, while its phosphate groups, surrounding metal counterions, face towards the particle interior. The most ordered part of this density was first observed in canine parvovirus (CPV) virions, where it corresponds to 11 nucleotides per asymmetric unit, which make 15 putative hydrogen bonds with capsid amino acids (Chapman and Rossmann, 1995). These interactions are highly conserved in MVM, so that they effectively identify an icosahedrally repeated DNA-recognition site on the capsid interior, which changes conformation slightly upon introduction of the DNA. In CPV, analysis of the DNA electron density indicated preferences for particular bases in parts of this binding site, allowing a weak consensus sequence to be derived that corresponds to around 30 thymidine-rich sequences of 11 nucleotides in the CPV genome. Thus, optimal packaging or virion stability appears to require, or be facilitated by, reiterations of certain types of degenerate, thymidine-rich, DNA motifs in the chromosome.

When compared with cellular DNA, parvoviral genomes have a relatively high content of G+C nucleotides (41–53 percent), in part reflecting their high density of transcriptional regulatory elements. Members of some other virus families encode protein in multiple overlapping reading frames, but in parvoviruses this complexity is enhanced by the fact that transcriptional promoter and myriad splicing signals also lie embedded in the same primary sequence. Thus, for example, the MVM P38 promoter is specified by a DNA sequence that also encodes the carboxyterminal domain of NS1 and the major splice acceptor of the large intron. This inevitably imposes severe constraints on the primary nucleotide sequence, since mutations at many sites will affect multiple products or processes.

This apparent constraint makes the emergence of genetically disparate variants, such as the V9 and A6 isolates of human parvovirus B19, particularly remarkable, especially since the nucleotide sequence of these viruses diverge 11–12 percent from the reference isolate while their protein sequences remain highly conserved (Nguyen *et al.*, 2002). However, it is becoming increasingly apparent that parvoviral genomes can be highly heterogeneous *in vivo*, and frequently occur in infected tissues as a complex mixture of more-or-less viable quasispecies. Thus, Gottschalck *et al.* (1991, 1994) showed that Aleutian mink disease virus (AMDV) isolates derived from a single host animal could contain at least four distinct forms of the viral genome, and in a screen for endogenous AAV sequences Gao and colleagues (Gao *et al.*, 2003) cloned 12 VP1 sequences from the mesenteric lymph node of a single rhesus macaque. Even in experimental situations where genetically homogeneous viral stocks are administered, variants rapidly emerge, as

seen in a recent study by Almendral and colleagues (Lopez-Bueno *et al.*, 2003) who derived MVMi stocks directly by transfection from a plasmid clone of the genome to ensure their genetic homogeneity, but was subsequently able to isolate four genetically distinct monoclonal antibody-escape mutants of the virus from a single mouse. Thus viral replication *in vivo* appears to promote the generation of diversity. This is somewhat surprising since the genomes are replicated by a subset of the DNA synthetic machinery of their host cell, so that mutation rates would be expected to be low. However, viral replication involves a unidirectional, strand-displacement mechanism that may well exhibit lower fidelity than is normally associated with the bidirectional replication of cellular genes, and template strand switching appears to be highly prevalent. Moreover, within their host cell, viruses replicate through a series of concatemeric duplex intermediates, so that the possibility for efficient inter- and intramolecular recombination is high. Finally, and perhaps most significantly, because the virus is transmitted as a single strand, it carries with it no template for the correction of environmentally induced sequence changes. Thus viral genomes appear to be under intense selection, with their mode of replication and dissemination promoting diversity, but their constrained size, and hence their genetic complexity, severely restricting the types of modifications that can be tolerated.

Given this mode of replication, it is perhaps not surprising that many parvoviral genomes are highly repetitive. This is best documented for members of the genus *Parvovirus* where, for example, Bodnar (1989) showed that the nine-nucleotide sequence, 5'-AACCAACCA-3', makes up approximately 10 percent of the DNA of MVM. This sequence was later shown to be a simple form of the duplex DNA-binding site for NS1, which itself is a reiterated form of the tetranucleotide 5'-ACCA-3' (Cotmore *et al.*, 1995). While degenerate oligonucleotide selection (SELEX) studies suggest that optimal forms of this site should contain at least three tandem direct repeats of the 5'-ACCA-3' motif, many more-degenerate sites also show significant NS1-binding activity when assayed in the presence of ATP, which causes NS1 to assemble into multimers. NS1 binds asymmetrically over these motifs in the presence of ATP, protecting a 41–43 basepair sequence that approximately aligns with the 3' end of the (ACCA)<sub>2–3</sub> sequence but extends ~20 basepairs 5' of the consensus. Thus, inverted pairs of binding sites would be expected to show diminished binding, due to competition for space abutting the recognition site, but these actually bind NS1 with higher affinity than equivalent isolated sequences (Cotmore *et al.*, 2000). Such binding thus appears to promote the assembly of more stable, rearranged or higher order, NS1 complexes, suggesting that the tandem and inverted motif reiterations that are scattered throughout the viral genome could promote and modulate the binding of NS1 over the entire chromosome. The frequency and distribution of its binding sites thus suggests a role for NS1 in viral chromatin structure.

In contrast, genomes from members of the *Erythrovirus* and *Dependovirus* genera appear to have far fewer copies of their equivalent NS1 or Rep68/78 protein binding sites. For the human erythrovirus B19 the NS1 recognition sequence, 5'-(GCCGCCGG)<sub>2</sub>-3', is found only in the hairpins, although its NS1 does also bind with low affinity to a cognate Sp1 site in the viral P6 promoter (Christensen, 2002). In the dependoviruses AAV2 and AAV5, the Rep68/78 binding site consists of between 4 and 6 more-or-less degenerate direct repeats of the tetranucleotides 5'-GAGC-3' (Ryan *et al.*, 1996) and 5'-GCTC-3' (Chiorini *et al.*, 1999), respectively, which only occur in the viral hairpins and P5 promoters. Whether or not this implies a fundamental difference in the chromatin structure of the various genera remains to be determined, but studies of the size of nucleosomal elements protected from digestion by DNase1 suggest that there are differences. Thus, whereas adeno-associated virus (AAV) DNA gives the characteristic ~160 base nucleosome repeat pattern associated with cellular DNA (Marcus-Sekura and Carter, 1983), MVM does not, but, when cross-linked with psoralen, appears to exist in protein-associated domains of  $90 \pm 29$  nucleotides, perhaps reflecting the presence of higher-order NS1-DNA complexes (Doerig *et al.*, 1986). Recent X-ray crystallographic studies of the AAV5 amino-terminal nuclease domain complexed with oligonucleotides mimicking its reiterated 5'-GCTC-3' binding-site have gone a long way to clarify our understanding of how these proteins interact with their recognition motifs, and in particular to explain why binding appears modular and why sequence substitutions and insertions between the reiterations are tolerated (Hickman *et al.*, 2004). Specifically, the AAV5 Rep68 nuclease domain appears to bind as a hexamer, in which five Rep monomers bind five tetranucleotide direct repeats, each repeat being recognized by two Rep monomers from opposing faces of the DNA, and each monomer making contact with two nucleotides from each of two adjacent direct repeats (Hickman *et al.*, 2004). Whether or not higher-order Rep complexes can assemble *in vivo*, as suggested for MVM, remains to be addressed.

Defective genomes also provide fossil evidence for frequent sequence reiterations within the DNA. For example, internally-deleted 'type 1' defective MVM genomes (Faust and Ward, 1979) were shown to have recombined not only at sequences now recognizable as minimal NS1 binding sites (Hogan and Faust, 1984; Cotmore *et al.*, 1995), but also at degenerate forms of the NS1 nick site, 5'-CTWWTC-3 (Hogan and Faust, 1986; Cotmore and Tattersall, 1994) that are similarly scattered throughout the viral coding sequences. This tendency for sequence reiteration may well be the result of recombination or strand switching by the unidirectional replication forks during synthesis of long linear molecules, as discussed previously. Virus genomes also frequently contain somewhat larger blocks of duplicated sequence. In the prototype strain of MVM, MVMp, a 65 basepair (bp) sequence located near the hairpin at the right end of the genome is duplicated, while only a single copy of this sequence occurs

in the lymphotropic MVMi strain. In MVMp this insertion occurred at the position of a strong NS1 binding site, which now flanks the repeat. CPV also has a 60 bp tandem repeat overlapping the end of the capsid protein coding sequence, and the rodent virus H1 has a 55 bp repeat sequence positioned 23 nucleotides upstream from the start of the MVM repeat (Rhode and Iverson, 1990). Since these repeats are not located in identical positions, and some viruses lack them, they may well be generated by replication anomalies such as looping of the DNA between NS1 binding sites, and be tolerated or favored as long as the resulting genome approaches, but does not exceed, optimal packaging limits. Defective interfering particles of H1, generated by serial propagation at high multiplicity, were found to contain up to 10 copies of this repeat, suggesting that it is easily generated and that it may confer a selective cis-acting advantage on the molecule, since these genomes appeared to accumulate at the expense of wild-type DNA (Rhode, 1978). A positive role for these elements is also suggested by recent data, showing that the presence or absence of the MVM repeat is rapidly selected for, or against, during viral expansion in cells of particular differentiated phenotypes (Etingov *et al.*, 2004), although whether or not such repeats do benefit the replicon remains controversial (Tam and Astell, 1993; Cossons *et al.*, 1996).

The significance of another group of sequences from the region just inboard of the MVM right-end hairpin (commonly dubbed the 'Rsa' fragments, spanning nucleotides 4489 and 4695) remains similarly controversial. Specifically, these sequences appear to potentiate the replication and/or packaging efficiency of experimentally truncated genomes, dubbed 'minigenomes' by Tam and colleagues (Tam and Astell, 1993, 1994), or of some viral vector sequences in which DNA from this region has been substituted with foreign genes (Kestler *et al.*, 1999). However, the exact nature of these sequence elements and the role they play in replication remains equivocal. Astell and colleagues showed that DNA from this region binds a series of as yet unidentified cellular factors, and have mapped these interactions to three short DNA elements that also appear to promote replication (Brunstein and Astell, 1997). They postulate that the sequences constitute an 'internal replication sequence' (IRS) that promotes amplification by serving as a leading-strand replication origin. According to this scenario, DNAs initiating from this IRS would serve to destabilize extended-forms of the right-end hairpin, thus allowing it to reconfigure into 'rabbit-ear' structures that are essential for priming successive rounds of viral replication. This role appears unlikely, since NS1 is known to melt the telomere directly, in an ATP-dependent reaction, by binding to NS1 recognition sequences that immediately flank the axis of the palindrome (Baldauf *et al.*, 1997; Willwand *et al.*, 1997, 1998, 2002), but exactly what other role the identified sequence elements might play is unclear. Recent experiments, in which transgene-encoding viral vectors that replicated very poorly were rescued by insertion of

synthetic NS1-binding sequences into this region of the genome (Zhang and Tattersall, unpublished) suggest that sequestering NS1 near the right-end origin may substantially promote replication efficiency. However, the sequences mapped by Astell and colleagues are not NS1 binding sites. Possibly they contribute to the spacing of NS1 molecules along this region of the genome, as discussed previously, or interact with cellular factors that promote NS1 accumulation, and hence load this dominant origin with reservoirs of its essential, multifunctional, effector molecule.

## THE VIRAL TELOMERES

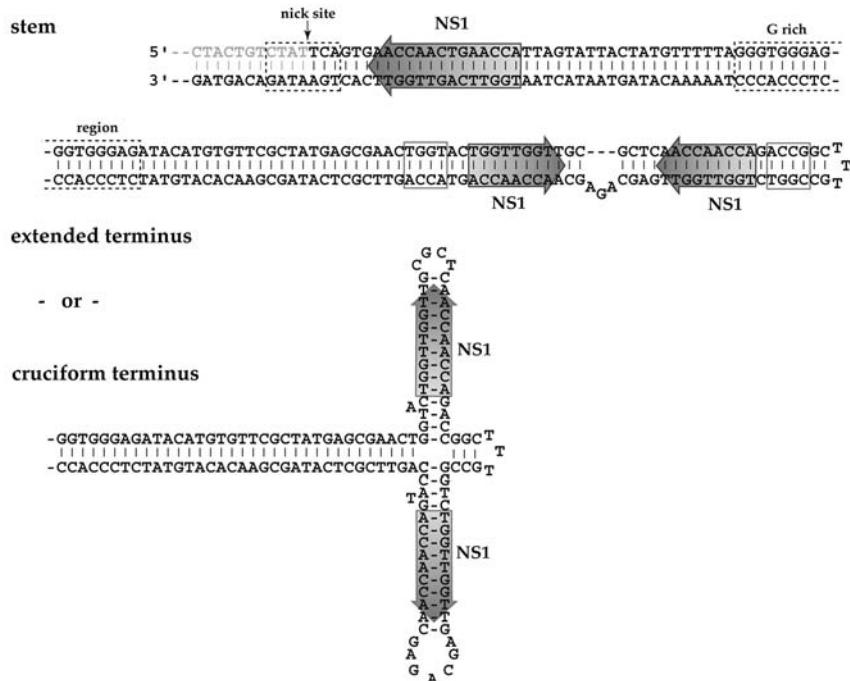
Short imperfect palindromes at each end of the genome fold back on themselves to form duplex telomeres in which a fully, or predominantly, basepaired stem region terminates in some sort of axial asymmetry, creating the secondary structures, illustrated in Figures 7.1 and 7.2. In these repeats the terminal nucleotide is paired to an internal base. Together with a few flanking nucleotides, these palindromes contain all of the cis-acting information needed to support viral DNA replication and packaging. Copying the extreme 5' end of linear DNA molecules is problematic because DNA polymerases synthesize in a 5' to 3' direction and absolutely require a basepaired primer. Parvoviruses solve this problem by using their terminal hairpins as primers for complementary strand synthesis, later cutting the palindrome at the inboard end of the original hairpin and copying it separately. The simplest form of this reaction, called 'terminal resolution', results in the inversion of the terminus with each round of replication, and since all parvoviral palindromes are somewhat imperfect, this generates hairpins in two forms, flip and flop, which are the inverted complements of each other. Viral replication mechanisms are reviewed in detail elsewhere in this book (Chapters 14 and 15). Within the cell replication proceeds through a series of concatemeric duplex linear intermediates, via a rolling-hairpin mechanism, in which the palindromes effectively act as hinges, allowing the direction of synthesis to be reversed at the end of each unit-length genome, and thus adapting the continuous, unidirectional rolling-circle replication strategy for the amplification of linear chromosomes. Their ability to act as hinges appears to depend upon the presence of the imperfectly, or alternately, basepaired regions surrounding the axis of each terminus, which likely provide an energetically favorable environment for unfolding and refolding the hairpin. These asymmetries commonly take the form of small internal palindromes, potentially creating Y- or T-shaped structures such as those found at the left end of MVM and in AAV (Figure 7.1). Alternatively, the termini may be severely asymmetric, as in the *Amdovirus* and *Iteravirus* genera, or simply contain one or more clusters of mismatched residues near the axis. For want of a better term, in this

article we have used the designation 'arrow-like' (Zadori *et al.*, 1995) to describe a wide range of variously mismatched axial regions that do not assume any easily described secondary structure, as in the B19 genome shown in Figure 7.1. As disposed in monomer or dimer duplex RF intermediates, sequences from each hairpin then create a viral replication origin, where the initiator protein (NS1 or Rep68/78) is able to nick the DNA via a trans-esterification reaction, separating unit-length genomes and allowing the termini to be duplicated through the establishment of new replication forks.

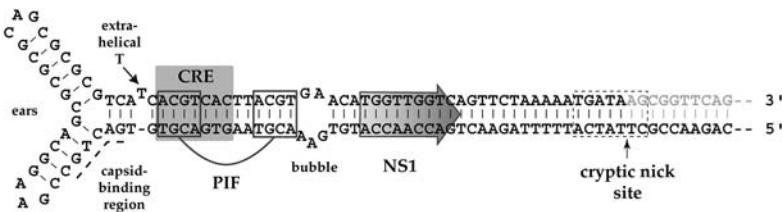
Thus all hairpins have at least two functions: to create a replication origin that will allow the DNA to be nicked and used as a DNA primer; and to function as a hinge that can be used to reorient the replication fork at the end of the linear genome, allowing forks to roll back and forward along the linear DNA. Additionally, hairpins may contain transcriptional control elements for nearby viral promoters, and/or sequences or structures that promote interactions between the viral DNA and the capsid. In many genera the palindromes are part of inverted terminal repeats (ITRs), so that the sequences at both ends of the genome are closely related, but in other genera they are disparate. As a general rule, viruses with identical termini replicate both hairpins by terminal resolution, while viruses with unique termini replicate the right end by terminal resolution and the left by an asymmetric mechanism called junction resolution. Where present, this asymmetry appears to have become essential for the virus (Burnett and Tattersall, unpublished), possibly because it allows sequences in the outboard arm of the left hairpin to be dedicated to replication (Cotmore and Tattersall, 1994) while those in the inboard arm are fine-tuned for transcriptional control of the adjacent early promoter (Gu *et al.*, 1995; Perros *et al.*, 1995; Fuks *et al.*, 1996; Deleu *et al.*, 1999). In this case, the decision to use left-end sequences arranged in a particular configuration for nicking or transcription is mediated by the binding of cellular factors, which either interact with nearby transcription initiation complexes or stabilize the binding of NS1 (Christensen *et al.*, 2001), allowing it to melt the duplex and nick the origin.

As illustrated in Table 7.1, known hairpins range in size from 117 to 418 nucleotides. In some genera the terminal repeats and hairpins are both large, in others they are both small, or the repeat may be large, but the hairpin small, and even within a single genus (the *Dependoviruses*) hairpins may be minimally small (125 bases in AAV2) or the largest currently on record (418 bases in MDPV). These differences are difficult to understand, but relatively few of the hairpins have been dissected experimentally to determine which sequences are required for viability or what alternate functions the various elements may fulfill. Below we review the structure and function of the more-extensively studied termini from MVM and AAV, and then briefly explore the diversity of these elements through the parvoviral genera.

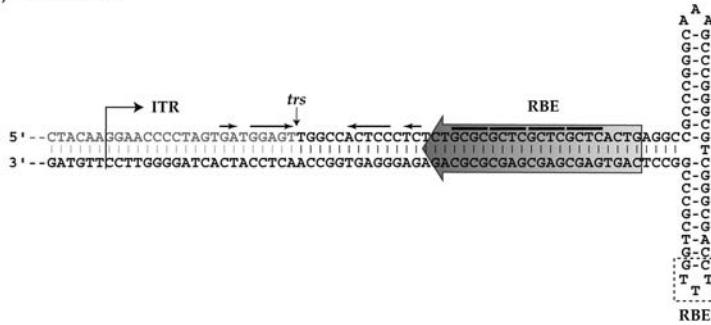
## (A) MVM RHE



## (B) MVM LHE



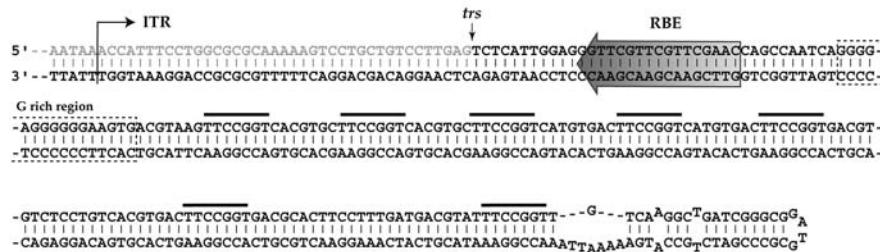
## (C) AAV2 RHE



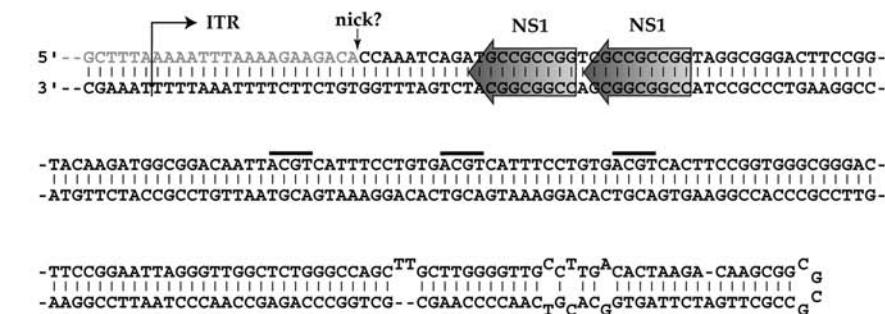
**Figure 7.2** The telomeres of the Parvoviridae. DNA sequences and predicted secondary structure for the terminal hairpins of representatives of each of the nine genera of Parvoviridae. For those genomes with inverted terminal repeats (ITR), the whole ITR is shown, except for the ~550 nucleotide ITR of GmDNV. For genomes with ITRs, only the right-hand end hairpin is depicted, in the 'flip' configuration, and the inboard end of the ITR is marked by a rightward arrow. Where known, the binding sites for the replicator protein, NS1 or Rep, are indicated by the shaded arrow boxes, and their sites of nucleolytic cleavage are represented by vertical arrows. For left-hand termini that are not nicking substrates in their hairpin configuration, this site is denoted as cryptic. Where the nick site is not experimentally established, the vertical arrow indicates the end of the sequenced genome.

Abbreviations: AaeDNV, Aedes aegypti densovirus; AMDV, Aleutian mink disease virus; BmDNV, Bombyx mori densovirus; BPV, bovine parvovirus; CRE, cyclic AMP response element; LHE, left-hand end; MDPV, Muscovy duck parvovirus; PfDNV, Periplaneta fuliginosa densovirus; PIF, parvoviral initiation factor; RBE, Rep-binding element; RHE, right-hand end; trs, terminal resolution site; other acronyms as described in the legend for Figure 7.1.

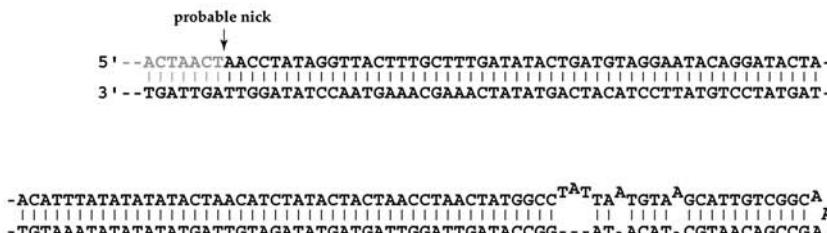
## (D) MDPV RHE



## (E) B19 RHE



## (F) AMDV RHE



## (G) AMDV LHE

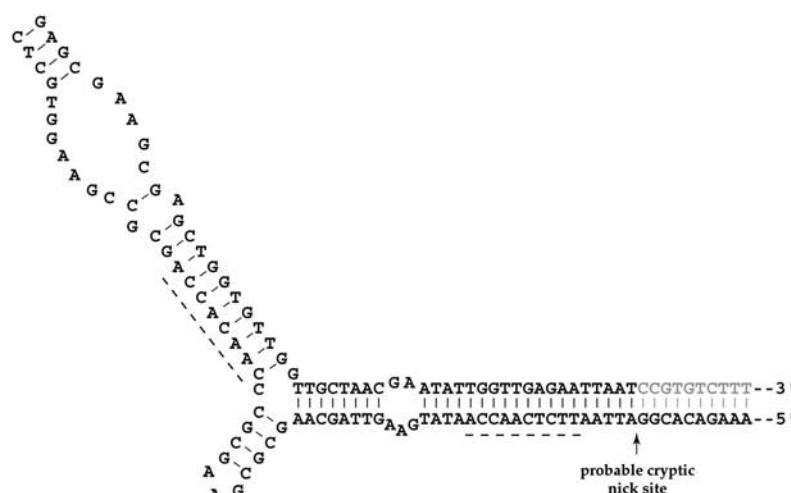
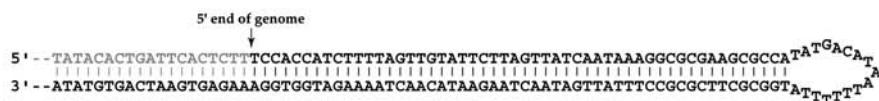
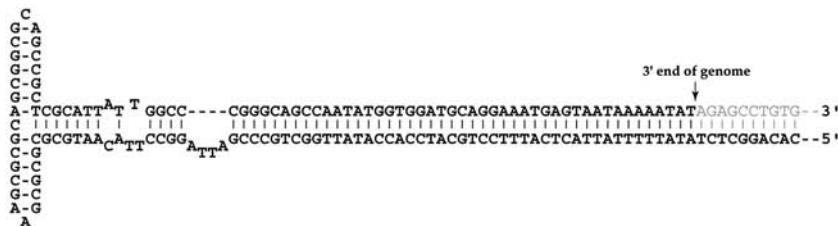


Figure 7.2 (continued).

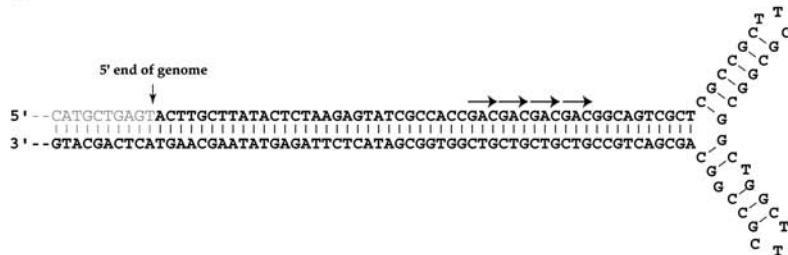
(H) BPV RHE



#### (I) BPV LHE



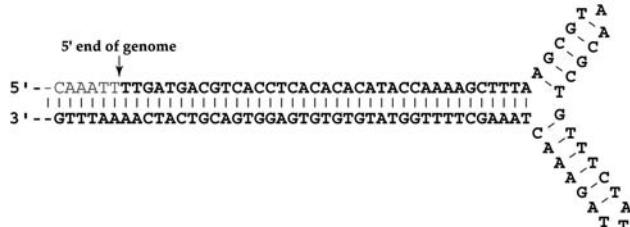
(J) **GmDNV RHE**



(K) **BmDNV RHE**



(L) AaeDNV RHE



**Figure 7.2** (continued).

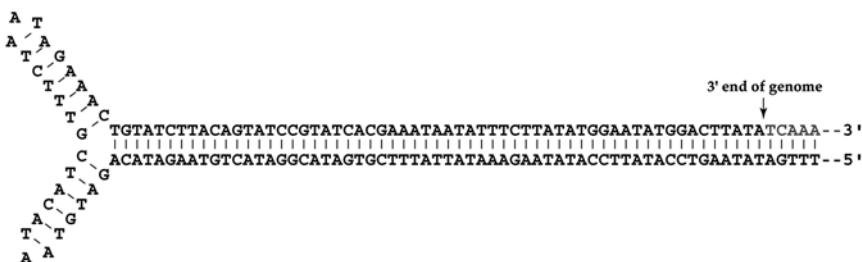
(M) *Aae*DNV LHE(N) *Pf*DNV RHE

Figure 7.2 (continued).

Table 7.1 Genomic characteristics of representative members of Parvovirus genera

Genus/Virus	Genome size*	Sense	Packaging polarity	ITR*	LEH*	REH*	Telomere structure	Gene bank accession number
<b>Parvovirus</b>								
MVMp	5170	mono	~5/95		121	248	Y-shaped Linear/cruciform	J02275
Lulli	5136	mono	50/50		121	248	Y-shaped Linear/cruciform	M81888
<b>Erythrovirus</b>								
B19	5592	mono	50/50	383	365	365	Arrow-like	M13178
<b>Dependovirus</b>								
AAV2	4780	mono	50/50	145	125	125	T-shaped	J01901
AAV5	4642	mono	50/50	145	125	125	T-shaped	AF085716
MDPV	5132	Mono	50/50	457	418	418	Arrow-like	X75093
<b>Amdovirus</b>								
AMDV	4748	mono	~5/95		117	242	Y-shaped Arrow-like	M20036
<b>Bocavirus</b>								
BPV	5517	mono	10/90		150	121	T-shaped Arrow-like	M14363
<b>Densovirus</b>								
GmDNV	6039	ambi	50/50	550	136	136	Y-shaped	L32896
<b>Iteravirus</b>								
BmDNV	5076	mono	50/50	230	159	159	Asymmetric J	AY033435
<b>Brevidensovirus</b>								
AaeDNV	3978	mono	15/85		146	164	T-shaped T-shaped	AY160976
<b>Pefudensovirus</b>								
PfDNV	5454	ambi	50/50	201	122	122	Arrow-like	AF192260

Abbreviations: ITR, inverted terminal repeat; LEH, left-end hairpin; REH, right-end hairpin.

\* Genome, ITR, LEH and REH lengths reported in nucleotides.

Packaging polarity: percent +ve strands/-ve strands in virion.

## Parvovirus: the right-end hairpin of MVM

At 248 nucleotides the right-end hairpin of MVM is approximately twice the size of many parvoviral hairpins, including its own left-end telomere (Table 7.1). As illustrated in Figure 7.2A, this palindrome is most favorably arranged as an almost perfect duplex with just three unpaired bases at the axis and a single mismatched region positioned in the stem 20 nucleotides from the axis. Here a three-nucleotide insertion (AGA or TCT) on one strand separates opposing pairs of NS1 binding sites, creating a 36 basepair palindrome that could potentially assume an alternate cruciform configuration with little change in free energy, as shown in Figure 7.2A, p. 78. Whether or not this cruciform rearrangement is required for viability has yet to be demonstrated experimentally, but it would be expected to contribute significantly to the instability of the duplex that allows it to function as a hinge. This hairpin exists in both flip and flop orientations, indicating that it is replicated by a hairpin transfer mechanism as described in Chapter 14.

Both the hairpin and fully basepaired linear forms of this sequence function as NS1-dependent replication origins *in vitro* (Cotmore and Tattersall, 1998; Cotmore *et al.*, 2000), and almost the entire 248 base sequence of the telomere is required for this activity. At least three specific recognition elements are essential for origin function, as shown in Figure 7.2A. These elements are:

- a duplex NS1 recognition sequence in the stem of the hairpin, that orients an NS1 complex over the adjacent nick site;
- the nick site consensus, 5'-CTWWTCA-3', positioned seven nucleotides upstream from the NS1-binding site, and which must ultimately be exposed in a single-stranded form, perhaps stabilized by a minimal stem-loop structure; and
- a second NS1 binding site located immediately next to the axis of the hairpin, outboard of the AGA asymmetry. This latter site is positioned ~120 bp from the nick site, but is essential for NS1-mediated cleavage.

Interactions between NS1 molecules bound to these various recognition elements are mediated by a cellular DNA-bending protein from the HMG1/2 family, which is absolutely required for formation of the cleavage complex (Cotmore *et al.*, 2000). In the closely related virus LuIII, an additional AT dinucleotide is present immediately upstream of the nick site, which dampens the efficiency of this otherwise dominant viral origin. This modification allows LuIII to displace and package positive-sense DNA in addition to the negative-sense strands encapsidated by all other members of the *Parvovirus* genus (Cotmore and Tattersall, 2005a).

The ability of the axial region to reconfigure into a cruciform does not appear to be critical for right-end origin function, since a third NS1 recognition sequence, positioned

immediately inboard of the AGA asymmetry, can be mutated without impairing nicking efficiency *in vitro*, even though this mutation completely disrupts the potential cruciform structure (Cotmore *et al.*, 2000). Cleavage is also absolutely dependent upon the correct spacing of the various elements, since insertions or deletions of more than one base at an AflIII site in the stem are lethal, but sequence substitutions at this position are permissible. DNase I footprinting studies show that NS1 protects all three binding sites in the wild-type origin, with the NS1 complexes disposed asymmetrically over their recognition elements. Addition of the essential co-factor HMG1 induces a change in the conformation of the DNA, causing a double helical loop to be extruded in the vicinity of a characteristic G-rich element in the stem, indicated in Figure 7.2A, p. 78. Between this sequence and the nick site there is also a stretch of five thymidine residues, which would be expected to make the duplex more rigid, and a region of alternating A and T residues, which sequences characteristically facilitate melting and are a common feature of eukaryotic origins. Whether or not these elements are required for initiation is uncertain, but any mutant origins which fail to reconfigure into a double helical loop upon addition of HMG, also fail to nick, suggesting that the structure of this entire telomere is critical (Cotmore and Tattersall, unpublished results).

Although replicated by a simple hairpin transfer mechanism, cellular replication forks are unable to unfold and copy this hairpin when acting alone (Baldauf *et al.*, 1997). However, addition of NS1 allows unfolding to proceed, suggesting that it directly mediates this reaction *in trans* via an as yet unidentified interaction. Similarly, the ability of duplex extended-form copies of the right-end telomere to melt out and fold back on themselves (i.e. reconfigure into so-called 'rabbit-ear' structures) is greatly enhanced by NS1 (Willwand *et al.*, 1998), and in this situation it has been shown that the NS1-binding sites at the tip of the hairpin, immediately next to the axis, are required (Willwand *et al.*, 2002). *In vitro* this latter reaction remains extremely inefficient, and it seems likely that other (as yet unidentified) cellular factors are required to promote the rearrangement (Cotmore and Tattersall, unpublished).

When propagated as part of a bacterial plasmid, extended forms of the MVM right-end hairpin are prone to suffer a series of deletions that remove the axial NS1 binding sequences, and these are lethal for the virus. Many other parvoviral hairpins sustain similar, or worse, deletions upon propagation in *coli*, and it is thus important to note that several bacterial strains are available that impede such recombination. Boissy and Astell (1985) first identified a recBCsbcBrecF host, JC8111, that fulfilled this requirement, although more-recently the commercially available Sure strains (Invitrogen) have also proven effective.

It was noticed early on that the right-end hairpin of MVM virion DNA is often ~20 basepairs shorter than the sequence shown in Figure 7.2A, p. 78. This is because the

extreme 5' end of the palindrome, with its covalently-attached NS1 molecule, always remains outside the viral particle, attached to the protected bulk of the genome via a short 'tether' nucleotide sequence that projects through the capsid wall (Cotmore and Tattersall, 1989). Mature virions are released from the parental cell in this form, but the exposed 5' end of the DNA is not required for infectivity and is susceptible to nucleolytic attack both in the external environment and during entry, so that it is frequently missing from virion populations. A similar situation likely exists for the 5' ends of all packaged parvoviral DNA, but it is more conspicuous in viruses like MVM, which only package single-sense strands, since the same end of the DNA is always exposed and cleaved.

### **Parvovirus: the left-end hairpin of MVM**

The 121 base left-end hairpin of MVM can assume a Y-shaped configuration containing a 43 basepair duplex stem and small internal palindromes giving rise to the 'ears', as illustrated in Figure 7.2B, p. 78. Interrupting the stem is a mismatched 'bubble' sequence, where a GA dinucleotide in the outboard arm of the hairpin is paired with a GAA triplet, and the outboard arm also contains a single asymmetric thymidine residue positioned near the ears. In negative-sense virion DNA this hairpin is only expressed in one orientation, dubbed flip, as shown, and it is unusual because it cannot function as a replication origin in its hairpin configuration, as discussed in Chapter 14. Sequences from this hairpin are involved in both replication and transcriptional control, with the elements involved in these two processes effectively segregating in the outboard and inboard arms of the hairpin, respectively.

The minimal linear duplex origin derived from this hairpin is ~50 basepairs long, contains the dinucleotide, 'GA' sequence from outboard form of the bubble, and extends from the two 5'-ACGT-3' motifs spaced five nucleotides apart seen adjacent to the hairpin ears in Figure 7.2B, p. 78, to a position some seven basepairs beyond the nick site (Cotmore and Tattersall, 1994). While the actual sequence of the 'GA' doublet is relatively unimportant, insertion of any third nucleotide here inactivates the origin, indicating that the bubble is a critical spacer, rather than a recognition element in its own right, and that it is this bubble asymmetry which prevents the equivalent sequences from the inboard arm of the hairpin, and the hairpin configuration itself, from functioning as a replication origin. As in the right-end hairpin, this origin contains three essential recognition sequences: (1) the modular NS1 binding site that orients the NS1 complex over (2) the consensus nick site, 5'-CTWWTCA-3', and (3) the two ACGT half sites. The latter bind a heterodimeric cellular factor called PIF, for parvovirus initiation factor (PIF) (Christensen *et al.*, 1997, 1999) which, in the active form of the origin, interacts with

and stabilizes NS1 molecules bound to the reiterated 5'-ACCA-3' NS1 recognition motif, allowing them to unwind the DNA of the nick site prior to nicking. PIF is unable to establish a similar interaction with NS1 over the trinucleotide bubble sequence present in duplexes derived from the inner arm of the hairpin, so that these sequences remain refractory to nicking (Christensen *et al.*, 2001).

The left-end hairpin also contributes upstream transcriptional control elements to the viral P4 promoter. As seen in the Figure 7.2B, a cyclic AMP-responsive element (CRE) overlaps the distal PIF half site in this origin, which has been shown to contribute to basal levels of P4 promoter activity and to the upregulation of P4 in ras-transformed cells (Perros *et al.*, 1995, 1999). Hypothesizing that factors binding to the PIF and CRE sites would compete with each other during infection, Burnett and Tattersall separately mutated residues that were known to contribute to one or other binding site (Burnett and Tattersall, 2003). As expected, destroying the PIF binding site was lethal, and led to the accumulation of 10 kb duplex dimer RF, although mutant genomes were fully able to initiate infection as measured by expression from the P4 promoter following transfection. In contrast, mutants lacking the CRE site were viable but impaired, with P4 initiation efficiencies 10-fold lower than normal in murine A9 cells, but 100-fold lower than wild-type in the transformed human cell line 324K, suggesting that a functional CRE plays a significant role in initiating infection in general, but may be particularly important in certain transformed cell types. Interestingly, a mutant lacking the single unpaired thymidine residue positioned near the hairpin ears (Figure 7.2B, p. 78), showed a similar pattern of cell-specific P4 impairment, suggesting that this asymmetry is also implicated in transcriptional control, although it is also feasible that it simply exerts its effect by modifying the three-dimensional structure of the hairpin.

Finally, Willwand and colleagues identified a third type of recognition element in the MVM left-end hairpin. These authors showed that hairpin forms of the terminus bind to empty capsid particles, and they used hydroxy-radical footprinting to map this interaction to the branch point between the hairpin stem and the ears, as indicated in the figure (Willwand and Hirt, 1991). A remarkably strong interaction of this type has also been observed when attempting to use cellular factors to induce MVM virions to uncoat *in vitro* (Cotmore and Tattersall, unpublished), but the assays for this interaction are not yet robust, and are reported to be difficult to reproduce in other parvoviral systems (King *et al.*, 2001). Willwand and colleagues suggested that these interactions could direct the 3' to 5' DNA packaging process. Alternatively, they could serve to keep the genome associated with the capsid after entry into the host cell cytoplasm, since capsid transitions that expose the VP1 amino-terminus have occurred by this stage (Vihinen-Ranta *et al.*, 2002), and *in vitro* such transitions also expose the viral DNA (Cotmore *et al.*, 1999).

## Dependovirus: the termini of AAV

The 4679 base AAV2 genome has ITRs of 145 nucleotides, the first 125 of which form an imperfect palindrome that can assume a ‘T’-shaped hairpin when folded to maximize basepairing (as shown in Figure 7.2C, p. 78). In this configuration only seven bases remain unpaired, six at the axes of the arms and the other separating the arms. Hairpins from AAV 1 through 4 are >95 percent identical, and while AAV5 has a slightly different resolution site, it is otherwise very similar. Recently, new AAV-like avian (Bossis and Chiorini, 2003) and snake (Farkas *et al.*, 2004) viruses have been isolated and cloned. These have genomes of 4694 and 4432 nucleotides and terminal repeats of 142 and 154 nucleotides, respectively, and both have distinct T-shaped hairpins of 122 nucleotide. The ITRs of AAV5 are around 60–62 percent homologous with those of serotypes 1 through 4, and 48 percent homologous with serotype 5. AAV hairpins are among the smallest known to be replicated by hairpin transfer, but other members of the *Dependovirus* genus, the helper-independent avian viruses, have much larger termini of 407–418 nucleotides, described below (Figure 7.2D, p. 79). Whether or not these autonomous virus termini provide additional signals or functions that are not required by AAV, remains uncertain.

The AAV hairpins serve as origins when presented in their hairpin form at the end of a duplex replicative-form DNA intermediate, but these sequences also function, albeit less efficiently, when presented in a simple linear configuration, as discussed in detail in Chapter 15. For these viruses just two sequence elements are absolutely required for origin function: a 22 basepair rep-binding element (RBE, equivalent to the MVM NS1 recognition motif), centered around the core tetranucleotide repeat, 5'-GAGC-3', that positions Rep68/78 complexes on the DNA in the correct orientation and position for cleavage; and a terminal resolution site (*trs*, equivalent to the MVM consensus nick site), positioned 16 nucleotides inboard of the RBE, where RBE-bound Rep complexes cleave an exposed single-strand of DNA. A third recognition element 5'-GTTTC-3', called the RBE', is positioned at the tip of the hairpin arm opposite the nick site (Ryan *et al.*, 1996). While not absolutely required for nicking, this motif is contacted by one monomer from the Rep complexes positioned on the RBE (Hickman *et al.*, 2004), and enhances origin efficiency by 20- to 100-fold (McCarty *et al.*, 1994). Short palindromic sequences flanking the *trs*, indicated in Figure 7.2C by arrows above the sequence, then stabilize exposure of the critical phospho-diester bond by folding into a stem-loop structure (Brister and Muzyczka, 1999) that also effectively moves the cleavage site towards the active site of the RBE-bound Rep complex. Initiation efficiency at this origin can be enhanced by, but is not dependent upon, the presence of the cellular DNA bending protein, HMG1 (Costello *et al.*, 1997). Although the hairpin exists in two forms, flip and flop, at both ends of the genome, the RBE' sequence is present in the same position relative to the

nick site in both forms. Since its juxtaposition to the Rep complex is determined by the hairpin configuration of the telomere, linear origin sequences, found at the end of many duplex replicative-form DNA intermediates *in vivo*, present suboptimal substrates for rep. The precise position of the nick appears slightly flexible *in vivo*, plus or minus one base, with approximately 50 percent of virion DNA being cleaved between the nucleotides indicated in Figure 7.2C.

While the minimal requirements for replication initiation are an RBE and an appropriately spaced *trs*, the hairpin ears are required both to create a three-dimensional structure that optimizes this reaction and, critically, to allow the hairpin to function efficiently as a toggle switch. Surprisingly deletion of almost an entire hairpin (<113 nucleotides) from one end of the genome is tolerated, because the missing sequences can be repaired to wild type using the other terminal repeat (TR) as a template (Samulski *et al.*, 1983). However, deletion of 55 nucleotides from both termini, which resects into the base of the outer ear, is lethal, indicating that these asymmetries are essential for hairpin function. Similarly, deletion of an 11 base symmetrical sequence from hairpins at both ends of the genome, effectively removing one of the cross-arms of the T, is lethal, while substitution of an alternative 8 or 12 base sequence that differs in sequence but maintains the symmetry of the palindrome, results in viable AAV carrying the mutation. This suggests that the structure, rather than the sequence, of the hairpin axis is of primary importance for viability (Lefebvre *et al.*, 1984, Bohenzky *et al.*, 1988), although undoubtedly during evolution the primary sequence of these ears has become optimized for rep-mediated activation. Binding of Rep to the RBE is probably also essential for both unfolding and refolding the duplex during hairpin transfer. Rep possesses 3'-to-5' helicase activity, which is activated when Rep binds to an RBE (Zhou *et al.*, 1999), so that it likely serves to unwind the duplex, while the hairpin ears provide the necessary hinge element. Certainly the *trs* region is not required for such conformational changes (Ward and Berns, 1995), and whether or not additional rep-mediated contacts in the ears promote these reactions appears uncertain. Such additional contacts are clearly required for the larger MVM right-end hairpin, as discussed previously.

## Dependovirus: the termini of the helper-independent avian viruses

Helper-independent avian viruses from the genus *Dependovirus* (goose parvovirus, GPV, and muscovy duck parvovirus, MDPV) have inverted terminal repeats of 444 and 457 bases respectively of which the distal 407 and 420 nucleotides can fold into hairpins expressed in both flip and flop sequence orientations (Zadori *et al.*, 1995, Yoon *et al.*, 2001). These telomeres have been described as assuming an ‘arrow-like’ structure, in which a perfectly duplex stem, of 181 and 187 basepairs respectively, terminates in less

symmetrical axial regions of 44 and 46 nucleotides where pairing is intermittent, as shown in Figure 7.2D, p. 79. As in AAV, an RBE sequence, made up of a series of tetranucleotide repeats, aligns the Rep1 initiator complex over the *trs*. However, the sequences of the RBE (5'-GTTC-3') n and *trs* are somewhat different from those of AAV2, and the RBE is spaced 12 nucleotides from the nick site, compared with 16 nucleotides in AAV2. Yoon *et al.* (2001) showed that site-specific binding to the RBE and initiation by GPV Rep1 or AAV2 Rep78 was origin-specific *in vitro*, and used Rep1/Rep78 chimeras to show that this specificity mapped to the 235 and 232 amino-terminal residues of Rep1 and Rep78, respectively. Despite efficient binding, chimeric origins comprising the GPV RBE and the AAV *trs* were poorly cleaved by Rep1 or the Rep1/Rep78 chimera, so that the *trs* sequence and/or the spacing of these elements also exhibits species specificity.

As seen in Figure 7.2D, p. 79, just outboard from the RBE in the MDPV hairpin there is a G-rich element that resembles a sequence involved in loop-formation in the MVM right-end origin. In MDPV this is followed by a remarkable series of direct repeats. For example, the heptanucleotide, 5'-TTCCGGT-3', occurs seven times in the remaining hairpin stem, with the first five of these being separated by perfect or almost perfect copies of the sequence 5'-CACGTGC-3. Notably, this latter repeat element contains the 5'-ACGT-3' motif of the PIF half-site that is essential in the left-end origin of MVM, but this is a common recognition motif and also contributes to a large number of potential transcription factor binding sites that appear to be distributed through the stem. Not surprisingly, these hairpins are prone to deletion when replicated as bacterial plasmids, but proved sufficiently stable in the Sure strain to allow infectious viral clones to be constructed (Zadori *et al.*, 1995). The significance of most of these sequences remains untested *in vivo*, but only the *trs* and RBE are absolutely required for origin function *in vitro* (Yoon *et al.*, 2001).

## Erythrovirus: the termini of B19

Parvovirus B19 is 5592 nucleotides in length and has inverted terminal repeats of 381 residues, of which the terminal 361 can be folded into hairpins expressed in the two alternate, flip and flop, sequence orientations (Deiss *et al.*, 1990, Zhi *et al.*, 2004b). An NS1-binding site, made up from two copies of the GC-rich octanucleotide 5'-GCCGCCGG-3', positions the initiator over the nick site (Christensen, 2002). Like the avian dependoviruses, this telomere consists of an arrow-like structure in which a perfect duplex stem of 145 bps terminates in an axial region of 71 nucleotides, where pairing remains substantial but is more intermittent, as seen in Figure 7.2E, p. 79. Given to multiple sequence deletions when amplified as part of a bacterial plasmid clone, as detailed by Boissy and Astell (Boissy and Astell, 1985), plasmids containing this genome have recently been propagated without

deletion in Sure2 cells grown at 30° (Zhi *et al.*, 2004b), and shown to give rise to detectable levels of infectious virus when transfected into UT7/Epo-S1 cells. Whether or not these telomeres contain upstream control elements for the single viral transcriptional promoter (P6, see Figure 7.1, p. 74) remains uncertain, but they do contain reiterated 5'-ACGT-3' half sites that may serve as binding sites for the hypoxia-inducible transcription factor, HIF-1 (Pillet *et al.*, 2004).

## AMDOVIRUS

Recently reclassified as the sole member of the new *Amdovirus* genus, Aleutian mink disease parvovirus (AMDV) packages predominantly negative sense DNA, is ~4748 nucleotides in length, and has disparate palindromic termini of 117 nucleotides and >242 nucleotides at the left and right ends of the genome respectively. The right hairpin forms a perfect duplex stem of ~100 bps that terminates in a less perfectly-paired 44 nucleotide loop (Bloom *et al.*, 1990), as shown in Figure 7.2F, p. 79, while the left-end hairpin can be folded into a highly-asymmetric Y-shaped configuration, shown in Figure 7.2G, p. 79. The latter is less stable than the Y-shaped MVM left end, with a calculated T<sub>m</sub> of 37.3°C for the tissue-culture adapted AMDV-G strain as compared with 48.7°C for MVM (Bloom *et al.*, 1988). However, as seen in the figure, the hairpin stem is shorter and one ear is longer than its MVM counterpart. Within this sequence are some degenerate reiterations of the tetranucleotide 5'-ACCA-3', which in MVM is the NS1 recognition motif, and these are also present through the 'stem' of the longer ear, suggesting that AMDVs NS1 may use a similar recognition motif and make multiple contacts throughout the structure. However, similar sequences are difficult to identify in the right-end hairpin, and the projected nick sites appear different in the two telomeres, so at present we have no information about how these termini function. Infectious plasmid clones of the tissue-culture-adapted AMDV-G strain do yield infectious virus when transfected into Crandell feline kidney (CRFK) cells, so that functional analysis of the hairpins would be possible.

## BOCAVIRUS

Genomes of the bocaviruses, bovine parvovirus (BPV) and canine minute virus (CnMV), are around 5.5 kb and have non-identical termini. BPV is unusual because it packages approximately 90 percent minus-sense and 10 percent positive-sense DNA. Its right-end hairpin is ~120 nucleotides in length, occurs in two sequence orientations in both strands, and forms a 52 basepair duplex stem with a 17 nucleotide axial unpaired loop (Figure 7.2H, p. 80) (Chen *et al.*, 1986, 1989). The hairpin at the left-end of the BPV genome is approximately 150 nucleotides in length and occurs in two sequence orientations, but in negative-sense virion DNA there is a 10-fold excess of one orientation

(flip) while in positive-sense progeny the two forms appear equimolar. This terminus can be folded into a T-shaped hairpin, with a cluster of asymmetries in the stem, rather than a single bubble mismatch as seen in genus *Parvovirus* (Figure 7.2I, p. 80). The termini of CnMV have not been sequenced to date (Schwartz *et al.*, 2002).

## DENSOVIRUS

Viruses from the genus *Densovirus*, *Junonia coenia* densovirus (*JcDNV*), *Galleria mellonella* densovirus (*GmDNV*) and *Mythimna loreyi* densovirus (*MlDNV*) have viral genomes of around 6 kb, with long ITRs (>500 nucleotide) of which the terminal ~130 nucleotides fold into simple Y-shaped hairpins that exist in two sequence orientations (Tijssen *et al.*, 2003; Abd-Alla *et al.*, 2004; Fédière *et al.*, 2004). In the 6039 nucleotide *GmDNV* genome the ITRs are 550 nucleotides, the distal 136 of which fold into hairpins with a 52 basepair fully-duplex stem and two small, GC-rich, internal ears, as shown in Figure 7.2J, p. 80. These are ambisense viruses, encoding the NS and capsid genes on different DNA strands from transcripts that start ~23 nucleotides downstream of their respective ITRs, and with the TATA boxes and all upstream promoter elements located in the ITR, and hence equivalent for the two promoters. A recombinant form of the NS1 protein of *JcDNV* has been shown to bind site-specifically to the sequence (GAC)<sub>4</sub>, found both in its terminal hairpins and in those of *GmDNV* (Figure 7.2J, p. 80), and to nick single-stranded forms of a presumed initiation site, 5'-GTATTG-3', *in vitro* (Ding *et al.*, 2002). However, the 5' end of the genome in cloned forms of *GmDNV* lies 20–23 nucleotides beyond these projected nick sites, suggesting that *in vivo* nicking may occur substantially inboard of the proposed sequence.

## ITERAVIRUS

The genomes of *Bombyx mori* densovirus (*BmDNV*) and *Casphalia extranea* densovirus (*CeDNV*) are 5048 and 5002 nucleotides long respectively (Li *et al.*, 2001, Fédière *et al.*, 2002), with ITRs of 225 and 230 nucleotides and terminal hairpins of 153 and 159 residues. These hairpins are predicted to assume unusual J-shaped configurations, as shown in Figure 7.2K, p. 80, with a duplex stem of 58 basepairs terminating in an imperfect J-shaped palindrome that exists in flip and flop sequence orientations. The J-structure is relatively conserved between the two viruses, being identical at 42/43 nucleotides whereas the stem sequences of this terminus are only 67 percent homologous.

## BREVIDENSOVIRUS

To date brevidensovirus have only been isolated from mosquitos (and mosquito cell lines), and from shrimp. These viruses have small (~4 kb) genomes with unique hairpins at either end. The type species, *Aedes aegypti* densovirus (*AaeDNV*), has palindromes of 146 and 164 nucleotides at

the left and right ends of the genome respectively, both of which fold to form T-shaped structures, as shown in Figures 7.2L, p. 80, and 7.2M, p. 81 (Afanasiev *et al.*, 1991). This virus packages both positive and negative sense DNA, although negative-sense strands predominate (~85 percent of the total).

## PEFUDENSOVIRUS

The single species classified in this genus, *Periplaneta fuliginosa* densovirus (*PfDNV*), has an ambisense genome of around 5.5 kb, with ITRs of 201 nucleotides and arrow-like hairpins of 122 nucleotides, shown in Figure 7.2N, p. 81 (Guo *et al.*, 2000). The virus is remarkable because its PLA<sub>2</sub> motif is located near the carboxy-terminus of the small capsid protein open reading frame, rather than in the amino-terminus of VP1, as in all other viruses, except AMDV, where it is absent.

## SEQUENCES INVOLVED IN THE REGULATION OF GENE EXPRESSION

During lytic infection, DNA replication generally precedes gene expression, since complementary strand synthesis is needed for efficient conversion of incoming single-stranded genomes into duplex transcription templates. AAVs, and perhaps some other viruses, can gradually evade this constraint when delivered at high multiplicities to certain post-mitotic tissues *in vivo*, generating circular duplex monomeric and concatemeric DNAs by mechanisms that appear to depend on recombination within the terminal repeat sequences and on either cellular repair pathways or the annealing of incoming strands of opposite polarity (discussed in Chapter 38). However, during productive infection there appear to be no classical 'early' genes. Instead, there is brief phasing of the various gene cassettes, commencing with transcription from a promoter near the left end of the genome that programs expression of the NS1 (or large Rep) proteins, which then commonly proceed to modulate transcription from all viral promoters, via mechanisms that involve binding to their specific recognition motifs positioned at one or more sites in the genome. The restricted size of their genome means that parvoviruses must encode proteins in multiple overlapping open-reading frames, with signals for the control of transcription, splicing and polyadenylation embedded in coding sequence, so that even apparently silent mutations can sometimes induce major changes in gene expression. Timing and expression levels of individual viral polypeptides are orchestrated during the viral life cycle by a wide variety of mechanisms, including promoter phasing, the use of initiation codons of differing efficiency, alternative splicing patterns, and differential protein cleavage, stability, or post-translational modification, although details of these control pathways differ markedly between genera.

In all parvoviruses, the single-stranded coding region contains an NS or *rep* gene in the left half of the genome and a VP or *cap* gene in the right half, accessed from between one to three transcriptional promoters, as illustrated in Figure 7.1, p. 74. The human *Erythrovirus* B19, which uses a single promoter at map unit 6 (P6), appears to have an additional open reading frame (ORF) downstream of its capsid gene, encoding an 11 kDa protein with Src homology 3 (SH3) ligand sequences, which is thus presumed to be involved in perturbing cellular signaling pathways (Fan *et al.*, 2001). This ORF has recently been shown to be essential for viral viability in UT7-Epo-S1 cells (Zhi *et al.*, 2004a). Until recently, parvoviruses were classified into distinct genera predominantly on the basis of physical characteristics such as the number and positions of promoters and polyadenylation signals used to orchestrate gene expression (Siegl *et al.*, 1985). However, it became increasingly apparent that such characteristics do not inevitably follow phylogeny. Thus in the newer sequence-based taxonomy, expression strategies may differ even within a single genus, while genetically disparate viruses may exhibit similar control mechanisms, suggesting a flexibility in coding strategy that was previously unrecognized. Below we briefly examine examples of the signals and mechanisms used to access coding sequences in the *Parvoviridae*, while detailed descriptions of transcription and RNA processing strategies employed by each genus are presented in Chapter 18 of this volume.

## Control of gene expression in the genus *Parvovirus*

MVM accesses the coding potential of its genome via three size classes of co-terminal mRNAs expressed from two promoters located at map units 4 and 38, called P4 and P38, respectively (Pintel *et al.*, 1983). The P4 promoter contains critical E2F, ETS-1, and Sp1 binding sites, positioned immediately upstream of its canonical TATA box in the single-stranded region of the genome (Faisst *et al.*, 1994), while NF-Y and MLTF sites in the proximal stem of the left-end hairpin (Gu *et al.*, 1995) coincide with an NS1 binding sequence, suggesting that there maybe competition for binding to this region. As discussed previously, competition between factors for specific recognition elements is even more apparent distal to the bubble sequence in the hairpin stem, where a cyclic AMP responsive element (CRE), which has been shown to contribute to basal levels of P4 activity and to the upregulation of P4 in ras-transformed cells (Perros *et al.*, 1995), overlaps with one of two 5'-ACGT-3' half sites need to bind parvovirus initiation factor (PIF), the heterodimeric cellular transcription modulator that is essential for stabilizing NS1 binding to the origin in such a way as to allow it to nick the DNA and thus initiate replication (Christensen and Tattersall, 2002). These two processes, transcription and replication, thus compete with each other for this site during infection. Both the CRE and the

ETS-1 binding site have been implicated in the upregulation of P4 activity in transformed cells (Perros *et al.*, 1995; Fuks *et al.*, 1996), which is believed to be one of the major mechanisms allowing MVM-mediated oncolysis. Accordingly, viral mutants in which the affinity of the PIF site was slightly impaired grew normally in untransformed cells, but appeared more successful than the wild type virus in transformed cells, suggesting that competition for the upstream element was particularly intense in the latter (Burnett and Tattersall, 2003).

P4 gives rise to unspliced nuclear transcripts from which three alternate forms of the small central intron are removed, generating transcripts that encode the single large ( $\text{Mr } 83\,000$ ) pleiotropic NS1 protein, using coding sequences that terminate immediately upstream of this small intron. During infection, phosphorylation of specific residues in NS1 modulates its enzymatic potential, and certain cleaved forms of the protein accumulate, as discussed in Chapter 19. A second size class of P4-derived mRNAs encode a group of smaller NS-2 proteins ( $\text{Mr } \sim 25\,000$ ), of which there are at least three carboxy-terminal variants (commonly referred to as isoforms). These transcripts are generated by excision of a large second intron from transcripts already carrying one or other form of the small splice. Thus NS2 molecules share 84 amino acids of amino-terminal sequence with NS1, but are then spliced into an alternate reading frame, which remains open through the first donor of the central splicing region. In this small central splice region both alternate donor and acceptor splice sites are used to varying extents, so that three forms of NS2 carrying slightly different carboxy-terminal extensions, NS2P, NS2Y, and NS2L, are generated in diminishing concentration. These proteins are differentially phosphorylated at specific sites, and serve multiple roles in the viral life cycle that are predominantly involved with the establishment of a favorable intracellular environment. NS2 is essential for viral DNA replication in cells of rodent origin (Naeger *et al.*, 1990; Brownstein *et al.*, 1992), although it is expendable in some transformed cell lines from other species, where the resulting mutant merely exhibits a virion export deficiency (Cotmore *et al.*, 1997). Recent studies, in which the presence or expression levels of the individual isoforms were modulated genetically, showed that one particular isoform, NS2P, is required to activate DNA replication in murine cells (Ruiz *et al.*, 2004). Since transcripts encoding NS2 are derived from those encoding NS1 by additional splicing, ratios of NS2/NS1 proteins can be varied by modifying the efficiency of the large splice. The fibrotropic (prototype) and lymphotropic strains of MVM, MVMp and MVMi, differ in this respect, mediated via a single nucleotide in the putative branch point of the splice acceptor, so that MVMp generates relatively more transcripts encoding NS2 and fewer encoding NS1, than does MVMi (Choi *et al.*, 2004). Mutations bringing about this splicing modification occur frequently when virus stocks are given the opportunity to grow on cells of the alternate differentiated phenotype (D'Abramo *et al.*,

2005), indicating that the regulation of NS1 and NS2 expression levels is of major importance for successful infection of specific cell types. Although the coding strategy of CPV closely resembles that of MVM, and CPV encodes and expresses a partial homologue of the rodent parvovirus NS2 protein, this appears to be expendable for viral growth in either cell culture or dogs (Wang *et al.*, 1998).

NS1 upregulates transcription from the P38 promoter approximately 100-fold, generating a third size class of mRNAs that encode the capsid proteins. This transcriptional upregulation is mediated by the acidic carboxyterminal domain of NS1, which acts as a classical transcriptional activator (Legendre and Rommelaere, 1992), which can be delivered to the promoter via a number of different interactions. First, modulation of P38 transcription is particularly potent when NS1 is bound to a 19 nucleotide sequence containing inverted copies of its recognition motif, previously called the transactivation response (tar) element, which is positioned 116 nucleotides upstream of the RNA initiation site (Rhode, 1985; Christensen *et al.*, 1995). However, NS1 can also potentiate P38 transcription when it is bound to one of several copies of its cognate binding site that are scattered throughout this region of the viral genome (Lorson *et al.*, 1996). Finally, the P38 promoter contains an essential Sp1-binding site, and NS1 has been shown to interact directly with Sp1 and with various basal transcription factors (Kraday and Ward, 1995; Lorson *et al.*, 1998) so that secondary interactions of this type likely also enhance gene expression. All P38 transcripts include one or other form of the small splice, which allows them to encode two distinct sizes of capsid protein in precise stoichiometric ratios. Of these, the minor VP1 species, Mr 83 000, is encoded from a transcript that employs the downstream splice donor and acceptor sites and initiates at a methionine codon positioned between the two potential donor sites, while the major VP2 species (Mr 63 000), is expressed from transcripts using the stronger upstream 5' splice site, which consequently lack the methionine used to initiate VP1 expression. Translation from such mRNAs initiates around 400 nucleotides downstream of the splice. VP1 and VP2 polypeptides thus differ only by the presence in VP1 of a 142 amino acid N-terminal extension, and are produced at ratios of around 1:5. A third, N-terminally truncated, form of the capsid protein, VP3 (Mr 52 000), is generated by proteolytic cleavage of VP2 molecules in full, but not in empty, capsid particles following progeny virus release from its host cell (Cotmore and Tattersall, 1989).

### Control of gene expression in the genus *Dependovirus*

During lytic infection AAV2 generates three size classes of co-terminal mRNAs from three promoters located at map units 5, 19, and 40 in the genome (P5, P19, and P40), giving rise to two forms of the large Rep initiator protein (Rep68

and Rep78), two small Rep proteins that share sequence with the carboxy-terminal regions of the large Reps (Rep40 and Rep52), and three capsid proteins (VP1, VP2, and VP3), respectively (Marcus *et al.*, 1981). Transcriptional regulation from these promoters is complex and is modulated by the presence or absence of adenovirus proteins, as discussed in detail elsewhere in this volume (Chapter 17). The critical element in this control is a Rep68/78-binding element (RBE) at position -20 in the P5 promoter, which acts in concert with cellular proteins to repress P5 transcription in the absence of helper virus coinfection. It has been estimated that under these conditions between 100–400 Rep molecules are expressed per cell, which is enough to catalyze integration of the genome into a specific site at 19q13.3-qter in the human genome. Adenovirus co-infection counteracts P5 repression, inducing transcription from the promoter by a mechanism that appears to involve interaction with the YY1 transcription factor and its cognate site in the P5 promoter, and leads to enhanced Rep68/78 expression. This then binds to the P5 RBE, enhancing transcription from P19 and P40, while repressing the activity of P5 itself. Thus Rep68/78-binding to the RBE does not act as a typical upstream activation signal, but rather represses nearby transcription while activating transcription at a distance. The P19 and P40 promoters have critical binding sites for the host transcription factor Sp1, and Rep bound to the P5 has been shown to form a loop with Sp1 at P19. These observations suggest that the RBE is an architectural element that acts by bringing P5 and P19 promoters together during transcription to orchestrate synthesis of the various RNAs (Lackner and Muzychka, 2002).

Each RNA size-class contains a small intron, with a single splice donor and two alternative splice acceptor sites, and is polyadenylated at a single site near the right end of the genome. Unspliced transcripts encode Rep78 and Rep52, while Rep68 and Rep40 are derived from spliced mRNAs. As a consequence, Rep78 and Rep52 have distinctive carboxy-terminal zinc-finger domains that mediate interactions with cellular kinases PKA and PRKX (Di Pasquale and Stacey, 1998; Di Pasquale and Chiorini, 2003), while the carboxy-terminal peptides of Rep68 and Rep40 interact with members of the 14-3-3 family (Han *et al.*, 2004), suggesting that these alternate forms of each Rep size class mediate unique interactions with cellular signalling pathways. However, in all other respects the splice variants of each size class appear functionally redundant, while in AAV5 only a single form of each class is generated because polyadenylation of P5 and P19 transcripts at a site within the small intron effectively eliminates this splice (Qiu *et al.*, 2004). In both serotypes all capsid proteins are generated from spliced transcripts driven by the P40 promoter, but since the downstream acceptor is more efficient than the upstream acceptor, major and minor spliced species are generated. RNAs with the major form of the splice direct synthesis of the major capsid protein VP3

(Mr ~62 000) from an AUG codon, and the minor capsid protein VP2 (Mr ~73 000) from an upstream in-frame ACG codon. The minor spliced message includes an upstream AUG that allows translation of the entire open reading frame to produce VP1 (Mr 87 000). Thus the *Parvoviruses* and dependoviruses differ significantly in the control of VP1 synthesis, accessing the initiating methionine codon by using alternative donors in the case of MVM, but alternate acceptors in AAV2. AAV2 capsid proteins accumulate in the approximate ratio of 1:1:10 for VPs 1–3 respectively, each having identical carboxy-terminal sequences but with VP1 being 137 residues longer than VP2, which is in turn 65 residues longer than the most common species, VP3. During infection unspliced P5 transcripts, encoding Rep78, are expressed first, allowing DNA amplification to initiate, but these are subsequently overtaken by products from the other promoters so that, late in infection, the relative strength of P5, P19, and P40 are 1:3:18, allowing high level production of the capsid polypeptides at late times and preferential accumulation of the shorter Rep species needed to drive packaging of progeny DNA (Mouw and Pintel, 2000).

As mentioned above, the transcription profile of AAV5 is somewhat different (Qiu *et al.*, 2004). Transcripts encoding the Rep proteins are predominantly cleaved and polyadenylated at a site within the central intron, while P41 transcripts primarily read through to the distal, right-end, polyadenylation site allowing them to be spliced like their AAV2 counterparts. Like many other viruses, including MVM, the central intron of AAV2 also contains a consensus polyadenylation signal, but this does not function in the absence of a downstream element (DSE) that overlaps the major acceptor site of the intron in AAV5. P9 promoter of GPV similarly uses an internal polyadenylation signal controlled by a DSE in the splice acceptor. Since the avian viruses replicate in the absence of a helper virus, it is presumed that early Rep1 expression will not repress P9 expression in these viruses, but details of their transcriptional control have yet to be explored.

## Control of gene expression in the genus *Erythrovirus*

As seen in Figure 7.1, p. 74, human parvovirus B19, the type species of the genus *Erythrovirus*, has a single promoter at six map units, which it uses in conjunction with two functional polyadenylation signals to phase expression of NS1, capsid proteins, and two small, less well-understood, polypeptides (Ozawa *et al.*, 1988a, 1988b). Control of gene expression from this promoter is complex and poorly understood, but a detailed account of the available information is presented in this volume in Chapter 3. While some of B19's erythroid tissue specificity may derive from the structure of its promoter, differences in RNA splicing and polyadenylation appear more significant, and these are

discussed in both Chapter 3 and Chapter 18, so that here we provide a minimal overview. Attempts to unravel details of B19 gene expression have been greatly impeded by the absence of any fully-productive host cell line, which has meant that expression patterns associated with abortive infection have frequently been studied. Nevertheless, some features of the promoter have been detailed. Thus, a region upstream of the TATA box (B19 nucleotides 113–210) appears to be highly conserved among different B19 isolates, and deletion of this element in reporter constructs led to a 90 percent drop in transcription, suggesting that it contains at least some of the relevant elements. In particular, a core binding motif for members of the ETS family of transcription factors is situated 208–201 nucleotides upstream of the initiation site, which binds hGARb when incubated with nuclear extracts from the semipermissive UT7-Epo cell line. This site had a synergistic effect with an Sp1-binding site (at nucleotides -200 to -195) when assayed in drosophila SL2 cells, while a nearby YY1 binding site (-220 to -212) diminished this effect (Vassias *et al.*, 1998). This region also contains binding sites for the transcription factor Oct-1 (Raab *et al.*, 2001). Recently there have been reports that P6 can be activated in anoxic environments by HIF-1alpha complexes, which may bind to reiterated 5'-ACGT-3' half sites in the nearby ITR (Caillet-Fauquet *et al.*, 2004; Pillet *et al.*, 2004).

To date, B19 is known to code for three proteins that are homologous to those encoded by other parvoviruses: the NS1 initiator protein, encoded by a transcript that is cleaved and polyadenylated in the middle of the genome, and the two capsid polypeptides VP1 and VP2, which are identical to each other except for the presence of an amino-terminal 226 amino acid extension in VP1. NS1 itself upregulates P6 transcription, but the mechanism is uncertain. This protein binds to duplex binding sites in the ITRs, and with low affinity to the P6 Sp1 site, but it also binds directly to Sp1, and some accounts argue in favor of an indirect effect mediated through Sp1 and a cAMP response element-binding protein (Gareus *et al.*, 1998). The VPs are encoded by transcripts that have a single or double splice, which remove most of the sequences from the left side of the genome. These proteins are produced in the VP1:VP2 ratio of 1:20, apparently by differential splicing and initiation-interference mediated by an AUG-rich sequence upstream of the VP1 start. Two small open reading frames are also accessed by extensively spliced transcripts, encoding 7.5 and 11 kDa proteins that have been detected during abortive infection in tissue culture. The 7.5 kDa protein is encoded in the NS gene, but the 11 kDa species is encoded downstream of the capsid, and appears to be the product of a third distinct viral gene. This protein contains three proline-rich regions that conform to consensus SH3 (Src homology 3) ligand sequences (Fan *et al.*, 2001), and is reported to be essential for viral propagation from an infectious plasmid clone in UT7-Epo-S1 cells (Zhi *et al.*, 2004a).

## Unique aspects of expression in the *Amdovirus* and *Bocavirus* genera

Aleutian mink disease virus (AMDV) uses two promoters, P3 and P36, and two polyadenylation signals, at map units 53 and 92, but the transcription profile is complex. Five transcripts with characteristic splice patterns have been identified, with those originating from the P3 promoter encoding NS1 and NS2 proteins similar to those of MVM, and an NS3 species that only encodes the N-terminal peptide that is also shared by NS1 and 2, but NS3 has yet to be detected *in vivo* (Alexandersen *et al.*, 1988). In AMDV, full-length NS1 molecules are not transported into the cell nucleus. However, the virus causes its host cell to enter apoptosis, which induces caspases that cleave at two sites in NS1, releasing a carboxy-terminal domain that will not only undergo nuclear translocation by itself, but will also effectively co-transport full length NS1 molecules into the nucleus (Best *et al.*, 2002, 2003). Thus induction of apoptosis is essential for productive infection, and creates a truncated NS species that would be expected to lack site-specific DNA binding activity, but carry the carboxy-terminal helicase domain of NS1, and would thus be analogous to the Rep52/40 packaging helicases encoded from the AAV P19 promoter.

Detailed transcription maps are not available for the bocaviruses BPV or CnMV, although NS1 and a nested set of capsid polypeptides are predicted from sequence homologies and protein expression studies. Remarkably, in addition to NS1 and NS2, these viruses appear to encode an abundant 25 kDa nuclear phosphoprotein, NP1, of unknown function (Lederman *et al.*, 1984; Schwartz *et al.*, 2002), discussed further in Chapters 33 and 34.

## Gene expression in the subfamily Densovirinae

Relatively little is known about the regulated expression of coding sequences among the myriad viruses of the subfamily Densovirinae. Those mechanisms that have been identified are reviewed in detail elsewhere in this volume (Chapter 5), and tend to reflect strategies commonly used in their insect hosts, and are hence rather different from those discussed previously. To date the best studied are viruses from the genus *Densovirus*, which use an ambisense coding strategy with the non-structural and capsid polypeptides expressed from RNAs that start just downstream (23 nucleotides in GmDNV) of their respective terminal repeats. Thus the promoters and control elements for both transcripts are identical, and phasing seems unlikely. Three NS products have been identified for GmDNV and JCDNV, of which NS2 is encoded entirely within the NS1 coding sequence but from a different open reading frame, while a novel NS3 protein is encoded upstream of NS1/2. Although splicing is relatively rare in insect cells, most NS transcripts

are spliced to remove the upstream NS3, allowing leaky scanning translation of NS1 and NS2. In contrast, NS3 is translated from an unspliced RNA, and in JcDNV this protein has been shown to be expressed early and to be essential for viral DNA replication. VP transcripts are not spliced, but a nested set of four VP proteins are generated from a single transcript class again by leaky scanning translation initiation (Tijssen *et al.*, 2003; Abd-Alla *et al.*, 2004).

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# Phospholipase A<sub>2</sub> domains in structural proteins of parvoviruses

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It can be estimated from the atomic structure of parvoviruses that structural proteins of 25–30 kDa would suffice to form a capsid to protect the viral genome. However, this minimum size is often greatly exceeded. The smallest structural proteins of parvoviruses, from densoviruses in the *Breviadensovirus* genus, are about 40 kDa, whereas the smallest from other parvoviruses, such as the members of the *Parvovirus* genus, may be 60 kDa, and the maximum size of these structural proteins is about 85–100 kDa. Although two to five different proteins, depending on the parvovirus species, can be distinguished by electrophoresis through polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS-PAGE), they share common sequences (Tijssen and Kurstak, 1981). Essentially, 60 copies of a single protein are required for the T = 1 capsid formation but some of these may have N-terminal extensions. The parts of the structural proteins in excess of 30 kDa may be required for a number of processes in the viral cycle, such as nuclear localization, polymerization, DNA packaging and the various steps in exit, escape of immune surveillance, entry, and DNA delivery.

Most proteins are composed of an ‘in-line’ arrangement of modules, often independently folded, which have evolved by the joining of preexisting domains during a domain shuffling process. Parvoviruses have evolved different strategies to express these domains, such as the use of alternative splicing, leaky scanning and/or proteolytic cleavage. The

use of alternative splicing is exemplified by members of the *Parvovirus* and *Dependovirus* genera, and serves to start translation at alternative initiation codons. The largest structural protein (VP1) usually represents about 10 percent of the total protein mass in the particle. The dependoviruses also use a leaky scanning mechanism, in which an ACG codon in a favorable context is used to initiate VP2 polypeptides, although most translation starts from the next AUG. Densoviruses from the *Densovirus* and *Iteravirus* genera also use leaky scanning to generate about four structural proteins. The larger proteins are minor species, which is consistent with the leaky scanning model in which the downstream start codons display increasing strength. Each additional peptide extension is thought to fulfill at least one unique function in the viral cycle. These strategies also allow the virus to express the different domains to different optimal amounts.

The focus of this chapter is the phospholipase A<sub>2</sub> (PLA<sub>2</sub>) domain that we have identified in the minor structural protein of almost all parvoviruses. The exceptions are the *Breviadensovirus* members, which have relatively small structural proteins, and Aleutian mink disease virus (AMDV). Since the *Parvoviridae* are the first virus family in which such an activity has been described, we will also give a short review of PLA<sub>2</sub> enzymes. For more detailed reviews on non-viral PLA<sub>2</sub>s the reader is referred to Gelb *et al.* (1994, 1995, 1999, 2000a,b), Jain *et al.* (1995), Berg *et al.* (2001), Balsinde *et al.* (2002), Fuentes *et al.* (2002), Kudo and

Murakami (2002), Murakami and Kudo (2003), and Sun *et al.* (2004). The first reports on parvoviral PLA<sub>2</sub> are listed in Box 8.1.

## OVERVIEW OF PHOSPHOLIPASE A<sub>2</sub>

Phospholipids consist of a glycerol backbone with a fatty acid at the *sn*-1 position that is usually saturated, a fatty acid at the *sn*-2 position that is usually unsaturated (except in lipid rafts), and an esterified phosphate group at the *sn*-3 position (Figure 8.1). Anionic and zwitterionic phospholipids can be distinguished. The two most common glycerophospholipids, phosphatidylcholine and phosphatidylethanolamine, are zwitterionic, whereas phosphatidylserine, phosphatidylglycerol, diphosphatidylglycerol (cardiolipin) and phosphatidylinositol are anionic. Phosphatidylcholine usually has palmitic or stearic acid at its *sn*-1 position, and oleic, linoleic or linonelic acids at the *sn*-2 position. Phosphatidylethanolamine usually contains palmitic or oleic acid at its *sn*-1 position but a long chain polyunsaturated fatty acid, such as arachidonic acid, at the *sn*-2 position.

Phospholipases are classified according to the ester bond that they hydrolyze (Figure 8.1). These enzymes play fundamental roles in a large number of biological processes, including the homeostasis of cellular membranes, lipid digestion, and the production of potent lipid mediators that in turn exert key functions in normal and pathological states, such as atherosclerosis, blood coagulation, and cancer (MacPhee *et al.*, 1995; Mounier *et al.*, 2000; Tietge *et al.*, 2000; Murakami and Kudo, 2003; Niessen *et al.*, 2003). In contrast to the intracellular phospholipase C and D, at least 20 distinct intracellular and extracellular PLA<sub>2</sub> enzymes can be distinguished, which points to the multitude of functions these PLA<sub>2</sub> enzymes perform. This growing family of PLA<sub>2</sub>-type acylhydrolases can be subdivided into groups based on their enzymatic and structural characteristics (Table 8.1). There are three types of PLA<sub>2</sub> with respect to Ca<sup>2+</sup>-dependence (Fuentes *et al.*, 2002): those that require mM concentrations of Ca<sup>2+</sup> for catalysis (extracellular or secretory, sPLA<sub>2</sub>), those requiring submicromolar concentrations of Ca<sup>2+</sup> for membrane translocation (cytosolic, cPLA<sub>2</sub>), and the Ca<sup>2+</sup>-independent PLA<sub>2</sub>s (iPLA<sub>2</sub>s). In this respect, parvoviral PLA<sub>2</sub>s resemble the sPLA<sub>2</sub>s. Hundreds of sPLA<sub>2</sub> sequences are currently available in protein databanks.

sPLA<sub>2</sub>s often exhibit species- and tissue-specific expression suggesting that their cellular functions differ (Valentin *et al.* 2000a,b; Schadow *et al.* 2001; Gurrieri *et al.*, 2003). The typical sPLA<sub>2</sub>s (groups I/II/V/X) have low molecular mass (12–19 kDa) and a rigid tertiary structure. The number of disulfide bridges that configure this structure, usually 6–8, was used in the classification of these sPLA<sub>2</sub>s (Table 8.1). Group IB and X enzymes have an N-terminal prepropeptide and need proteolytic cleavage for activation, whereas the group IIIB, group XII and XIII sPLA<sub>2</sub>s are domains in

### Box 8.1 First reports of parvoviral phospholipase A<sub>2</sub> research

Zádori *et al.*, 2001

Identification of PLA<sub>2</sub> domain in VP1 of parvoviruses

Experimental proof of PLA<sub>2</sub> activity in B19, PPV, and GmDNV and determination of their  $k_{cat}/K_M$ . Sequences outside PLA<sub>2</sub> domain contribute to catalytic activity

Link between enzyme activity and infectivity, requirement in *cis*

PLA<sub>2</sub> required during entry between late endosome/lysosome and nuclear entry

Li *et al.*, 2001

Experimental proof of PLA<sub>2</sub> activity in BmDNV and determination of its  $k_{cat}/K_M$

Fédière *et al.*, 2002

Experimental proof of PLA<sub>2</sub> activity in CeDNV and determination of its  $k_{cat}/K_M$

Girod *et al.*, 2002

Experimental proof of PLA<sub>2</sub> activity in AAV2. Link between enzyme activity and infectivity, requirement in *cis*

PLA<sub>2</sub> required during entry between late endosome/lysosome and nuclear entry

Dorsch *et al.*, 2002

Confirmation of B19 PLA<sub>2</sub> activity  
Confirmation that sequences outside PLA<sub>2</sub> domain contribute to catalytic activity

Tijssen *et al.*, 2003

Experimental confirmation of PLA<sub>2</sub> activity in GmDNV  
Low  $k_{cat}/K_M$  may be at least partially related to poor stability of expressed protein

Suikkanen *et al.*, 2003

Experimental proof of PLA<sub>2</sub> activity in CPV  
Evidence of externalization of its PLA<sub>2</sub> domain in endosomal compartments  
Role of CPV PLA<sub>2</sub> in lysosomal exit

Fédière *et al.*, 2004

Experimental proof of PLA<sub>2</sub> activity in MlDNV

Canaan *et al.*, 2004

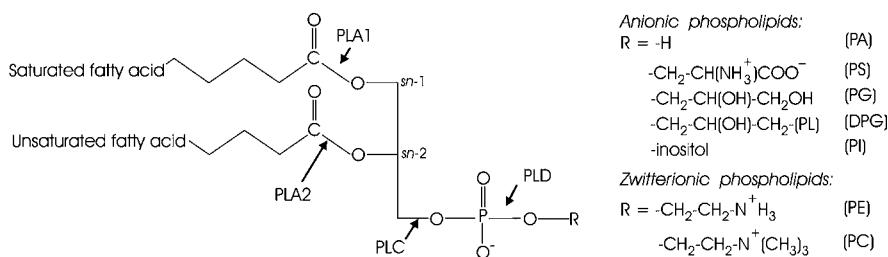
Enzyme characteristics of B19, PPV and AAV2 PLA<sub>2</sub>s

Determinations of kinetic parameters to different substrates

Determinations of affinity constants to membranes

Determinations of affinity constants to calcium ions

Effect of classical inhibitors on enzyme activity  
Arachidonic acid release by cells after incubation with parvoviral PLA<sub>2</sub>

**Figure 8.1** Structure of phospholipids. Phospholipase A<sub>1</sub>, A<sub>2</sub>, C, and D have specificity for different ester bonds in the phospholipid.

Abbreviations: DPG, diphosphatidylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine;

PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine. PLA<sub>2</sub> yields a fatty acid and a lysophosphatidyl product.**Table 8.1** Major groups of phospholipase A2

Group		Source (example)	Size (kDa) and type	Ca <sup>2+</sup>	C-C	Characteristics	Gene bank accession number
I	A	Kraits, cobras	~14 (sPLA2)	mM	7	HD	P15445
	B	Pancreas	~14 (sPLA2)	mM	7	HD, elapid loop	NP_000919
II	A	Rattlesnake	~14 (sPLA2)	mM	7	HD, carboxyl extension	NP_000291
		Synovial fluid					
III	B	Gaboon viper	~14 (sPLA2)	mM	6	HD, carboxyl extension	PSBGA
	C	Mouse testes	~15 (sPLA2)	mM	8	HD, carboxyl extension	NP_032894
	D	Pancreas/spleen	~14 (sPLA2)	mM	7	HD, carboxyl extension	NP_036532
	E	Brain/heart/uterus	~14 (sPLA2)	mM	7	HD, carboxyl extension	AAF36541
	F	Testis/embryo	~16 (sPLA2)	mM	7	HD, large carboxyl extension	AAF04500
	A	Bee, lizard	~17 (sPLA2)	mM	5	HD	P00630
		Human	~55 (sPLA2)	mM	?	HD, N- and C-terminal extensions	
IV	A	Rat kidney	~85 (cPLA2)	μM		Ser228, Arg200, Asp549	P47712
	B	Pancreas/liver	~114 (cPLA2)	μM			AAD32135
	C		~60 (cPLA2)			Prenylated	AAC32823
V		P338D <sub>1</sub> macrophages	~14 (sPLA2)	mM	6	HD	NP_000920
		Heart/lung					
VI	A	CHO cells	~80 (iPLA2)			GxSxG motif, complex	AAD41722
	B	Heart/skeletal muscle	~90 (iPLA2)			Membrane bound	BAA94997
VII		Plasma	~45 (iPLA2)			GxSxG motif (PAF)	Q99487
VIII		Brain	~26 (iPLA2)			Ser47/HD	Q15102
IX		Marine snail venom	~14 (sPLA2)	<mM	6	HD	AAB33555
X		Spleen/thymus	~14 (sPLA2)	mM	8	HD, carboxyl extension	NP_003552
XI	A	Rice	~12 (sPLA2)	mM	7	HD	CAB40841
	B	Rice	~13 (sPLA2)	mM	7	HD	CAB40842
XII	A	Th2 cells	~20 (sPLA2)	mM	7	HD	AY007381
	B	Human/mouse	~20 (sPLA2)	mM	7	Inactive, H replaced by L	AF349540
XIII		Parvovirus	~85 (vPLA2)	mM	0	HD, multifunctional protein	AF375296
XIV		<i>Baculovirus</i>	~15 (vPLA2)	mM	0/1	HD	AY349019
		Symbiotic fungus	~13–19	mM	2	HD	E08479
		<i>Streptomyces</i>					

multifunctional proteins. The structure of bee venom group IIIA sPLAs can also be distinguished from the typical sPLAs.

Group I sPLAs have been subdivided into two subgroups, those that are found in the venom of cobra and kraits (group IA) and those (group IB) that are abundantly expressed in the pancreas as an inactive zymogen and, after secretion into the intestinal lumen and activation by tryptic

cleavage, function as digestive enzymes for dietary phospholipids. Group IB enzymes are also present at low concentrations in non-digestive organs, where they may be regulatory proteins acting via the M-type sPLA<sub>2</sub> receptor (Lambeau *et al.*, 1994).

sPLAs in group II are divided into several subgroups of non-pancreatic sPLAs that are widely distributed in

mammalian tissues and are enriched in platelets and in inflammatory exudates. (Although they have many characteristics in common with Group I PLA<sub>2</sub>s, and 70 percent sequence identity, most also display unique properties such as a preference for phosphatidylethanolamines, affinity for heparin, or synthesis as a mature protein that is stored in intracellular granules – Gijón and Leslie, 1997; Enomoto *et al.*, 2000). In addition to their roles in inflammation (Murakami *et al.*, 2002a,b) group IIA sPLA<sub>2</sub>, like other cationic sPLA<sub>2</sub>s, has a potent Gram-positive bactericidal activity, for example directed against staphylococci, and their level is elevated in tears and seminal fluids (Weinrauch *et al.*, 1996; Koduri *et al.*, 2002; Koprivnjak *et al.*, 2002).

sPLA<sub>2</sub>s display widely different affinities for membranes, depending on the composition of the enzyme surface and the membrane at the interface. The zwitterionic phosphatidylcholine is enriched in the outer leaflet of the plasma membrane of quiescent cells, and sPLA<sub>2</sub>s depend on direct binding to the membrane to release fatty acids (activity of PLA<sub>2</sub>s in groups X > V > IIF > III > IB >> IIA; while IIA, IIC, IID, IIE, and XII are inactive via this route) (Bezzine *et al.*, 2002; Pan *et al.*, 2002). In contrast, cationic (heparin-binding) group II subfamily sPLA<sub>2</sub>s, such as groups IIA, IID, and IIE, show a marked preference for anionic phospholipids, and use heparan sulfate proteoglycan in the caveolae or rafts of activated cells to mediate their internalization and translocation in vesicles to the perinuclear region where downstream cyclooxygenases (COX-1,2) are located (Kim *et al.*, 2002; Murakami *et al.*, 2003).

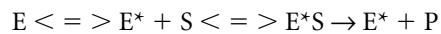
There are also natural sPLA<sub>2</sub> variants that are devoid of enzymatic activity because they have point mutations in their active sites (Gutierrez and Lomonte, 1995; Kini and Chan, 1999; Verpy *et al.*, 1999; Rouault *et al.*, 2003). Nevertheless, they may still have important biological activities, e.g. affinity-related functions. Some of the non-active sPLA<sub>2</sub>s in snake venom are still venomous, and human group XIIB sPLA<sub>2</sub> expression was found to be dramatically reduced in tumours in several tissues (Rouault *et al.*, 2003). The catalytically inactive sPLA<sub>2</sub> protein otoconin-95 is, atypically, only detected in the inner ears (Verpy *et al.*, 1999).

The PLA<sub>2</sub> receptor (PLA<sub>2</sub>R), a type I transmembrane glycoprotein related to the mannose receptor, also regulates a variety of biological responses elicited by specific types of sPLA<sub>2</sub>s. Group IB sPLA<sub>2</sub> acts as an endogenous PLA<sub>2</sub>R ligand to induce cell proliferation, cell migration, and lipid mediator production. In the circulation, the soluble form of PLA<sub>2</sub>R is constitutively present as an endogenous inhibitor of sPLA<sub>2</sub>s, and is involved in their clearance to suppress their potent enzymatic activities (Hanasaki and Arita, 2002).

## THE CATALYTIC SITE OF SECRETORY AND PARVOVIRAL PHOSPHOLIPASES

PLA<sub>2</sub>s work only on aggregated substrates, such as lipid membranes. The enzymatic process of PLA<sub>2</sub>s – so-called

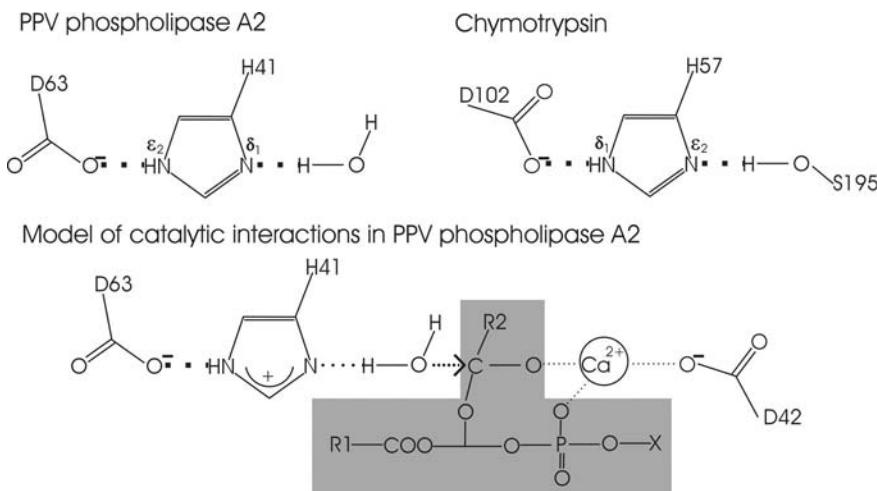
interfacial catalysis – is more complicated than that of enzymes that operate in a homogeneous aqueous environment. Interfacial catalysis can be dissected into the binding of the aqueous-phase enzyme to the lipid interface to gain access to its substrate and into events that occur in the catalytic site. The enzyme kinetics can be described with the equation



The enzyme (E) binds to the interface (E\*) and can capture a substrate molecule (S) in its active site to give the Michaelis complex (E\*S) which then can yield products (P) (Yu *et al.*, 1997).

Analyses of crystal structures of sPLA<sub>2</sub>s with transition-state analogs have shown that their active site is composed of a histidine-aspartic acid (HD) dyad (White *et al.*, 1990; Scott *et al.*, 1990a,b 1991; Annand *et al.*, 1996; Sekar *et al.*, 1998). The catalytic site histidine (His) is assisted by another aspartic acid (Asp) to polarize a bound water molecule, which then attacks the carbonyl group (Figure 8.2) of the fatty acid in the *sn*-2 position. The Asp in the catalytic dyad aids the required Ca<sup>2+</sup> ion to stabilize the transition state. This Ca<sup>2+</sup> ion is bound in a conserved Ca<sup>2+</sup> binding loop. The enzyme does not seem to be dissociated from the membrane after each catalytic event (as in the so-called ‘hopping mode’) but rather moves to another substrate molecule (the ‘scouting mode’) (Yu *et al.*, 1997). The structure of the enzyme is rigid and the substrate is believed to move into the catalytic site after the enzyme is bound to the membrane (Yu and Dennis, 1992).

Despite their low overall sequence identity, sequence comparisons revealed a highly-conserved domain in the structural proteins of most vertebrate and insect parvoviruses, suggesting that this domain was crucial in the viral life cycle (Tijssen and Bergoin, 1995). Interestingly, this sequence, in the unique portion of VP1 (VP1up), was interrupted by an intron in the case of PPV but not in the case of GmDNV. MVM VP1up mutants were found to be non-viable (Tullis *et al.*, 1993). Analysis of the conserved domain in the VP1up sequences of 34 parvovirus revealed that 31 contained a conserved HDXXY motif and aYXGXG motif that are found in the catalytic site and the conserved Ca<sup>2+</sup> binding loop respectively of sPLA<sub>2</sub>s (Zádori *et al.*, 2001). Conservation between VP1up and sPLA<sub>2</sub> is poor outside these 2 motifs, restricted to a few amino acids (an Asp at -5 and a proline (Pro) at -11 in the HD catalytic dyad), and parvoviruses lack the typical disulfide bridges of sPLA<sub>2</sub>s (Table 8.1). The position of the PLA<sub>2</sub> domain within the VP1up can also vary considerably, for instance for MVM and members of the *Iteravirus* genus it is located at the N-terminus, for CPV it is preceded by an NLS and starts only with Leu-33. For the AAVs, the domain starts at about leucine (Leu)44/45 whereas for the related waterfowl parvoviruses it starts at about phenylalanine (Phe)53. For members of the *Erythrovirus* and *Densovirus* genera the



**Figure 8.2** Comparison of the HD catalytic dyad of PPV PLA<sub>2</sub> (with a catalytic water molecule) and the DHS catalytic triad of chymotrypsin. Proposed model of the interactions between the catalytic residues in the active site of PPV PLA<sub>2</sub> and Ca<sup>2+</sup>, with the tetrahedral transition state generated from the water molecule and the phospholipid (shaded) carbonyl group (Dennis 1994; Poi et al., 2003).

		Ca-binding loop	Catalytic site	
CeDNV:	9	HNYLGP <span style="font-family: monospace;">GSDNFKKQPVDEDDAIARAHDL<span style="font-family: monospace;">DYDKASSDKDIFKADKQARDE</span></span> 56		
		H YLG G++ + P+DEDD IA HDLDY A+S DI AD+ A +		
NeleNPV:	12	HRYLGI <span style="font-family: monospace;">GNELYCGEPIDE<span style="font-family: monospace;">DDDKIAFYHDLDYTLATSADDIMLADQTAVQK</span></span> 55		
CeDNV:	57	FFSSFVHSGNLHSLIGGLGLGAKNLVEEHVLGKSLY-GMGKRKSTEKD 104		
		FF+ N+H LI G L K ++E+ LG+ Y + K +EK+ +		
NeleNPV:	56	FFA---LRNIHGLIAGSLLHLKYIIIEKR-LGRIIYPNINKYTESEKN 103		

**Figure 8.3** Alignment of group XIII A and XIII B viral phospholipases. Examples used are CeDNV VP1up (group XIII A, accession number AF375296 [structural protein], Fédière et al., 2002) and NeleNPV (group XIII B, accession number AY349019 [ORF 81], Lauzon et al., 2004).

PLA<sub>2</sub> domain is located in the C-terminal part of VP1up. Interestingly, we recently observed that the PLA<sub>2</sub> domain in *Ad*DNV is maintained in some of the translation products by alternative splicing of mRNAs when expressed by baculovirus (unpublished results).

PPV, B19, and *Gm*DNV VP1ups, and portions thereof, were expressed in bacteria, and the isolated peptides shown to exhibit PLA<sub>2</sub> activity when the mixed micelles assay with radiolabeled phospholipids as substrate was used (Zádori et al., 2001). Striking observations included the following:

- the conserved PLA<sub>2</sub> domain by itself had relatively low activity and required additional VP1up sequences to achieve high activity;
- the PLA<sub>2</sub> activity of different parvoviruses could differ by as much as about 1000-fold, with that of PPV being as active as the most active sPLA<sub>2</sub>;
- the PLA<sub>2</sub> activity of PPV was located within the capsid (in agreement with the observation of Cotmore et al. (1999) that the VP1up of MVM resides within the virion).

Potentially critical amino acids were mutated in both the expressed VP1up PLA<sub>2</sub>s as well as in the infectious clone of PPV (Zádori et al., 2001). Although all mutations had an effect, both enzyme activity and viral infectivity decreased dramatically (>10 000-fold) when the amino acids of the HD catalytic dyad were mutated. When 11 mutations were analyzed, a correlation between enzyme activity and viral infectivity was observed, with a correlation coefficient of about 0.9.

Recently, we also identified a PLA<sub>2</sub> motif in a baculovirus (ORF 81 of NeleNPV). It shares the critical Ca<sup>2+</sup>-binding loop (YxGxG) and catalytic dyad (HD) motifs of PLA<sub>2</sub> (Figure 8.3). In contrast to parvoviruses, this putative PLA<sub>2</sub> is not part of a multifunctional protein, but seems to be a stand-alone protein, like most sPLA<sub>2</sub>s. However, we cannot rule out the possibility that it is spliced to the vp91 capsid protein, which is immediately downstream in the genome (Lauzon et al., 2004), and thus that it conforms to the parvovirus strategy. This is the first viral PLA<sub>2</sub> to be identified outside the parvoviruses. It does not seem to be present in lepidopteran baculoviruses, but may be generally present in those of hymenopteran baculoviruses.

## SUBSTRATE SPECIFICITY AND pH-RATE PROFILES OF VP1up PLA<sub>2</sub>s

Recombinant viral VP1up PLA<sub>2</sub>s from PPV, AAV2, and B19 were shown to display maximum activity in the pH 6–7 range using an assay where co-vesicles of 1-palmitoyl-2-oleoyl phosphatidylcholine containing 1-palmitoyl-2-[9,10-<sup>3</sup>H] palmitoyl phosphatidylcholine were incubated in a variety of buffers and liberated free [<sup>3</sup>H]-palmitic acid was quantified (Canaan *et al.*, 2004). The increase in activity from pH 5 to 6 may be associated with the deprotonation of the His in the active site. Typical sPLA<sub>2</sub>s display a similar shift in activity with increasing pH, with an apparent pK<sub>a</sub> value of 6–7 (Gelb *et al.*, 1995; Bayburt and Gelb, 1997).

The specific activities of PPV VP1up and its truncated form M33-VP1up (amino acids 2–128) are about 300 and 625 mol/min-mg, respectively, in a fluorimetric assay with anionic glycerophospholipids (Dudler *et al.*, 1992). This exceeds the 250 mol/(min-mg) activity reported for human group IIA PLA<sub>2</sub>s (Singer *et al.*, 2002), and puts the viral PLA<sub>2</sub>s among the most active currently known. Human group IIA PLA<sub>2</sub>s bind poorly to zwitterionic phosphatidylcholine vesicles, resulting in very low activity when compared with their activity on anionic membranes. However, the build-up of anionic reaction products (fatty acids) in the zwitterionic membranes improves the binding of human group IIA PLA<sub>2</sub>s to these vesicles and accelerates the reaction (Bezzine *et al.*, 2002). In contrast, PPV VP1up PLA<sub>2</sub>, like human group X sPLA<sub>2</sub>s, binds equally well to zwitterionic and anionic vesicles and has high specific activity on both. Since PPV VP1up and its truncated form M33-VP1up have a similar molar specific activity, it can be deduced that the 10 kDa C-terminal domain of the VP1up does not contribute to its PLA<sub>2</sub> activity and probably has another function.

The specific activities of PLA<sub>2</sub>s from the B19, GmDNV, and AAV2 VP1up were about 100 to 1000-fold less active than PPV VP1up and M33-VP1up (Zádori *et al.*, 2001; Canaan *et al.*, 2004). A similar variability in specific activities is observed among sPLA<sub>2</sub>s: for instance the specific activities of human groups IID, IIE, and XIIA sPLA<sub>2</sub>s are also two to three orders of magnitude lower than the most active sPLA<sub>2</sub>s (Gelb *et al.*, 1995). Like the PPV and human group X PLA<sub>2</sub>s, B19 and AAV2 VP1up PLA<sub>2</sub>s bind equally well to anionic and zwitterionic membranes. The limited data obtained to date seems to indicate that within each viral genus specific PLA<sub>2</sub> activities do not vary very much. For example, the activities of GmDNV and M'DNV PLA<sub>2</sub>s, from the *Densovirus* genus, are about 25 times lower than those of BmDNV and CeDNV, from the *Iteravirus* genus (Li *et al.*, 2001; Fédière *et al.*, 2002, 2004; Tijssen *et al.*, 2003). The reasons why subsets of these PLA<sub>2</sub>s have relatively low activity is not obvious from a comparison of the amino acid sequences of these enzymes. Perhaps the physiological substrates for these low-activity enzymes have structures that

are distinct from those of standard phospholipids. It is also interesting that the highly active PPV PLA<sub>2</sub> is buried inside the viral capsid during most of the viral cycle, where it would not be toxic, whereas the VP1up PLA<sub>2</sub> of B19, exposed near or on the viral surface (Rosenfeld *et al.*, 1992; Kawase *et al.*, 1995), has much lower activity.

We also determined the specificity of the VP1up proteins for the *sn*-2 fatty acyl chain of phosphatidylcholine using a dual-radiolabel approach with 1-stearoyl-2-[1-<sup>14</sup>C]arachidonoyl-phosphatidylcholine and 1-palmitoyl-2-[9,10-<sup>3</sup>H]palmitoyl phosphatidylcholine (Canaan *et al.*, 2004). The relative ratios of the  $k_{cat}/K_M^*$  for the two substrates (asterisks denote kinetic constants for the action of enzymes at an interface) was not statistically different from unity for VP1up PLA<sub>2</sub>s from PPV, B19, and AAV2. This suggests that the parvoviral PLA<sub>2</sub>s do not display specificity for *sn*-2 saturated versus polyunsaturated fatty acyl chains, as is typical for most sPLA<sub>2</sub>s (Gelb *et al.*, 1995).

The head groups of phospholipids may also impact PLA<sub>2</sub> activity. Phosphatidylglycerol, phosphatidylcholine, and phosphatidylserine vesicles were found to be the best substrates for the viral PLA<sub>2</sub>s, whereas phosphatidylinositol vesicles were a poor substrate (Canaan *et al.*, 2004). The specific activities of sPLA<sub>2</sub>s on vesicles are a reflection of both their binding to the vesicle surface and their catalytic efficiency at hydrolyzing different phospholipids (Gelb *et al.*, 1995). Therefore, we also studied the substrate specificity of viral PLA<sub>2</sub>s on mixed vesicles, and incorporated HPLC-electrospray ionization mass spectrometry to quantify the amount of each lysophospholipid produced before 20 percent of the total phospholipid in the mixed substrate vesicles had been hydrolyzed. In this experiment, substrate specificity is not influenced by the differential binding affinity of the PLA<sub>2</sub> to vesicles of different phospholipid composition, and it thus gives an indication of the intrinsic substrate specificity of the active site for phospholipids with different polar headgroups. This experiment demonstrated that all four VP1up proteins hydrolyze phosphatidylglycerol, phosphatidylcholine, and phosphatidic acid with high efficiency, that phosphatidylethanolamine and phosphatidylserine are slightly less preferred substrates, and that phosphatidylinositol is hydrolyzed only very poorly (Canaan *et al.*, 2004). The active site of parvoviral PLA<sub>2</sub>s, therefore, does not seem to be configured in a well-defined pocket for binding phospholipid polar head groups with high specificity.

## AFFINITY OF VIRAL PLA<sub>2</sub> FOR CALCIUM IONS AND PHOSPHOLIPID MEMBRANES

The affinity of the viral PLA<sub>2</sub>s for calcium and membranes was further investigated in order to establish whether these could be responsible for the large differences in specific activities. Zádori *et al.* (2001) observed no PLA<sub>2</sub> activity when

calcium was omitted from the assay (in the presence of 1 mM EGTA). Recently, we determined the apparent dissociation constant for Ca<sup>2+</sup>, K<sub>d</sub>, for several parvoviral PLA<sub>2</sub>s (Canaan *et al.*, 2004). The data obtained fit a standard hyperbolic binding equation for a single Ca<sup>2+</sup> site on the protein. The obtained values of the K<sub>d</sub> are 1 ± 0.3 mM for PPV VP1up and its truncated form, 0.24 ± 0.06 mM for AAV2 VP1up, and 0.14 ± 0.05 mM for B19 VP1up. Identical values of K<sub>d</sub> for both PPV VP1up PLA<sub>2</sub>s indicated that the 58 amino acid C-terminal extension present in PPV VP1up has no role in the affinity of the protein for calcium. Also, B19 and AAV2 VP1ups have higher affinity for calcium than PPV VP1up, although the K<sub>d</sub> values determined in that study were only apparent values. The binding of a single phospholipid in the active site of sPLA<sub>2</sub>s requires Ca<sup>2+</sup>, and active site phospholipid binding also requires that the enzyme be bound to the interface of phospholipid vesicles since long-chain phospholipids have virtually no solubility in the aqueous phase (Yu *et al.*, 1993). The dependence of the viral PLA<sub>2</sub> enzyme activity on the calcium concentration is typical of the behavior of classical sPLA<sub>2</sub>s (Gelb *et al.*, 1995).

The binding of VP1up proteins to phospholipid vesicles was determined by binding VP1up proteins to sucrose-loaded phospholipid vesicles, which could then be pelleted by ultracentrifugation. We used a molar ratio of total phospholipid to VP1up proteins that was in excess of 200:1, to avoid protein crowding on the vesicles. Also, as binding studies were carried out in the presence of sufficient Ca<sup>2+</sup> to saturate the enzyme, we used diether phospholipids (dioleyl) to avoid hydrolysis of the vesicles by the bound VP1up proteins. The amount of VP1up PLA<sub>2</sub> remaining in the supernatant after pelleting the vesicles by centrifugation was then established. The equilibrium constant for enzyme dissociation from the vesicles, K<sub>d</sub>, was expressed in terms of millimolar phospholipid in the outer leaflet of the vesicles (half of the total phospholipid in the binding reaction). The K<sub>d</sub> of PPV VP1up was found to be about 0.5 mM for dioleyl phosphatidylcholine vesicles containing either 10 percent or 30 percent dioleyl phosphatidylserine (Canaan *et al.*, 2004). AAV2 VP1up binds 2- to 5-fold more tightly to these vesicles than PPV VP1up, so that poor interfacial binding cannot explain the lower catalytic activity of AAV2 VP1up relative to its PPV VP1up counterpart.

The human group X sPLA<sub>2</sub> binds to 10 percent dioleyl phosphatidylserine/dioleyl phosphatidylcholine vesicles with a K<sub>d</sub> of 0.13 mM (Bezzine *et al.*, 2002), a value similar to that of AAV2 VP1up. These authors also showed that human group IIA sPLA<sub>2</sub> has a dramatic dependence on the presence of >10–20 percent anionic phosphatidylserine for binding to vesicles, which is consistent with its function as a Gram-positive bactericidal agent. As in the case of substrate specificity, parvoviral PLA<sub>2</sub>s act like human group X sPLA<sub>2</sub>s with respect to interfacial binding.

## CLASSICAL PHOSPHOLIPASE A<sub>2</sub> INHIBITORS AND PARVOVIRAL PLA<sub>2</sub>s

Parvoviruses with point mutations in the PLA<sub>2</sub> catalytic residues of their VP1up PLA<sub>2</sub> proteins are not, or are poorly, infectious (Zádori *et al.*, 2001; Girod *et al.*, 2002). Catalytic inhibitors, therefore, are expected to display antiviral activity.

Tetracain and oleyloxyethylphosphorylcholine were found to delay infection (Zádori *et al.*, 2001) but the possibility of side effects from these agents *in vivo* cannot be excluded. Nine potent and active site-directed inhibitors of classical sPLA<sub>2</sub>s were also studied *in vitro* as potential inhibitors of VP1up PLA<sub>2</sub>s. Remarkably, all of these inhibitors displayed little or no potency against the VP1up PLA<sub>2</sub>s (Canaan *et al.*, 2004). The potent LY311727 inhibits human group IIA PLA<sub>2</sub> with an IC<sub>50</sub> of about 0.1 μM, but failed to inhibit the VP1up PLA<sub>2</sub>s at 25 μM. Some inhibition was seen with phospholipid analogs (MJ33, MJ50, HK30, HK38, HK61), but it was much weaker than that seen with classical sPLA<sub>2</sub>s. No inhibition of B19 VP1up was observed.

The active sites of the viral PLA<sub>2</sub>s may contain some features that are distinct from those of classical sPLA<sub>2</sub>s, such as greater flexibility because of the absence of disulfide bonds. Work is in progress to determine the high-resolution structure of VP1up proteins of PPV and B19.

## LIBERATION OF ARACHIDONIC ACID BY VIRAL PLA<sub>2</sub>

Arachidonic acid is an essential, unsaturated, 20 carbon fatty acid that humans use to synthesize prostaglandin and thromboxane mediators. This fatty acid can be produced from membrane phospholipids by PLA<sub>2</sub> and is then very rapidly metabolized into eicosanoids. For example, the COX (cyclooxygenase) enzyme pathway metabolizes it into various prostaglandins, while the lipoxygenase enzyme pathway generates leukotrienes. Both prostaglandins and leukotrienes are highly pro-inflammatory, bronchospastic, and vasodilatory.

It was expected that the viral PLA<sub>2</sub>s would be able to act on the outer leaflet of the membranes of mammalian cells, which are rich in zwitterionic phospholipids, since viral PLA<sub>2</sub>s are able to readily hydrolyze phosphatidylcholine vesicles. CHO-K1 cells were preincubated for 24 hours with radiolabeled arachidonic acid, washed and VP1up protein was added. The release of arachidonic acid was then assessed by scintillation counting. As little as 100 ng/ml of exogenously added PPV VP1up led, after 2 or 4 hours, to measurable release of [<sup>3</sup>H]-arachidonic acid into the culture medium (Canaan *et al.*, 2004). Due to the low specific activity of B19 and AAV2 PLA<sub>2</sub>s on phospholipid vesicles, no release of arachidonic acid could be measured with these enzymes.

Not surprisingly, human group IIA sPLA<sub>2</sub> fails to release arachidonic acid when added exogenously to mammalian cells at concentrations of up to 1 µg/ml, since it binds weakly to phosphatidylcholine-rich membranes (Gelb *et al.*, 1995; Bezzine *et al.*, 2000). Again, the property of these viral PLA<sub>2</sub>s to liberate arachidonic acid from CHO-K1 cells is reminiscent of human group X PLA<sub>2</sub>, which readily hydrolyzes phosphatidylcholine and consequently is also able to release arachidonic acid (Bezzine *et al.*, 2002).

## ROLE OF VIRAL PLA<sub>2</sub> IN THE VIRAL CYCLE

Data obtained with PLA<sub>2</sub>-negative mutants indicated that, after transfection of infectious PPV clones, DNA replication, protein expression, capsid formation, packaging, and cellular exit are not affected. However, within 2–4 hours of entering new host cells via the classical endocytic pathway, these mutants were seen to accumulate in perinuclear LAMP-2 positive late endosomes/lysosomes (LAMP-2 is a membrane protein marker) (Zádori *et al.*, 2001). Baculovirus-expressed capsids lacking VP1up altogether also accumulated in the same location. This suggested that parvoviral PLA<sub>2</sub> acted at this or an upstream event.

The interfacial binding and specific activities of the VP1up proteins are consistent with the behavior of the human group X PLA<sub>2</sub>s, and suggest that they act on host cell membranes, which are typically not rich in acidic phospholipids. VP1up PLA<sub>2</sub> activity requires submillimolar to millimolar calcium concentrations, about 1000-fold higher than those routinely present in the cytosol, suggesting that they act either in the extracellular fluid or inside an intracellular organelle containing extracellular fluid. VP1up proteins lack disulfide bonds, in contrast to the classical sPLA<sub>2</sub>s, which contain several disulfides and thus are not expected to survive the reducing environment of the mammalian cell cytosol. In contrast, the common part of the GmDNV VPs contains disulfide bonds (Tijssen and Kurstak, 1981).

The VP1up of MVM, CPV and PPV is inside the virion (Cotmore *et al.*, 1999; Vihtinen-Ranta *et al.*, 2000; Zádori *et al.*, 2001; Canaan *et al.*, 2004) and must be externalized, or the virion become dissociated, before PLA<sub>2</sub> can get access to its substrate. The PPV virus particle can undergo a heat-induced structural transition that externalizes the VP1up PLA<sub>2</sub> domain, while the virus remains intact, which greatly enhances its PLA<sub>2</sub> enzymatic activity (Zádori *et al.*, 2001; Canaan *et al.*, 2004). However, the viral genome becomes accessible to externally applied DNase after heat shock (Cotmore *et al.*, 1999). It was suggested (Zádori *et al.*, 2001) that the PPV viral capsid may be induced, at physiological temperature, to undergo a similar coordinated structural transition *in vivo* during virus entry into host cells, which would expose its PLA<sub>2</sub> domain when it encountered a specific environment (e.g. in an endosome) or a particular receptor, at exactly the time and location that this enzyme

activity is required. Indeed, Suikkanen *et al.* (2003) recently reported that CPV treated with low pH (4–6) buffer became PLA<sub>2</sub>-active. In contrast, the VP1up of B19 virus is apparently always exposed at the particle surface (Rosenfeld *et al.*, 1992; Kawase *et al.*, 1995), so that its low PLA<sub>2</sub> activity may be required to avoid the deleterious side effects that would be associated with higher activities.

Suikkanen *et al.* (2003) provided evidence, using VP1up antibodies, that the VP1up becomes exposed in the endosome. Co-internalization studies of CPV using alpha-sarcin or rhodamine-labeled dextrans demonstrated that the virus could penetrate the membrane without major changes in permeability, i.e. the endosomal membrane retained alpha-sarcin or  $M_r$  10 000 dextran but not  $M_r$  3000 dextran during CPV infection (Parker and Parrish, 2000; Suikkanen *et al.*, 2003). Since the larger dextrans could not escape, it was suggested that PLA<sub>2</sub>, which becomes exposed in the acidic lysosome, is responsible for egress from this organelle in conjunction with another factor (Suikkanen *et al.*, 2003). In this respect, it should be noted that PLA<sub>2</sub> and infectious DNA should be supplied in *cis*, since co-infection of PLA<sub>2</sub>DNA<sup>+</sup> particles with PLA<sub>2</sub><sup>+</sup>DNA<sup>−</sup> particles did not result in an infection (Zádori *et al.*, 2001). Therefore, no bulk transport from the lysosome was expected. *In situ* hybridization demonstrated that no viral DNA was transferred to the nucleus in PLA<sub>2</sub> negative mutants (Zádori *et al.*, 2001).

## ANTIPHOSPHOLIPID-PROTEIN COMPLEX ANTIBODIES AND PARVOVIRUSES

The location of the B19 VP1up on the outside of its virion, which allows it to bind phospholipids, could explain the presence of antiphospholipid-protein complex antibodies in some B19-related syndromes. Similarities between systemic lupus syndrome (SLE) and B19-related lupus erythematosus (BLE) are striking, both clinically and serologically. Whether B19 triggers or mimics SLE remains controversial (Severin *et al.*, 2003). Von Landenberg *et al.* (2003) observed that antiphospholipid antibodies are preferentially detected in children with juvenile idiopathic arthritis and persistent infection of B19, and that adults with antiphospholipid antibodies have a high incidence of persistent B19 infections.

## CONCLUSIONS

Sequence comparisons of vertebrate and insect parvoviruses revealed conserved HDXXY and YXGXG motifs in the unique part of a minor structural protein (VP1up) that are found, respectively, in the catalytic site and conserved Ca<sup>2+</sup> binding loop of sPLA<sub>2</sub>s. While the conservation between

VP1up and sPLA<sub>2</sub> is poor or absent outside these two motifs and parvoviruses lack the typical disulfide bridges of sPLA<sub>2</sub>s (Table 8.1, p. 97), a recombinant protein containing this domain displayed clear PLA<sub>2</sub> activity. This is the first virus family for which an sPLA<sub>2</sub>-like activity has been found, and confirms the classification of vertebrate and arthropod parvoviruses into the same family even though these two groups of viruses otherwise share only very limited sequence identity and use different expression strategies.

The conserved PLA<sub>2</sub> domain by itself has relatively low activity and requires additional VP1up sequences to achieve full activity. This could be a reason why the PLA<sub>2</sub> activity of the different parvoviruses can differ by up to about 1000-fold despite high sequence identity within the conserved domain, with that of PPV being as active as the most active sPLA<sub>2</sub>. Interestingly, the PLA<sub>2</sub> activity of PPV, the most active identified so far, was located within the capsid. Viral infectivity of PPV decreased dramatically when amino acids in the HD catalytic dyad were mutated. It can also be expected that parvoviruses with low PLA<sub>2</sub> activity may rely less on this enzyme for infectivity. In addition to catalytic activity, viral PLA<sub>2</sub>s may, in analogy to sPLA<sub>2</sub>s, have supplementary functions such as in membrane binding.

Kinetic and interfacial binding analysis indicated that the parvoviral PLA<sub>2</sub>s most resemble the human group X sPLA<sub>2</sub>s. An apparent pK<sub>a</sub> value of 6–7 is consistent with the deprotonation of the His in the active site. The specific activity of PPV PLA<sub>2</sub> (amino acids 2–128 in VP1) is about 625 mol/min-mg) with anionic glycerophospholipids and is among the most active PLA<sub>2</sub>s (including snake, scorpion, and bee venoms) known. PPV PLA<sub>2</sub>, like human group X sPLA<sub>2</sub>s (Pan *et al.*, 2002), binds equally well to zwitterionic and anionic vesicles and has high specific activity on both. Although the specific activities of the parvoviral PLA<sub>2</sub>s can differ significantly, this may not be the case within the genera, at least for the densoviruses. Parvoviral PLA<sub>2</sub>s do not seem to have a preference for *sn*-2 saturated versus polyunsaturated fatty acyl chains, as is typical for most sPLA<sub>2</sub>s. In contrast, VP1up proteins prefer some head groups, since PLA<sub>2</sub>s from PPV, B19, and AAV2 hydrolyze phosphatidylglycerol, phosphatidylcholine, and phosphatidic acid with high efficiency, phosphatidylethanolamine and phosphatidylserine slightly less well, and phosphatidylinositol is hydrolyzed only very poorly. Parvoviral PLA<sub>2</sub>s need concentrations of calcium that are typically 1000–10 000 times higher than that found in the cytosol. The <sup>Ca</sup>K<sub>d</sub> of PPV PLA<sub>2</sub> is about 1 mM, and that of AAV2 and B19-VP1up about 0.25 mM and 0.15 mM, respectively. The interfacial binding K<sub>d</sub> of PPV VP1up was found to be about 0.5 mM for dioleyl phosphatidylcholine irrespective of its phosphatidylserine content, whereas AAV2 VP1up bound 2- to 5-fold more tightly to such vesicles. This range is typical for sPLA<sub>2</sub>s. Most classical PLA<sub>2</sub> inhibitors displayed little or no potency on the VP1up PLA<sub>2</sub>s, suggesting that the active sites of the viral PLA<sub>2</sub>s may contain some features that are distinct from those of classical sPLA<sub>2</sub>s.

Arachidonic acid, an important 20 carbon fatty acid that is used to synthesize prostaglandin and thromboxane mediators by the cyclooxygenase or lipoxygenase pathways, is efficiently released from the outer leaflet of the cellular membrane by PPV PLA<sub>2</sub>.

Evidence is accumulating that parvoviral PLA<sub>2</sub>s are required during late entry stages to escape perinuclear vesicles, probably the late endosome/lysosome. The VP1up becomes externalized and the substrate becomes then accessible to the viral PLA<sub>2</sub>. Co-internalization studies using molecules of different sizes demonstrated that the virus could penetrate the membrane without major changes in permeability. Co-internalization of recombinant parvoviral PLA<sub>2</sub> and PLA<sub>2</sub>-negative virus particles does not result in an infection.

A possible role of the parvoviral phospholipase in erythemaotosis remains to be investigated.

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# Atomic structure of viral particles

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Since 1991, we have learned much about the structure of parvoviruses. Those inclined towards outrageous claims might declare parvoviruses to be the first completed structural genomics project. We know structures for products of all (two!) genes. In fact the story is far from complete. Some components still have structures that are uncharacterized, and much remains in associating function with structure. However, much about function and mechanism can be learned from the available structures. This chapter will focus exclusively on the capsid, leaving structures of the replication proteins to be discussed elsewhere (Hickman *et al.*, 2002; James *et al.*, 2003, 2004; Yoon-Robarts *et al.*, 2004). This chapter will start from the molecular anatomy of the capsid, working towards function. The basis of the next chapter will be molecular virology and function as interpreted through a structural perspective.

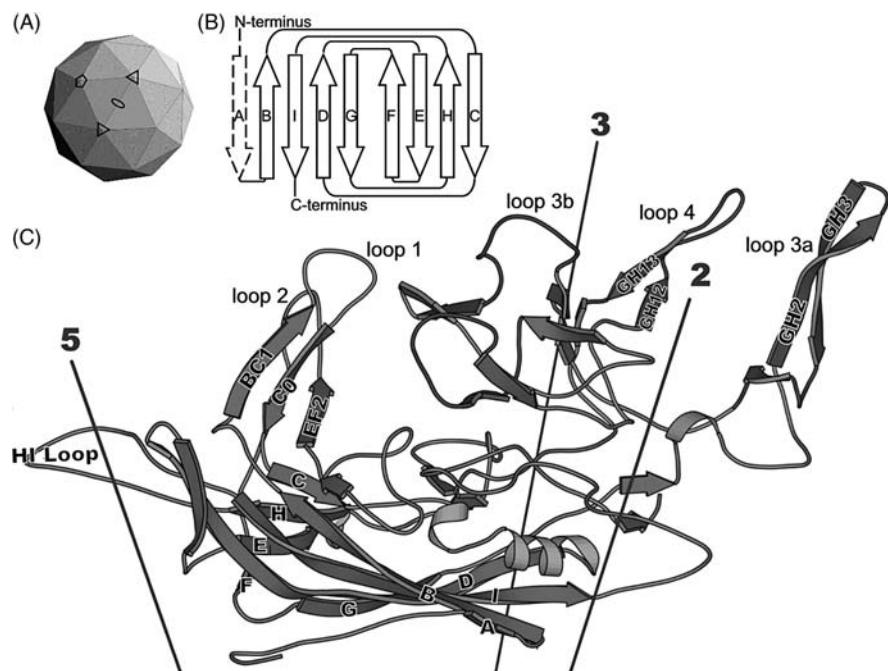
## **ICOSAHEDRAL VIRUS STRUCTURE**

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Parvoviruses are among the smallest of the spherical or isometric viruses at a diameter of  $\sim 255\text{ \AA}$ . A modest single-strand ssDNA genome of 5000 nucleotides is surrounded by a protein shell that is the main focus of this chapter. This protein capsid provides a protective coat to the DNA as it encounters environmental challenges in transmission from host to host, and cell to cell. A capsid may have several other functions: recognition of appropriate host cells, entry, intracellular transport, release of nucleic acid at the appropriate time and place, assembly of progeny virus and their release.

Enclosing itself in a proactive protein coat presents a challenge to a viral genome. The number of amino acids that can be encoded by a small viral genome is not sufficient to enclose it unless the same proteins are used repeatedly. The capsid must be modular, and largely self-assembling. These considerations led to the expectation that viruses would be assembled with the geometry of the platonic solids with multiple copies of the same facet. If the interactions of a subunit with several neighbors were self-encoded, formation of new associations could lead to self-directed assembly. The largest platonic solid is the icosadeltahedron with 60 identical faces (Figure 9.1). Caspar and Klug predicted that the simplest, so-called  $T = 1$  isometric viruses would be icosadeltahedra, and that larger viruses could be assembled with ‘quasi-equivalent’ modules with higher  $T$  numbers and greater numbers of protein subunits per icosadeltahedral facet (Caspar and Klug, 1962). These icosadeltahedra have the same point group rotational symmetry elements as the 20-sided icosahedron, leading to the common terminology of ‘icosahedral viruses’, although they contain 60 not 20 identical units. Although previously suspected, the icosahedral nature of parvoviruses was unequivocally established from symmetry detected in the preliminary characterization of canine parvovirus crystals (Luo *et al.*, 1988).

The 5-3-2 point group symmetry contains 31 rotational symmetry elements that intersect at the center: six 5-folds, ten 3-folds and fifteen 2-folds (double these numbers if you count separately the views from opposite directions). If the structure is defined for all atoms between two neighboring 3-fold axes, and one 5-fold, then all atoms of the capsid



**Figure 9.1** The architecture of a parvovirus. (A) Parvoviruses are  $T = 1$  icosahedral viruses, meaning that their near spherical capsid is comprised of 60 copies of the capsid protein. The surface can be divided into an icosadeltahedron of 60 equal triangles, each designating one protein. Of course the capsid protein is not triangular, so the surface triangle is comprised of a set of unique atoms from each of several neighboring subunits that combined would complete one triangle. The icosadeltahedron has vertices where either 5-fold or 3-fold axes pass towards the center of the virus, examples shown as a pentagon and triangles respectively. Midway between each neighboring pair of 3-fold axes passes a 2-fold (ellipse). Successive application of the rotational symmetries can generate an entire capsid from a single subunit. (B) The topology of each subunit is a viral jellyroll  $\beta$ -barrel. The connectivity of the strands (arrows) is indicated in a schematic representation that is not supposed to indicate the length or structure of the intervening loops. Strand A is dashed, because it is present in parvo- and a number of other virus families, but not all. One can imagine the nine strands as consisting of two antiparallel sheets of five and four strands respectively. There is a discontinuity in the hydrogen-bonding between strands F and G as the second sheet is folded over the first. The schematic is simplified by omitting embellishing secondary structures from the loops. (C) The 3D fold of the AAV-2 subunit. The  $\beta$ -sheets that are highly conserved among parvoviruses are shown in fuschia at the bottom with strands labeled as in (B). The loops between the strands are very different from the loops of other viruses, and even differ significantly between the different genuses of parvovirus. The loops are particularly convoluted in parvoviruses with the GH loop accounting for  $\sim 250$  residues. Most of these loops have extended chain structure, but there are additional secondary structural elements (red, orange, and purple), but these are not fully conserved, even among parvoviruses, and account for their unique properties in terms of antigenicity and association with cellular receptors. Loop secondary structures are labeled by the two letters of the bounding barrel strands and a number that increases towards the C-terminus. Other secondary structures are labeled with a single letter/number if they are nearly contiguous with a barrel strand. Thus CO immediately precedes strand C. ([C] is reproduced with permission from Xie et al., 2002, copyright of National Academy of Sciences, USA, 2002.) See also Color Plate 9.1.

can be generated by systematic application of all of the symmetry elements. These symmetry axes are often used as frames of reference for describing the locations of features in the structures. It is understood that the same features would be repeated near each of the identical symmetry axes.

## Icosahedral virus structure determination

At atomic resolution, parvoviral capsid structures have been the exclusive domain of X-ray crystallography. Electron microscopy has the potential for near-atomic resolution but,

with parvoviruses, has been applied only at lower resolution – 10 Å or less, sufficient to determine the surface topology, localization of components, and complexes of the virus with antibody or receptor fragments.

While the principles of viral crystallography are the same as protein crystallography, differences in the application of methods have implications for what can and cannot be visualized. The diffraction signal is weaker in proportion to the size of the viral complex. Averaging of the electron density between symmetry-related parts of the capsid is needed to ameliorate the poor signal/noise, and to help solve the phase problem. Diffraction amplitudes are Fourier amplitudes that,

**Table 9.1** Parvoviral atomic structure

Genus	Structure	Abbreviation	Host	PDB accession number	Resolution	R-factor	Citation(s)
<i>Densovirus</i>	<i>Galleria mellonella</i> densovirus	GmDNV	Wax moth	1DNV	3.7 Å	0.271	(Simpson <i>et al.</i> , 1998)
<i>Parvovirus</i>	Canine parvovirus	CPV	Dog	1DPV	2.9 Å	0.29	(Tsao <i>et al.</i> , 1991; Xie and Chapman, 1996) (Agbandje <i>et al.</i> , 1993; Simpson <i>et al.</i> , 2000)
	Feline panleukopenia virus	FPV	Cat	1FPV/1C8F	3.3/3.0 Å (1CF8)	0.285	(Agbandje-McKenna <i>et al.</i> , 1998)
	Minute virus of mouse	MVM	Mouse	1MVM	3.5 Å	0.327	(Agbandje-McKenna <i>et al.</i> , 1998)
	Porcine parvovirus	PPV	Pig	1K3V	3.5 Å	0.286	(Simpson <i>et al.</i> , 2002)
<i>Erythrovirus</i>	B19	B19	Human	1S58	3.5 Å	0.309	(Kaufmann <i>et al.</i> , 2004)
<i>Dependovirus</i>	Adeno-associated virus-2	AAV-2	Human	1LP3	3.0 Å	0.338	(Xie <i>et al.</i> , 2002)

if transformed, yield an image of the sample's electron density. Phases are also needed to synchronize the sinusoidal waves in the Fourier transformation, but conventional methods of experimentally determining phases by binding heavy atoms and/or changing the X-ray wavelength (Smith, 1991; McPherson, 2002) are difficult to apply to viruses (Tsao *et al.*, 1992). Fortunately the icosahedral symmetry can be applied as a constraint to improve crudely approximated phases. The starting approximations are usually calculated from related structures, electron microscopic images, or *ab initio* calculations (Rossmann 1972, 1995; Chapman, 1998; Chapman *et al.*, 1998). The implication of being forced to use the icosahedral symmetry during structure determination is that generally only those parts of the structure that adhere to the 60-fold symmetry will be seen.

(Kaufmann *et al.*, 2004), representing the *Erythrovirus* genus. Just when it was thought that all three Parvovirinae genera were represented by structures, taxonomic reclassification based on genomic differences has introduced two new Parvovirinae genera: *Amdovirus* (Aleutian mink disease virus, AMDV) and *Bocovirus* (bovine parvovirus and canine minute virus) (see Chapter 1). AMDV has been visualized by cryo-electron microscopy at 22 Å resolution (McKenna *et al.*, 1999), but there are no atomic structures for these new genera. Statistics for the atomic structures are summarized in Table 9.1. Unlisted are several variant CPV and FPV structures – full versus empty particles, mutants and different pHs (Wu and Rossmann, 1993; Llamas-Saiz *et al.*, 1996; Simpson *et al.*, 2000; Govindasamy *et al.*, 2003). CPV was the first structure to be determined (Tsao *et al.*, 1991), was pursued to the highest resolution refinement (Chapman and Rossmann, 1996), and most exhaustively interpreted (Xie and Chapman, 1996). It is therefore natural in this chapter to frame an overview around the CPV structure, and then to describe the differences of each of the other parvoviral structures and their implications relating to the viral life cycle.

Within each *Parvovirinae* genus, the capsid sequence identity may be as low as ~50 percent (e.g. Chapman and Rossmann, 1993; Rutledge *et al.*, 1998). This is a similar level of identity to that between different picornaviral genera. Even within a parvoviral genus, sequence might suggest an array of structural variations and diversity in host interactions as great as between, say, enteroviruses and rhinoviruses. Between the *Parvoviridae* genera, the capsid sequence identity ranges between 10 and 27 percent, much lower than between picornaviral genera. While overall subunit folds would be expected to be conserved, the low sequence identity might lead one to expect substantially different variations on a common structural theme, and perhaps several distinct ways that the virus might handle receptor-binding, immune evasion *etc.* In this light, the level of structural conservation that is seen is quite remarkable.

## PHYLOGENY AND ATOMIC STRUCTURES

The family *Parvoviridae* has two subfamilies – Parvovirinae whose members infect vertebrates, and Densovirinae whose members infect arthropods. There are four Densovirinae genera of which the wax moth (*Galleria mellonella*) densovirus (GmDNV) from the genus *Densovirus* is the sole structural representative (Simpson *et al.*, 1998). All Parvovirinae genera infect vertebrates. Genus *Parvovirus* is the best characterized with structures of canine parvovirus (CPV) (Tsao *et al.*, 1991), feline panleukopenia virus (FPV) (Agbandje *et al.*, 1993), minute virus of mouse (MVM) (Agbandje-McKenna *et al.*, 1998) and porcine parvovirus (PPV) (Simpson *et al.*, 2002). Genus *Dependovirus* is currently represented solely by adeno-associated virus serotype 2 (AAV-2) (Xie *et al.*, 2002), but good progress is being made on other serotypes which we hope will follow soon. The most recent addition is the structure of a B19 capsid, recombinantly expressed in insect cells from a baculovirus vector

## OVERVIEW OF THE STRUCTURE

### Elements of the structures visualized

In the CPV structure, the capsid proteins are resolved starting at residue 22 of viral protein 2 (VP2) (Tsao *et al.*, 1991; Chapman and Rossmann, 1996; Xie and Chapman, 1996). The capsid gene actually encodes three capsid proteins that differ at their N-terminal ends. VP1 at 737 residues is the largest and is the product of different mRNA splicing that give it an additional N-terminal 153 residues (in CPV) relative to VP2, while VP3 is either another splicing product, or, as in CPV, a proteolytic cleavage product of VP2 (Clinton and Hayashi, 1976; Tattersall *et al.*, 1977), missing (in CPV) the N-terminal 15–20 residues. Whether VP2 or VP3 is the majority form in mature capsids depends on the species, but VP1 is always at about 10-fold lower concentration. In crystal structures, the protein becomes visible a few residues after the N-terminus of VP3 (or VP4 in Densoviridae), so the N-terminal regions distinctive to VP1 or VP2 are not seen.

Are the VP1/2 N-terminal regions actually present in the crystallographic samples? This has been most carefully examined for the adeno-associated virus-2 (AAV-2) crystals, which, by denaturing gel electrophoresis, has an identical 1:1:8–10 complement of VP1, 2, and 3 to that of fresh viral preparations (Rose *et al.*, 1971; Johnson, 1984; Xie *et al.*, 2004). VP1 is likely present in many of the parvoviral crystals with the exception of those, like B19, that are recombinantly expressed. Successful crystallization suggests an internal location for VP1, because there are too few copies to be symmetrically arranged on the outer surface, and any heterogeneity in lattice contacts usually foils crystallization. The C-terminal ~570 residues of VP1, 2, and 3 are of the same sequence and structurally indistinguishable. The distinctive parts of the VP1/2 N-termini are present at well below the 30 percent occupancy generally needed to observe crystallographically, and thus it is not surprising that these ‘minority’ components are not seen.

The DNA that is enclosed by the capsid does not have 60-fold symmetry in its sequence. For the most part, its 3D structure will not conform to the 60-fold symmetry constrained during structure determination. It is likely to be seen only where the capsid imposes its symmetry in DNA-binding sites. Up to 28 percent of the genomic DNA has been seen in CPV and MVM, bound to the capsid, but in other structures, no DNA has been seen.

in several plant, then insect virus structures (reviewed in Rossmann and Johnson, 1990; Harrison *et al.*, 1996). It is an eight-stranded  $\beta$ -barrel, sometimes known as a beta sandwich to emphasize that it contains two curved (usually) 4-stranded sheets facing each other (Figure 9.1). In the prevalent configuration, the sheet containing strands B, I, D, and G is closest to the nucleic acid. Strands are labeled alphabetically, starting from the N-terminus. The strands are all antiparallel with strands B and D pointing towards the 5-fold axis, I and G away. Like all  $\beta$ -sheets, the sheet has a right-handed twist looking along the direction of the chains. The effect (for parvoviruses) is that the end of the BIDG sheet farthest from the 5-fold is tangential to the viral surface, but by the time it approaches the 5-fold the sheet is nearly edge on. Like many other viruses, parvoviruses have a fifth A strand hydrogen-bonded to the B strand. The sheet containing strands C, H, E, and F is shorter and closer to viral surface.

Comparing viral  $\beta$ -barrels from different viral families, it is at once surprising that the same motif can appear in so many guises, and to be expected, considering that there is no recognizable sequence similarity between many viruses that appear to share structural homology. The barrels have strands that vary in length up to 3-fold. Although they share the same topology, the strand lengths of CPV and bacteriophage  $\phi$ X174 (McKenna *et al.*, 1992), another ssDNA virus with comparable genome size, are poorly correlated ( $CC = 0.3$ ) (Xie and Chapman, 1996). By convention loops are named according to the strands between which they bridge, so that the BC loop is between strands B and C. The loops of different viruses have little in common, other than generally: loops BC, HI, DE, and FG, which are close to the 5-fold axis, tend to be shorter than the CD, EF, and GH loops. More surprising is the number of ways that this fold can be used. The prevalent orientation (as in parvoviruses) is with the strands approximately tangential to the virus. Within this prevalent form, the overall orientation varies by as much as  $19^\circ$  (Chapman and Liljas, 2003). Other examples use the same fold in a different context, such as the adenovirus hexon with its  $\beta$ -strands radial to the virus (Roberts *et al.*, 1986), or have added or tandem-repeat domains (reviewed in Chapman and Liljas, 2003). There have been several attempts to explain why the jellyroll has been found in so many viral structures, but none of these rationalizations has survived extended scrutiny.

When parvoviral structures first appeared, available virus structures were mostly restricted to small RNA viruses. The jellyroll barrel was near-universal, but the number of exceptions was beginning to grow. There were questions as to whether a single-stranded DNA virus would share homology with RNA viruses. There was also speculation, based on the length of the primary sequence, that the capsid protein could encode perhaps three barrels in a pseudo-T = 3 viral architecture. As we shall see, the latter proved to be incorrect.

## CAPSID SUBUNIT FOLD

### Viral jellyroll

One of the surprises from early viral atomic structures was the prevalence of the ‘viral jellyroll’ fold. It was first revealed

## $\beta$ -barrel of CPV and other parvoviruses

The CPV structure showed a single  $\beta$ -barrel domain per capsid protein (Tsao *et al.*, 1991) oriented in a standard way (Figure 9.1). For the most part, the barrels of parvoviral structures superimpose closely, but in GmDNV it is shifted outwards 10 Å and rotated 7° (Simpson *et al.*, 1998). Parvoviral VP3 is about three times the size of the smallest jellyrolls from other viruses, and the reason is long loops between the strands, as has also proved to be the case in microphage bacteriophages (McKenna *et al.*, 1992, 1996). Several loop nomenclatures are in use. Tsao *et al.* defined loops 1 through 4 according to their location in 3D space. In the AAV-2 structure, loop 3 needs to be divided into 'a' and 'b' regions. Russell and colleagues (Rutledge *et al.*, 1998) introduced 'variable domains' I through IV where the sequence varied most between different AAV serotypes. Regions I, II, and IV correspond respectively to loops 3a, 3b, and 4. From RNA virus literature, there is a more detailed nomenclature that will be used here, where loops are labeled according to the strands that flank them. Thus the BC loop links  $\beta$ -strands B and C.

The  $\beta$ -barrel is less regular than text-book schematics might imply, with strand lengths varying from 3 to 22 residues. Of the two sheets facing each other in the  $\beta$ -barrel, it is the BIDG sheet near the inner surface of the capsid that is more regular and with longer strands, averaging 15 compared with six for the CHEF sheet. The barrels, irregularities and all, are well conserved through the species of parvovirus. Lists of secondary structural elements in the protein data bank appear to indicate inexact structural homology, but this is an artifact. The lists are generated automatically through prediction of hydrogen-bonding (Hutchinson and Thornton, 1996), a process that works well for the 2.9 Å CPV structure (Chapman and Rossmann, 1996; Xie and Chapman, 1996), but not for the structures at lower (~3.5 Å) resolution. The ribbon diagrams drawn from manual designations of secondary structure give a truer impression of the high structural conservation of the barrel. There are a few detailed differences:  $\beta$ G is continuous in AAV-2, but broken into two in CPV with the insertion of a threonine; strand C is shorter in B19 than in other parvoviruses. However, these are small differences in a highly conserved structure. The structural conservation is remarkable considering the relatively low sequence identity which, for example, is 21 percent for the barrel strands of AAV-2 and CPV, no greater than the overall identity for VP2.

## EMBELLISHMENTS UPON THE BASIC BARREL

As in several other, but not all jellyroll virus structures, the BIDG sheet is actually 5-stranded with an N-terminal edge strand,  $\beta$ A. It is present in all parvoviruses. In all but *Densovirus*, there is a tight U-turn between  $\beta$ B and  $\beta$ A.

*Densovirus* differs with domain swapping of the first strand (Simpson *et al.*, 1998; Liu and Eisenberg, 2002).  $\beta$ A is an N-terminal extension of  $\beta$ B, extending, without a tight turn, to become the first strand of the BIDG sheet of a 2-fold related subunit, i.e. the first ( $\beta$ A) strands of two subunits have been swapped. The exchange of the  $\beta$ A- $\beta$ B intrasubunit hydrogen bonds of other parvoviruses for their inter-subunit equivalents in *Densovirus* helps to cement the association of dimers in the mature capsid. Domain-swapped is certainly the dominant configuration in most of the GmDNV VP4 subunits. If there were an unswapped configuration for minor components such as VP1, this would likely not be seen in the averaged electron density.

In CPV, the CHEF sheet is actually 6-stranded. At the N-terminal end of  $\beta$ A, the chain runs in an extended configuration without a turn, becoming, after a 3-residue gap, a 3-residue strand ( $\beta$ A0) that is a 6th strand of the CHEF sheet on the inner surface of the capsid (Xie and Chapman, 1996). On the outer edge,  $\beta$ EF2 from the EF loop 2 forms a first strand, an addition that is conserved in many parvoviruses.

## LOOPY STRUCTURES

VP3 is about three times larger than the most compact  $\beta$ -barrels of other viruses. Only ~80 of the ~530 VP3 residues are within the strands of the  $\beta$ -barrel. Before the CPV structure determination, it was expected that each subunit might contain tandem repeats of the barrel as in the pseudo-T = 3 insect family Comoviridae (Chen *et al.*, 1989; Chapman and Liljas, 2003), or might contain N- or C-terminal domains decorating the surface, as in the plant family Luteoviridae (Harrison *et al.*, 1978; Chapman and Liljas, 2003). It was a complete surprise, with the structure of CPV (Tsao *et al.*, 1991), to find just a single domain with the core secondary structure accounting for only 20 percent of the primary sequence. The rest of the sequence was contained in the loops between the strands, one of which, the GH loop, was an unprecedented 220 amino acids. This insertion within the  $\beta$ -barrel fold is itself equal to a medium-sized protein. Subsequently, large insertions have been found in capsid proteins of Microviridae bacteriophages (McKenna *et al.*, 1992, 1996; Bernal *et al.*, 2003; Chapman and Liljas, 2003), but none quite as big.

Taking CPV as an example, the loop sizes are 35 residues for the BC loop, 19 for CD, 17 for DE, 72 for EF, 5 for FG, 221 for GH, and 18 for HI. Thus, parvoviruses agree with the general finding that the turns between  $\beta$ -strands near the 5-fold axis have shorter insertions than those pointing away.

Among the turns near the 5-fold, the BC loop or 'loop 2' is the longest (35 residues in CPV). The B and C strands are on the edges of their respective sheets, and near the 5-fold axes they are twisted nearly edge on, such that the BC turn is the closest to the viral surface. The loop insertion turns

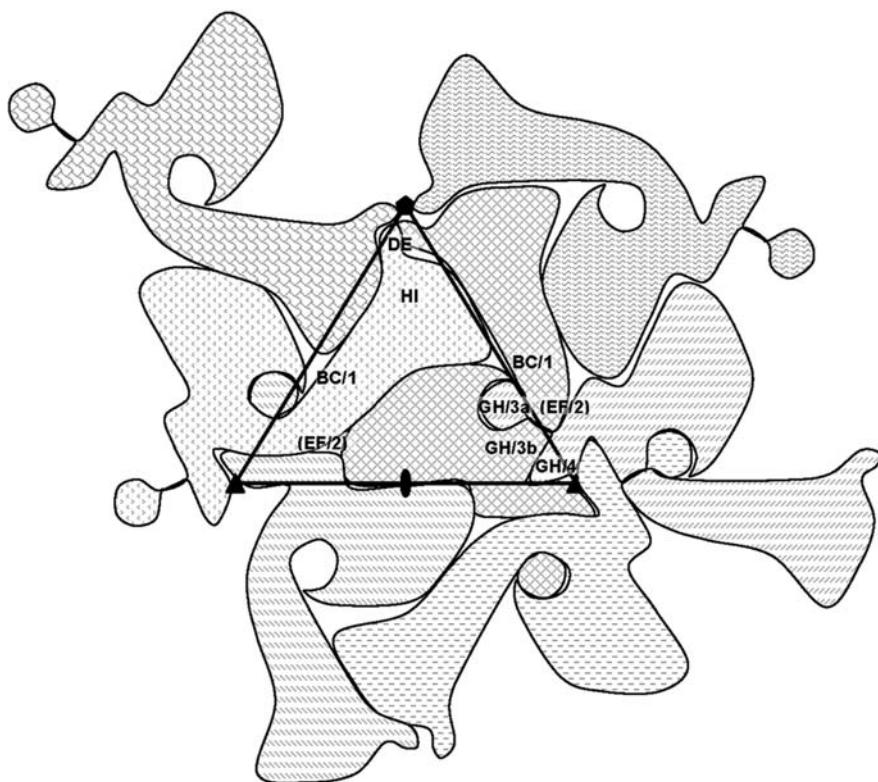
90° towards the capsid surface and away from the 5-fold to associate with the EF loop (loop 1) originating from the opposite ends of the sheets (Figure 9.1, p. 108). The tips of both of these loops (near CPV VP2 residues 93 and 225 respectively) are highly exposed on the outer surface. Several of the loops contain secondary structural elements additional to the barrel strands. The BC loop contains a  $\beta$ -ribbon – two antiparallel strands hydrogen-bonded together. Some parvoviruses have a relatively tight turn between the ribbon strands, whereas others have a longer loop containing a short  $\alpha$ -helix.

Next down from the surface is the HI turn. In all of the parvoviruses, the loop forms a loose turn of about 18 residues that interacts with a 5-fold-related subunit. The tip of this loop (near CPV VP2 520) forms much of the bottom of CPV's canyon, a ring of lower surface elevation encircling each 5-fold about 20 Å from the symmetry axis (Figures 9.2, 9.3 and 9.4). Although residues here are solvent accessible, they are not as exposed as the four protruding loops, and are perhaps not accessible to antibodies,

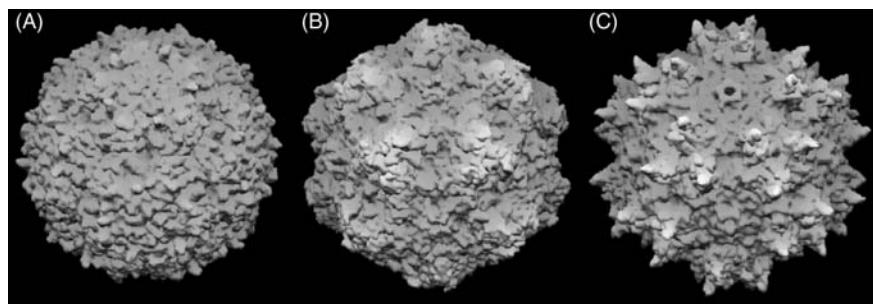
so the HI loop was not one of the numbered loops in the CPV structure.

The ends of the D and E strands are closer to the viral center. The ~17 residue loop connecting them forms a  $\beta$ -ribbon that extends parallel to the 5-fold axis to the viral surface where there is a tight turn near CPV VP2 residue 161. These two antiparallel strands are hydrogen-bonded with each other. Five of these ribbons surround each 5-fold axis. Although they have been referred to as a  $\beta$ -cylinder, the five ribbons are not quite close enough for the ribbons to hydrogen bond to each other. It is the ribbons of the symmetry-equivalent DE loops that line the 5-fold pore, which will be discussed later. Closest to the inner surface is the FG loop which is the shortest at five residues.

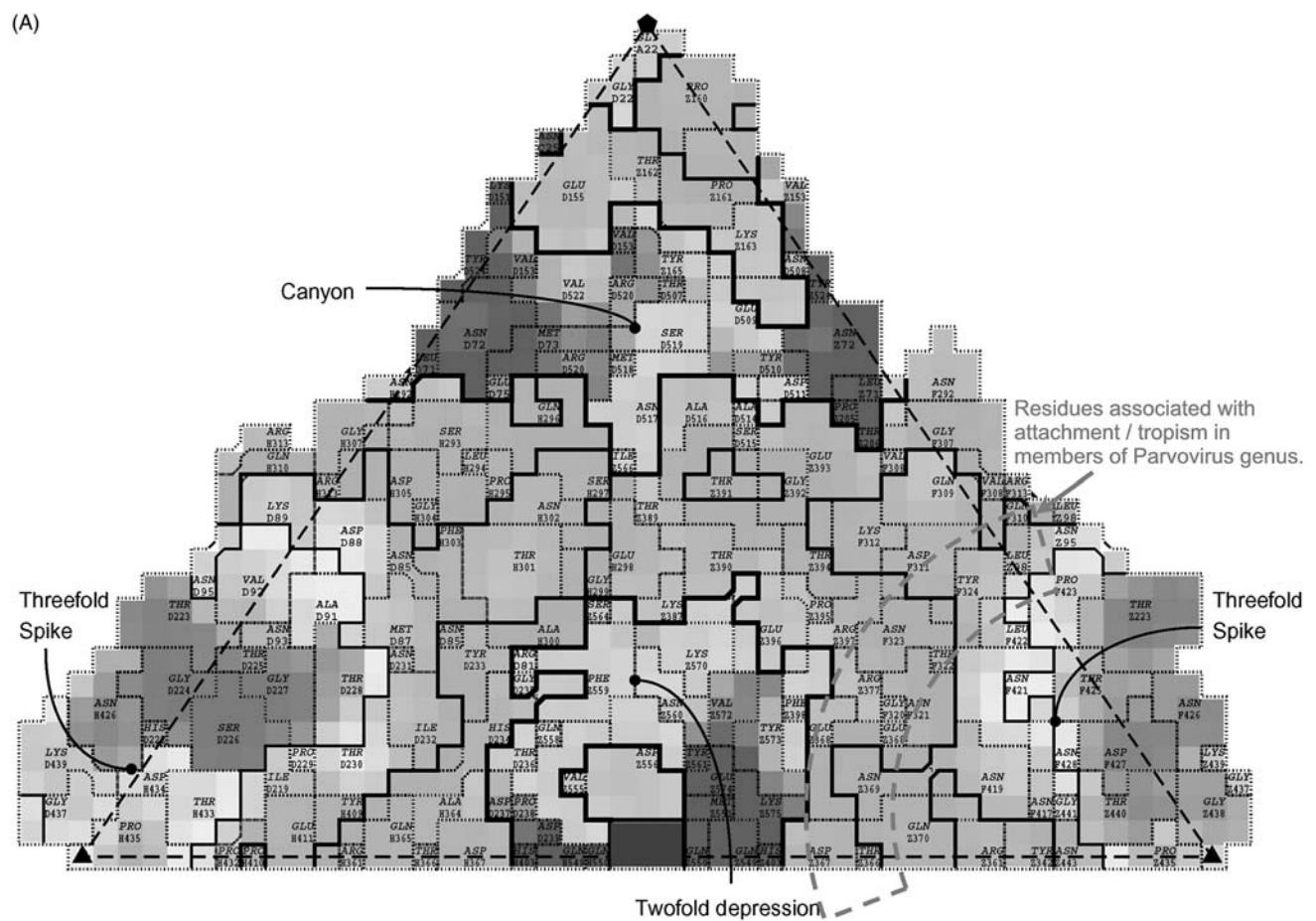
The 5-fold ends of the barrel  $\beta$ -strands are roughly aligned. As the CHEF sheet strands are much shorter than those of the BIDG sheets, the loops have to bridge a larger gap at the 2-fold end of the barrel. The short CHEF sheet remains mostly edge-on, but the longer BIDG sheet has twisted such that the strands are running tangentially to



**Figure 9.2** Surface composition of parvoviruses. The triangle shows one of the 60 identical faces of an icosahedron. As the subunit is not triangular, each face has contributions from several neighboring subunits, each shown with different shading. Near the 3-fold axes, the subunits are wrapped around each other intimately, helping to cement the assembly. Labeled are the barrel loops that contribute most to different parts of the surface. In many viruses these are named according to the flanking  $\beta$ -strands. In parvoviruses, the most surface-exposed loops have been given numbers 1–4 which are also shown. This schematic representation relates differences in the subunit primary/tertiary structure to the surface topology of the assembly. In AAV-2 and B19, loop 3a is particularly long, leading to prominent spikes (Xie et al., 2002; Kaufmann et al., 2004). In genus Parvovirus, the individual protrusions are not resolved, because an insertion in loop 4 adds bulk between the (shorter) symmetry equivalents of loop 3, filling the valleys of AAV-2. Loops 1 and 2 are longer in CPV and other members of the Parvovirus genus, leading to extension of the elevated region with a shoulder running towards the 5-fold axis.

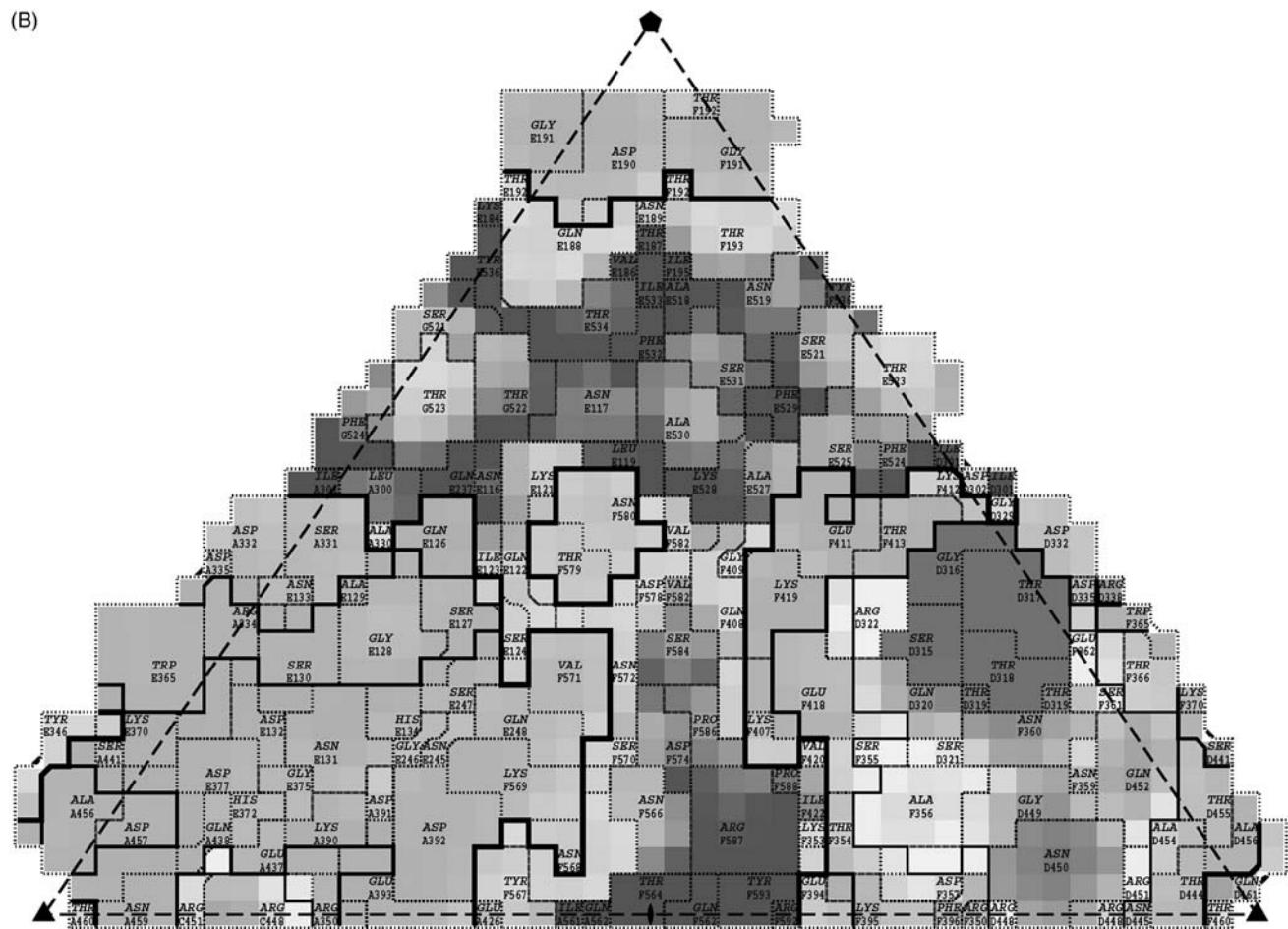


**Figure 9.3** Surface topology of parvoviruses **(A)** Densoivirus; **(B)** Parvovirus; **(C)** Dependovirus and likely Erythroivirus. GRASP (Nicholls, 1992) surface representations calculated from the atomic structures are shown to scale for representatives of parvoviral genera **(A)** (insect) densoivirus GmDNV (Simpson et al., 1998); CPV (Tsao et al., 1991) as a representative of genus Parvovirus – MVM (Agbandje-McKenna et al., 1998) and others would be very similar; and AAV-2 (Xie et al., 2002). B19, the Erythroivirus of known structure (Kaufmann et al., 2004) has an unseen disordered surface loop whose absence would distort a surface representation, but, in all other respects, its structure is most homologous to AAV-2 (Kaufmann et al., 2004), so it likely shares similar surface topology. The view is down a 2-fold axis (like Figure 9.2) with 3-fold axes left and right of center, and a 5-fold above center. See also Color Plate 9.3.

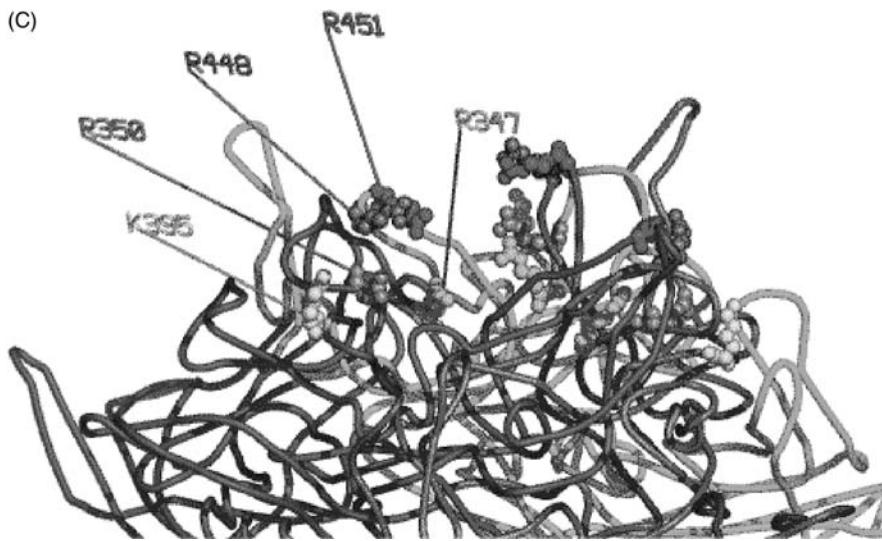


**Figure 9.4** Surface topologies and receptor-attachment of **(A)** CPV (Tsao et al., 1991) and **(B-D)** AAV-2 (Xie et al., 2002). **(A and B)** show Roadmap schematics (Chapman, 1993) of one of the 60 icosadeltahedral faces, bounded by the 5-fold (top) and two 3-folds (left and right), and viewed parallel to the 2-fold (bottom center). The solvent-accessible surface is colored like a topographical map with lower regions blue, elevated regions red. Surface residues are numbered with letter prefixes designating the subunit. (Different letters are used for CPV and AAV-2.) The region highlighted in **(A)** as associated with cell attachment and cell tropism represents a compilation from related parvoviruses aligned to the CPV surface. They include residues implicated in the cell tropism of MVM (Ball-Goodrich et al., 1991), PPV (Vasudevacharya and Compans, 1992), ADV (Bloom et al., 1988), and in the transferrin receptor-binding, cell specificity and hemagglutination of CPV and FPV (Parrish et al., 1988a,b; Barbis et al., 1992; Llamas-Saiz et al., 1996; Govindasamy et al., 2003; Hueffer et al., 2003). See also Color Plate 9.4.

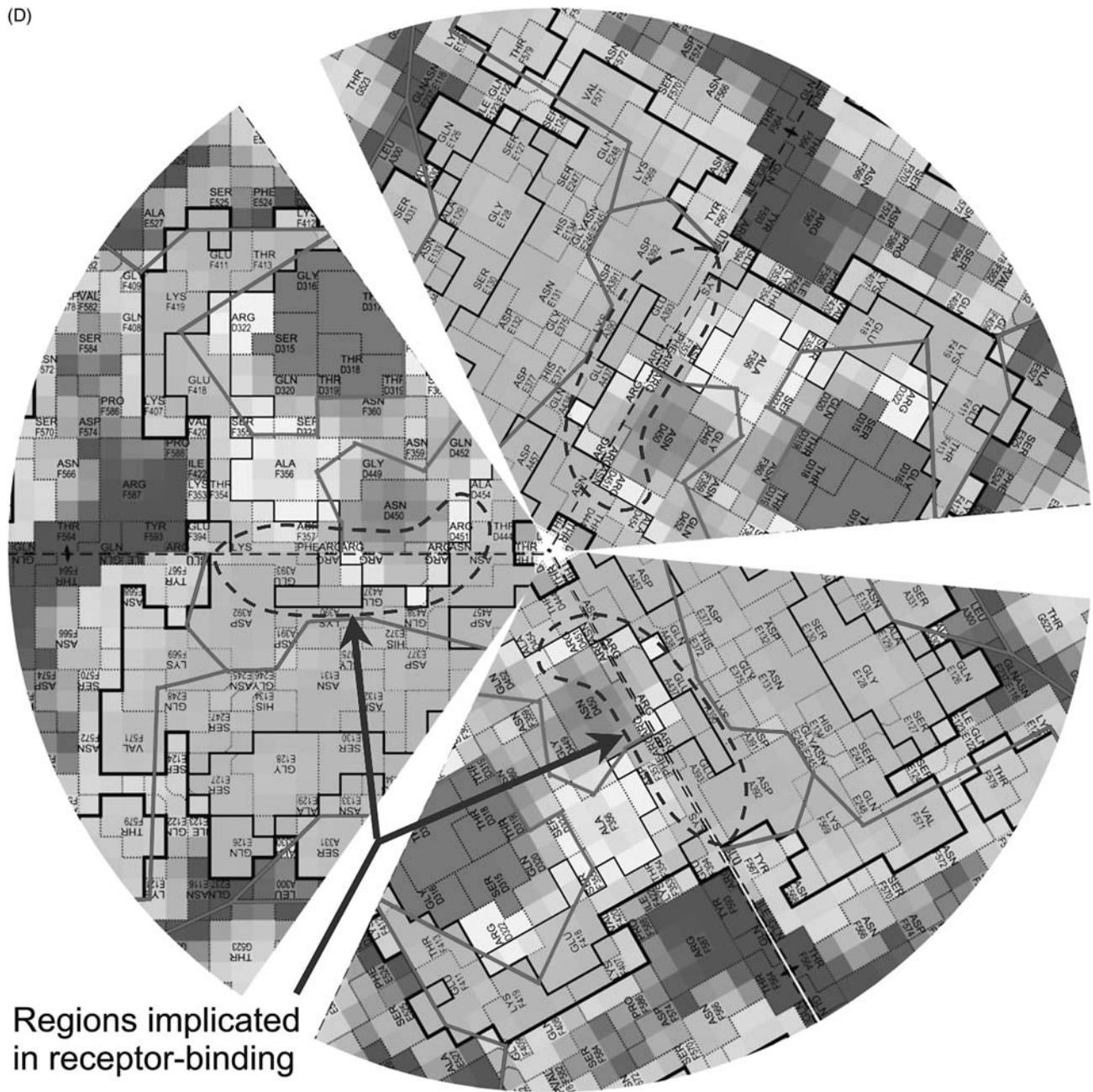
(B)



(C)



**Figure 9.4 (continued)** (C) Residues implicated by mutagenesis in the binding of AAV-2 to analogs of the heparan sulfate proteoglycan receptor and in cell entry (Kern et al., 2003; Opie et al., 2003) are shown (VP2 numbering) on a trimer from the AAV-2 structure (Xie et al., 2002) in which the backbones of subunits are in different colors. (D) The locations of these residues are mapped to a Roadmap surface of the region surrounding a 3-fold projected onto a plane. The region enclosing the implicated residues is shown with dashed blue lines, and lies between pairs of spikes, in the valley and on the side of one of the protrusions. These regions are repeated by the 3-fold symmetry, and one could imagine a heparan polysaccharide chain passing in through one valley, over the 3-fold and out through another. See also Color Plate 9.4.



**Figure 9.4 (continued)**

the viral center and the 2-fold ends of the BIDG sheet are all roughly the same distance from the viral center. Of the three loops, the CD loop is the shortest (~19 residues) forming a relatively direct connection with two  $\alpha$ -helices. The second and longer of these is buried at the dimer interface between subunits and among the most conserved parts of the capsid protein (Chapman and Rossmann, 1993). In the vertebrate parvoviruses, the CD loop runs below the GH loop that dominates the surface near the 3-fold axes.

The EF loop is 72 residues long in CPV and contains up to two  $\alpha$ -helices and four  $\beta$ -strands. Only one of the strands is conserved among all parvoviruses – a strand that

hydrogen-bonds with the ribbon of the BC loop to form a three-stranded antiparallel sheet, cementing the association between these two loops. The loop follows a similar path in all parvoviruses and its tip is a prominent part of the surface, but there are significant insertions and deletions in this loop as detailed later.

### The GH loop

The GH loop has held several surprises. As described above, it was of unprecedented length (221 residues) in

CPV. The GH loop of CPV can be divided into loop 3 and loop 4, reflecting that after ~80 residues, the chain has returned back close to the barrel before heading out to the surface again as loop 4 (Tsao *et al.*, 1991). Together they account for the large elevated area near the 3-fold axes. The second surprise in the CPV structure was that subloops from neighboring, 3-fold related subunits were interdigitated, greatly increasing the surface shared between adjacent subunits, and that the interdigitated subloops were stacked one on top of another. This indicated a sequential folding process that could only occur after the 3-fold related subunits had come together during assembly (Xie and Chapman, 1996). Glycines at the bases of the subloops, some of which are conserved, suggest flexible hinge points as these associations are made dynamically during assembly (Chapman and Rossmann, 1993; Xie and Chapman, 1996).

The third surprise came with the *Densovirus* structure (Simpson *et al.*, 1998). The reason that the capsid protein is smaller than the other parvoviruses is because it lacks loop 4. Loop 4 forms much of the bulk of what was called the 3-fold spike in Tsao *et al.* (1991), but might be more properly described as a flat elevated massif surrounding each 3-fold axis. Its absence in the arthropod parvoviruses leads to a much flatter surface topology (Figure 9.3, p. 113). The relative lack of surface features could perhaps be rationalized by a much different insect immune system that might not enforce as much selective pressure for change in the surface to escape immune neutralization. The fourth surprise came with the structures of AAV-2 and B19 (Xie *et al.*, 2002; Kaufmann *et al.*, 2004), and the extent of differences between the dependo- and erythroviruses compared with genus *Parvovirus*, as exemplified by CPV and MVM. In the vertebrate parvoviruses, the GH loops meander with little secondary structure other than some  $\beta$ -ribbons and small sheets that likely help stabilize the irregular fold. AAV-2 and CPV share a total of eight homologous  $\beta$ -strands, within buried regions of the loop, where the structures are very similar. AAV-2 has five additional  $\beta$ -strands, near the more exposed parts of the GH loop. Particularly striking is the inserted  $\beta$ -ribbon near AAV-2 VP2 317 that launches a ~20 residue 'finger' to the tip of a prominent protrusion, about 25 Å from the 3-fold.

## SURFACE TOPOLOGY

Three distinct outside surface topologies have been seen in the parvoviral structures. Common to two of these are the following features: an elevated region near the 3-fold axis, a depression between the 3-fold elevated regions at the 2-fold axis, and another depressed region encircling the 5-fold axis (Figures 9.3 and 9.4, p. 113). These two groups of viruses differ only in the shape of the 3-fold elevated region (see below). By contrast, a third type of topology was seen in *Densovirus* which is relatively spherical, flat, and featureless

(Simpson *et al.*, 1998). The absence of surface features that are antigenic in other parvoviruses has been suggested to be the result of the lack of an adaptive immune response (and its associated evolutionary pressure) in arthropods (Simpson *et al.*, 1998).

The characteristic feature of genus *Parvovirus* (CPV, MVM, PPV etc.) is the '3-fold spike' or elevated massif, 70 Å wide and 22 Å high, centered on each 3-fold axis. In comparing CPV (Tsao *et al.*, 1991), FPV (Agbandje *et al.*, 1993), MVM (Agbandje-McKenna *et al.*, 1998), and PPV (Simpson *et al.*, 2002), one is first struck by the overall similarity in the surface shape of all of these structures from the *Parvovirus* genus. The most antigenic regions of CPV (near residues 93 and 300 [Parrish *et al.*, 1988a]) lie on a spur of the massif running towards the 5-fold axis (Tsao *et al.*, 1991). The tips of the DE loops form a smaller elevated feature, forming a ring of 22 Å diameter surrounding each 5-fold axis that is about half the height of the massif (Tsao *et al.*, 1991). These features give rise to two regions of lower elevation. The first is an 11 Å wide valley, between the 5-fold ring and the 3-fold massifs, that, by analogy to the picornaviral structures, has been named the canyon (Figure 9.4, p. 113) (Tsao *et al.*, 1991). The second, named the 2-fold depression, is centered between two adjacent 3-fold massifs. The depression is implicated as the cell-receptor-binding locus in MVM through a recent crystallographic structure of MVM complexed with a fragment of its receptor, sialic acid (Lopez-Bueno *et al.*, submitted). This is the first direct visualization of virus-receptor interactions in genus *Parvovirus*. There is genetic evidence implicating neighboring, but distinct regions in several species. This includes mutants affecting CPV-binding to the transferrin receptor and affecting CPV/FPV host range (Govindasamy *et al.*, 2003). It also includes residues implicated in the cell tropism of MVM (Ball-Goodrich *et al.*, 1991), PPV (Vasudevacharya and Compans, 1992), and AMDV (Bloom *et al.*, 1988). The majority of the implicated amino acids cluster on the edge of the massif where it faces the 2-fold depression and 5-fold axis (Figure 9.4, p. 113). There are outliers to this clustering, as might be expected with the combined effects of:

- incomplete genetic characterization;
- different receptors for each virus; and
- cell tropism phenotypes mediated by primary or secondary external receptors, or those involved with intracellular targeting/transport.

More detailed discussions of the evidence and caveats are presented in the next chapter and elsewhere (Chapman and Rossmann, 1993; Hueffer and Parrish, 2003), but, overall, there is a compelling case that the same general region of the massif may be involved with receptor-binding in many members of the *Parvovirus* genus.

Genera *Dependovirus* and likely *Erythrovirus* have a similar surface topology that is distinct from that of *Parvovirus*.

In the B19 structure, a key surface loop is disordered and unseen, but with close homology in all other parts of the structure to AAV-2, all indications are that the *Erythrovirus* surface is similar to that of *Dependovirus* (Kaufmann *et al.*, 2004) with the minor exceptions detailed below. In both, the broad massif of CPV-like viruses is replaced by three distinct spike-like protrusions that rise to greater elevation in AAV and B19. Furthermore, the immediate vicinity of the 3-fold is not part of the elevated region as in CPV and MVM, but is at the lower ‘average’ elevation. Therefore the valleys between the symmetry-related spikes are connected at the 3-fold (Figure 9.3, p. 113). The B19 surface is subtly different from the AAV-2 (Xie *et al.*, 2002; Kaufmann *et al.*, 2004) with spur-like extensions from the spikes leading to more of a continuous rim at medium elevation surrounding the canyon (Kaufmann *et al.*, 2004).

Receptor-virus interactions have not yet been visualized directly for dependoviruses, but genetic evidence combined with structural inferences strongly implicate a region on the side of the spikes for AAV-2. Of panels of insertional and other mutants prepared and phenotypically characterized, a number were found to have reduced heparin-binding (Rabinowitz *et al.*, 1999; Wu *et al.*, 2000). Several disparate loci within the primary sequence had impact. Not all, but the preponderance of these loci cluster as different regions of the primary sequence are folded together and associated with neighboring subunits in the structure of AAV-2 (Xie *et al.*, 2002). The clustering is not perfect, but there are additional indicators that the cluster is the site of receptor-binding, and that other loci might have remote conformational effects. The dominant linear epitope of monoclonal C37-B attachment-inhibiting antibody was a neighboring surface peptide (Girod *et al.*, 1999; Xie *et al.*, 2002). Also, calculation of the Poisson–Boltzmann electrostatic potential shows the implicated surface on the side of the spike to be strongly positively charged (Nicholls, 1992; Gerstein *et al.*, 2001; Xie *et al.*, 2002), as expected for a protein binding a heparan-sulfate receptor (Summerford and Samulski, 1998; Mulloy and Linhardt, 2001).

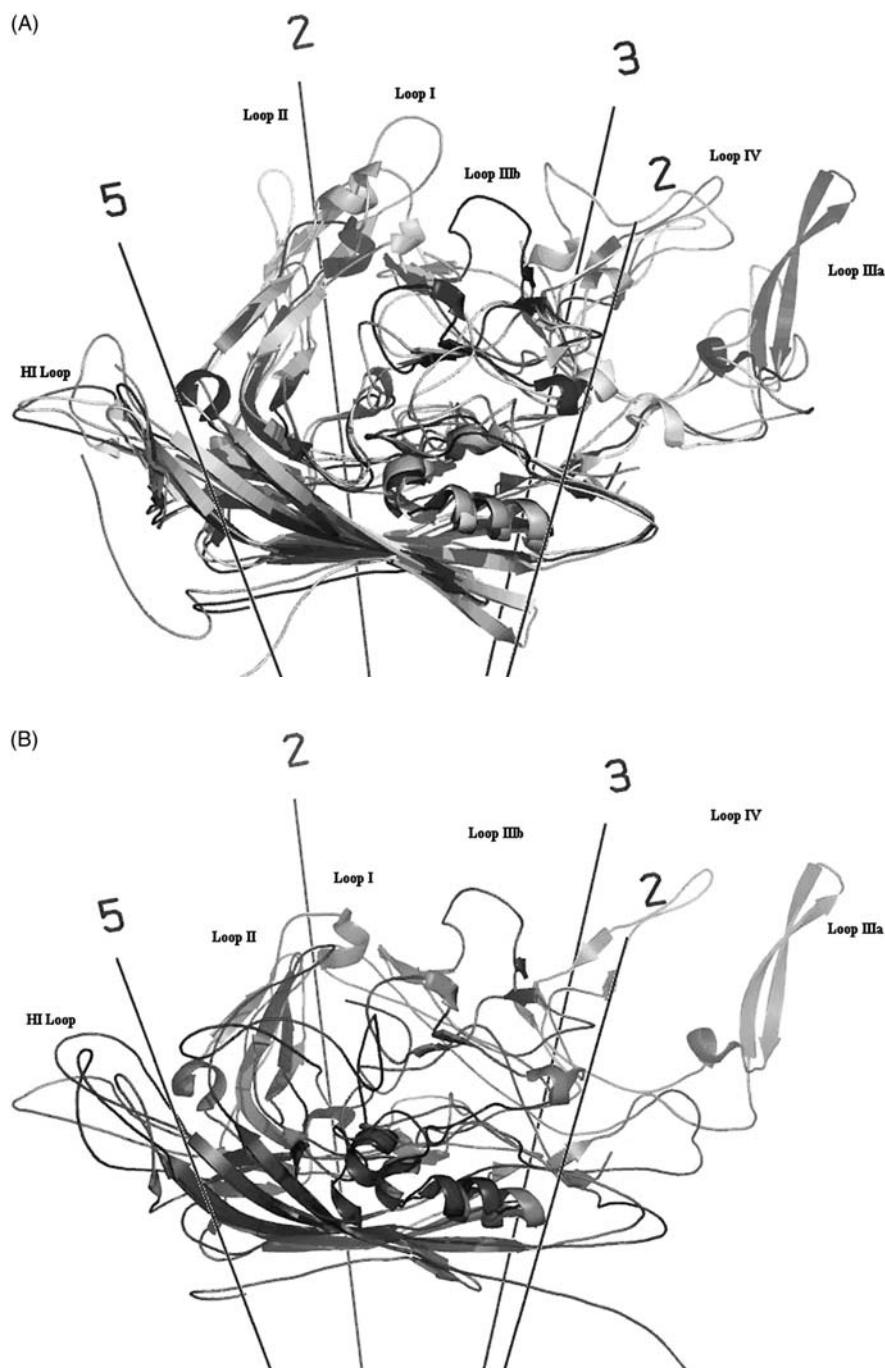
As for B19, there is a ~10 Å discrepancy between the EM-visualized globoside (receptor) binding site (Brown *et al.*, 1993; Chipman *et al.*, 1996) and the location of the B19 neutralizing epitopes (Sato *et al.*, 1991) that align structurally with residues implicated in AAV-2 receptor binding (Xie *et al.*, 2002; Kaufmann *et al.*, 2004). It is not yet clear whether the antibody footprint might (not implausibly) extend over the 3-fold to block the binding of the B19 receptor, or whether higher resolution imaging of the complex might lead to closer agreement. Abundantly clear is that the sides of the spikes, and the valleys running between the spikes and over the 3-fold axes, are a common general location of receptor-binding in dependoviruses and erythroviruses. As with genus *Parvovirus*, the surface topology of exposed protrusions appears to be key to understanding cell attachment and the antigenic characteristics of all parvoviruses.

One enigma might have been resolved recently. Members of the *Parvovirus* genus have similar surface topologies that are distinct from those of *Dependovirus* and *Erythrovirus*. AMDV’s surface, as seen in the cryo-electron microscopy reconstruction at 22 Å resolution (McKenna *et al.*, 1999) appears more like AAV-2 and B19 than the other members of the *Parvovirus* genus in which it was previously classified. The new taxonomy (Tattersall, reviewed in Chapter 1), reflecting genomic differences, has AMDV in a separate *Amdovirus* genus. The three types of surface topology in the *Parvoviridae* family are now more consistent with phylogenetic trees. The Densovirus subfamily has a relatively flat surface. Most of the Parvovirinae subfamily have three distinct peaks surrounding each 3-fold, with the exception of the *Parvovirus* genus where these are merged into a single massif centered about the 3-fold.

## RELATION OF SUBUNIT STRUCTURE TO SURFACE TOPOLOGY

The differences in the four surface loops, especially loops 3 and 4, lead to the three different surface topologies (Figures 9.2, p. 112, 9.3, p. 113, and 9.5, p. 118). The relatively featureless surface of densoviruses is due to the absence of a loop 4 that is the foundation for the elevated regions in other parvoviruses. For the elevated massif of genus *Parvovirus*, all four surface loops contribute. Subloops from the GH-loop (or loops 3 and 4) from adjacent subunits intertwine to form the center of the massif. In the dependoviruses and erythroviruses, subloops from adjacent subunits also interdigitate. However, with a number of sequence insertions and deletions, these subloops are deployed in a different manner. The subloops interdigitate with more of a radial orientation, not tangential. The contacts are similarly intimate, stabilizing the 3-fold associations, but the loops are not dynamically stacked on top of each other. The more radial subloop orientation leads to a well-separated spike that is repeated three times around the symmetry axis.

The exposed tips of the subloops come to the CPV and AAV-2 surfaces in slightly different positions. The most prominent part of the CPV-like massif superimposes on the valley between spikes in AAV-2 (Figure 9.3, p. 113). The prominence of the AAV-2 spikes is not only due to the extension of loops 3 and 4, but also to the truncation of loops 1 and 2, that in genus *Parvovirus* would build the edges of and fill in the valley between the spikes. The modest differences in surface topology between AAV-2 and B19 are due to longer BC and EF loops (1 and 2) in B19. Although of length more similar to CPV, the BC and EF loops of B19 come to the surface in positions different from CPV. They join a 10-residue insertion near the C-terminus to extend the spikes with a spur towards the 2-fold depression, helping to form a rim around the canyon (Kaufmann *et al.*, 2004).



**Figure 9.5** Superimposition of the subunit structures of: (A) CPV (green, Tsao et al., 1991), AAV-2 (red, Xie et al., 2002), and B19 (yellow, Kaufmann et al., 2004), and (B) DNV (blue, Simpson et al., 1998) and AAV-2 (red). Strands of the  $\beta$ -barrel superimpose well, as do the loops of AAV-2 and B19. The biggest differences are in loops 3 and 4, between CPV, DNV and a group including both AAV-2 and B19. DNV superimposes less well than the other parvoviruses (panel b). See also Color Plate 9.5.

## EMBELLISHING SECONDARY STRUCTURES

While the long barrel loops do not have recognizable folds and have less secondary structure than most protein domains, they have a few  $\alpha$ -helices, and a number of  $\beta$ -strands. In several cases (Figure 9.1, p. 108) the bases of

subloops are a pair of strands forming a  $\beta$ -ribbon, tying the ends together. In some cases the strands are part of small  $\beta$ -sheets, often bridging between neighboring subunits. Some of the details differ, but consider CPV where hydrogen-bonding and secondary structure has been analyzed exhaustively (Xie and Chapman, 1996). GH1, a small  $\beta$ -sheet with

strand lengths of 3–6 residues, consists of strands  $\beta\text{GH2}/\beta\text{GH3}$  from one subunit and  $\beta\text{GH8}/\beta\text{GH9}$  from a 3-fold neighbor. Sheet GH2 has strands of 3–5 residues:  $\beta\text{GH4}/\beta\text{GH6}$  from one subunit and  $\beta\text{GH1}$  from a 3-fold neighbor. Sheets BCE and GH3 are short 3- and 2-stranded sheets within their own subunits.

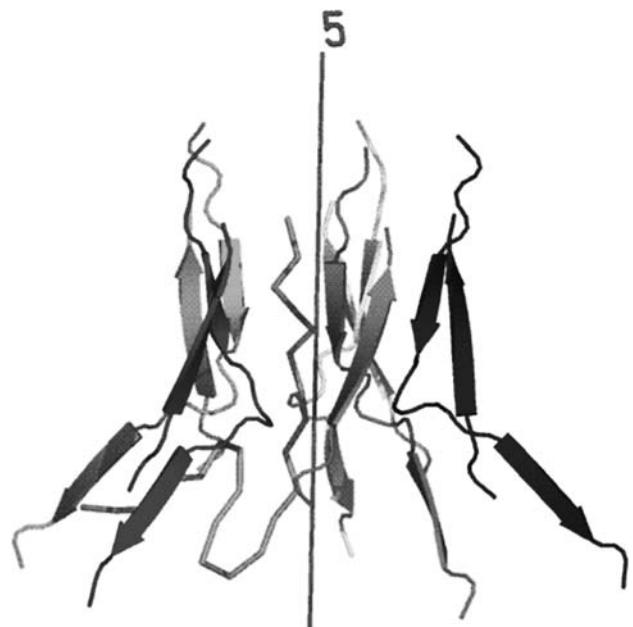
These additional secondary structures are not conserved in *Densovirus*, where the loops are absent or of very different structure. However, they are mostly conserved in our examples of *Erythrovirus* and *Dependovirus* (Xie *et al.*, 2002; Kaufmann *et al.*, 2004). They likely have two important functions. First they help solidify a foundation for the more exposed and variable tips of the loops that decorate the surface in a variety of configurations. Second, the hydrogen-bonding between strands from different subunits, helps to rigidify the interfaces.

## SUBUNIT INTERACTIONS IN THE CAPSID ASSEMBLY

Polar interactions, including hydrogen bonding, are unlikely to contribute to free energies of association, because lost solvation energy has to be subtracted from any favorable interaction energy (Kyte, 1995). Generally, assembly is driven by hydrophobic interactions that can be estimated semi-empirically from the change in (non-polar) molecular surface energy (Gerstein *et al.*, 2001). Calculations from the CPV structure (Xie and Chapman, 1996) show that the interactions between 3-fold related subunits is very favorable. The association appears very stable and unlikely to dissociate. It does not indicate whether trimers are, or are not, an assembly intermediate. The intertwining of the GH subloops accounts for more than half of the calculated interaction energy. The unassembled subunit structure is unknown, but without their neighbors, the loop structures would likely be very different, and the net change in solvent accessible area likely much less than when estimated just from the subunit structure in its assembled configuration. When the flexible GH subloops are completely omitted from the calculations, 3-fold interactions appear slightly more favorable than 5-fold, and then 2-fold. This is no more than a crude guide, not only because of the uncertain effect of flexible loops, but because estimated interaction energies speak to binding equilibria, but assembly pathways are likely kinetically controlled, and there is no data on the dynamics of relevant loop conformational changes. Experimental data for MVM is beginning to emerge from the laboratories of Almendral and others, so perhaps assembly pathways will soon be put on a firmer footing.

### The 5-fold pore

A tube of partially ordered electron density was found to run exactly along the 5-fold axis in CPV (Xie and Chapman,



**Figure 9.6** The 5-fold pore of CPV (Xie and Chapman, 1996). The  $\beta$ -ribbon of the DE loop is shown along with its symmetry equivalents (in different colors) surrounding the 5-fold. The tight turns of the ribbons on the external viral surface (top) are disordered and can not be modeled, but are visible in AAV-2. Up through the center, along the 5-fold, runs the glycine-rich sequence from one of the five neighboring subunits (green), so that up to one of five capsid protein N-termini can be on the external surface (top). Residues are numbered according to VP2, though it is not known whether it is VP1, 2 or 3 that is visualized crystallographically. See also color plate 9.6.

1996), down a channel formed by the DE loop  $\beta$ -ribbons of five symmetry-related subunits. The  $\beta$ -barrel starts on the inside surface of the capsid near the 5-fold axis. Following  $\beta\text{A}0$  backwards towards the N-terminus, the backbone trace diverges at Ser35-Gly34 with some conformers leading inwards and disappearing. About 1 in 8 conformers turn tightly and proceed outwards along the 5-fold axis through the disordered electron density (Figure 9.6). The pore is large enough to accommodate the peptide chain from one of the five surrounding subunits. The density level corresponds to two-thirds of the pores filled, but after icosahedral averaging, we cannot tell which, and details of the density are lost. All parvoviruses have a region rich in glycines, suitable for the tight turns, flexible configuration, and small size required to fit through the pore. In CPV, the chain could be traced towards the N-terminus to the site of VP2/3 cleavage, which is at the external surface near the 5-fold.

The length of the channel varies in different parvoviruses, but is correlated to the length of the glycine-rich sequence. In GmDNV, the glycine-rich sequence is five residues shorter than in CPV, but the channel is also only about half the length (20 versus 45 Å). B19 is intermediate (Kaufmann *et al.*, 2004). Among the various parvovirus structures, the pore is not uniformly filled. Structures determined

of empty capsids (including all that are recombinantly expressed) lack any electron density along the 5-fold. Such cases include B19 (Kaufmann *et al.*, 2004), the empty capsid forms of CPV (Wu and Rossmann, 1993), and FPV (Agbandje *et al.*, 1993), indicating a relationship between the presence of the DNA and the protein contents of the 5-fold pore. In B19 (Kaufmann *et al.*, 2004) the pore was narrower than in full capsids of CPV, with a constriction down to 9 Å (atom center–atom center). As is, this would be too narrow for passage of a polypeptide chain, but there is likely enough flexibility for room to be made. However, even among structures of DNA-containing capsids, the contents of the pore have not been uniform. The channel was filled in the structure of MVMi (Agbandje-McKenna *et al.*, 1998) as in CPV. The pore in AAV-2 contained only weak discontinuous density that could not be modeled, and the same was true in GmDNV (Simpson *et al.*, 1998). The pore density is expected to be weak, because at most one of the five surrounding subunits can have its N-terminal region extending up the pore to the outside. Within the pore, the structure visualized is the average of five different orientations, so parts of its density will be smeared. At the external surface, the polypeptide could take one of five equivalent (non-overlapping) routes, each of which would have occupancy less than detectable crystallographically. More mysterious is our failure to see the four of five N-terminal regions that remain internal at copy number that should be detectable. It suggests that the extreme N-termini of the VP2/3 that remain internal are of variable configuration.

A consequence of failing to resolve the VP2 N-termini is that there is not direct crystallographic evidence of whether it is VP1, 2, or 3 (or a mixture) that have external N-termini. It is assumed that VP1 must be externalized for its phospholipase activity to be put to use, but this could be the result of a receptor or entry-triggered conformational change. A variety of evidence indicates that some VP1 can be external in at least some parvoviruses. This included VP1-specific antigenicity in particles of CPV and B19 (Rimmelzwaan *et al.*, 1990; Kajigaya *et al.*, 1991; Rosenfeld *et al.*, 1992). Cryoelectron microscopy of AAV-2 empty particles gave a contrary indication, with ‘fuzzy globules’ suggesting a location on the inner capsid surface near the 2-fold (Kronenberg *et al.*, 2001). Weak and disordered density is expected for a flexible feature present in only a proportion of cases, but caution is needed in interpreting such features. Also, the location in these empty particles might differ from that in DNA-containing particles.

Crystal packing considerations argue that the VP1 are internal. Denatured crystals of AAV-2 show the expected ratios of VP1, 2, and 3 (Xie *et al.*, 2002, 2004) with insufficient VP1 to populate every 5-fold axis. If VP1-unique domains decorated some of the outside surface in a heterogeneous way, it is inconceivable that they would not disrupt the exact crystalline arrays in some of the now many parvovirus crystal forms. If the biophysical evidence for internal localization is to be reconciled with biochemical and immunological

evidence that it is external, the inescapable conclusion is that under some conditions the location can change dynamically. The pore is wide enough only for an extended peptide with small side chains. None of the existing structures give much indication of how the assembly could become plastic enough for the extrusion of an entire domain. (Structures solved under varying conditions, including DNA-containing and empty particles, show structures that are only subtly different [Wu and Rossmann, 1993; Xie and Chapman, 1996]). Similar structural reconfigurations are accomplished by other viruses, such as the externalization of VP4 in picornaviruses (Li *et al.*, 1994), so it is likely that parvoviruses are likely to reveal some interesting secrets in the future. Mutational studies on the phenotypes of pore-lining residues are beginning to give insights into their relevance to N-terminal location, DNA encapsidation, and capsid assembly (Farr and Tattersall, 2004; Reguera *et al.*, 2004).

There is independent evidence of dynamic conformational changes in parvoviruses. This includes tryptic susceptibility of VP2 in full, but not empty heat-treated MVM particles (in which it is assumed that the VP2-unique region might be sequestered internally) (Clinton and Hayashi, 1976; Tattersall *et al.*, 1977; Carreira *et al.*, 2004; Farr and Tattersall, 2004; Reguera *et al.*, 2004). This is consistent with the observation that only DNA-containing particles have filled 5-fold pores (see above). An interesting twist comes from considering the impact of domain-swapping in GmDNV (Simpson *et al.*, 1998). If all subunits are in the domain swapped configuration (and there is not a minority unswapped component), then the N-termini that fill *Densovirus* 5-folds must be threaded following at least partial assembly of the capsid, and can not be in place as pentamers are formed. One might also expect conformational changes associated with attachment, endosomal entry, nuclear targeting, and initiation of uncoating (Vihinen-Ranta *et al.*, 1998; Bartlett *et al.*, 2000; Seisenberger *et al.*, 2001). Attempts to characterize any conformational changes are just beginning. The structure of CPV at low pH showed conformational changes only in a surface loop (Simpson *et al.*, 2000), but there are indications that low pH is not the only endosomal trigger required for productive viral entry (Vihinen-Ranta *et al.*, 1998).

## DNA BINDING SITE

Although the inside surfaces of some other viruses are positively charged for interactions with DNA or RNA (Ban and McPherson, 1995), it was close to neutral in CPV (Xie and Chapman, 1996), consistent perhaps with ssDNA that remained (at least partly) single-stranded (rather than locally hairpin/duplex) (Chapman and Rossmann, 1995). With the icosahedral averaging, DNA was expected to be seen only when the capsid enforced this symmetry upon an otherwise unsymmetric genome (see above). Eleven nucleotide

fragments of CPV ssDNA were seen (Tsao *et al.*, 1991; Chapman and Rossmann, 1995), which, when multiplied over the 60 symmetry equivalent positions, accounted for about 13 percent of the genome. Its footprint consisted of mostly polar, but uncharged amino acids (Xie and Chapman 1996). The presence of DNA causes small conformational changes to the inside capsid surface (Wu and Rossmann, 1993). These are local to the DNA binding site, and there is no wholesale rearrangement communicating the presence of DNA to the outer surface. Of course we cannot comment about the unseen N-termini of VP1–3 that could have such a role. In MVMi, 23 nucleotides or 28 percent of the genome is visualized, bound in a similar location (Agbandje-McKenna *et al.*, 1998).

In CPV, the ssDNA has an unusual inverted-loop structure with phosphates chelated inside by 2 Mg<sup>++</sup>, and bases pointing outwards to interact with the capsid protein, four of them in a single-stranded stack. Characteristic features in the electron density showed that there was a weak but detectable preference for particular base types in some positions, and that the average image of 60 DNA fragments did not contain a random mixture of all possibilities (Chapman and Rossmann, 1995). A profile of sequences (Gribskov *et al.*, 1990), estimated to be consistent with the crystallographic electron density, was not an exact match to any one locus of the CPV genome, but was a closer match than random to a number of sequence elements (Chapman and Rossmann, 1995). Efficient packing dictates that 60 different regions of the sequence must be compatible with the symmetry-equivalent capsid-binding sites, and it may be that CPV selects its own DNA for encapsidation, not with a single highly specific recognition sequence, but through the multiplicative effect of many different regions of its genome each having modest similarity to a consensus binding sequence. A requirement to match only approximately a binding sequence would severely constrain the coding function of the genome.

Ten of MVM's 23 nucleotides seen correspond to those in CPV (Agbandje-McKenna *et al.*, 1998) and many of the others corresponded to weak uninterpretable density in CPV, suggesting that the DNA binding site is a conserved feature. In GmDNV, there is at best disordered density for some of the DNA, and it is not in a homologous position (Simpson *et al.*, 1998). Several of the structures were determined as empty capsids, including B19, FPV, and PPV, so of course no DNA is expected. One can only speculate why ordered DNA has not yet been seen in AAV – perhaps because unlike CPV and MVM, both positive and negative strands of the genomic DNA are packaged.

comparative analysis from which further functional inferences will emerge. Over the decade-plus of parvovirus structure, the combination of genetic and structural characterizations has greatly extended our understanding of selected examples, and now we can expect similar advances throughout the family. The atomic structures are providing a framework for the planning and interpretation of functional studies, which will be reviewed in the next chapter. Emerging studies of receptor and immune complexes offer particularly exciting prospects of advancing our understanding of the molecular bases and dynamic processes of viral-host interactions.

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## CONCLUSION

Structural studies have now extended to many of the parvovirus genera. This has opened a new chapter of

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# Correlating structure with function in the viral capsid

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MAVIS AGBANDJE-MCKENNA AND MICHAEL S. CHAPMAN

The multifunctional parvovirus coat protein	125	Antigenic properties of parvovirus capsids	133
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## THE MULTIFUNCTIONAL PARVOVIRUS COAT PROTEIN

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The simple parvovirus capsid protein is capable of performing a wide variety of structural and biological functions during the viral life cycle. Sixty copies of a common sequence from this protein make up the capsid shell, with polypeptides VP1 to VP4 (depending on the virus) sharing identical C-terminal domains, but with decreasing overlapping regions. The entire sequence of VP4 (found only in members of the densovirus genus) is contained within VP3, that of VP3 is contained within VP2, and VP2 is contained within VP1, which in turn has a unique N-terminal extension. These viral coat proteins are translated from the same messenger RNA (mRNA) codons by the use of alternative splicing and non-consensus start codons. In the *Parvovirus* genus, for example, Kilman rat virus, canine parvovirus (CPV), feline panleukopenia virus (FPV), minute virus of mice (MVM) and porcine parvovirus (PPV), VP2 to VP3 cleavage is a capsid maturation step that occurs in full, but not in empty, particles following their release from the parental host cell (Tattersall *et al.*, 1977; Cotmore and Tattersall, 1987; Cotmore *et al.*, 1999; Maroto *et al.*, 2000). Following DNA packaging, 15–18 N-terminal residues are proteolytically cleaved from the N-termini of VP2 to generate VP3. This cleavage event is not observed in all parvovirus genera, for example, the *Amdovirus* genus, illustrated by Aleutian mink disease parvovirus (AMDV) or the *Erythrovirus* genus, illustrated by human parvovirus B19. These viruses

contain only VP1 and VP2, translated from the same message using alternative start sites. For members of the *Dependovirus* and *Densovirus* genera, VP1, VP2, VP3, and VP4 are generated from overlapping RNA transcripts, and are not known to undergo a cleavage event. The VP1 capsid protein is always the minor component, while the smaller proteins, VP2, VP3, or VP4, depending on the virus, account for the highest percentage of the 60 copies of coat protein that make up the capsid.

The multitude of functional roles performed by the parvovirus capsid proteins includes host cell surface receptor recognition, pathogenicity determination, viral genomic encapsidation, self-assembly into capsids, maturation of virions to produce infectious virus progeny, nuclear import and export, escape from endosomes during infection, and host immune response detection and evasion. The relatively small parvoviral genome (~5000 ssDNA bases) has allowed the use of genetic manipulation to map functional domains of the viral proteins/capsid, which can be correlated to structural features of the particle.

Parvovirus capsids have been amenable to structural studies by X-ray crystallography and cryo-electron microscopy (cryo-EM). As detailed in the previous chapter (Chapter 9), high resolution 3D information is available for members of four main genera, namely *Parvovirus*, *Dependovirus*, *Erythrovirus*, and *Densovirus* (Tsao *et al.*, 1991; Agbandje *et al.*, 1993; Agbandje-McKenna *et al.*, 1998; Simpson *et al.*, 1998, 2000, 2002; Xie *et al.*, 2002; Govindasamy *et al.*, 2003; Kaufmann *et al.*, 2004). An 8 Å resolution structure is also

available for B19 determined by X-ray crystallography (Agbandje *et al.*, 1994). At lower resolution, the structure of *Junonia coenia* densovirus is available for the *Densovirus* genus (Bruemmer *et al.*, 2005); of empty capsids of adenovirus-associated virus (AAV) serotype 2 (Kronenberg *et al.*, 2001) and full capsids of AAV5 (Walters *et al.*, 2003) and AAV4 (Padron *et al.*, 2005) are available for the *Dependovirus* genus; of empty capsids of human parvovirus B19 (B19) made up of the major coat protein, VP2, and in complex with its infectious cellular receptor, globoside (Chipman *et al.*, 1996) are available for the *Erythrovirus* genus; of a CPV-Fab complex (Wikoff *et al.*, 1994) for the *Parvovirus* genus, and of VP2 capsids of AMDV (McKenna *et al.*, 1999) for the *Amdovirus* genus, determined using cryo-EM and image reconstruction.

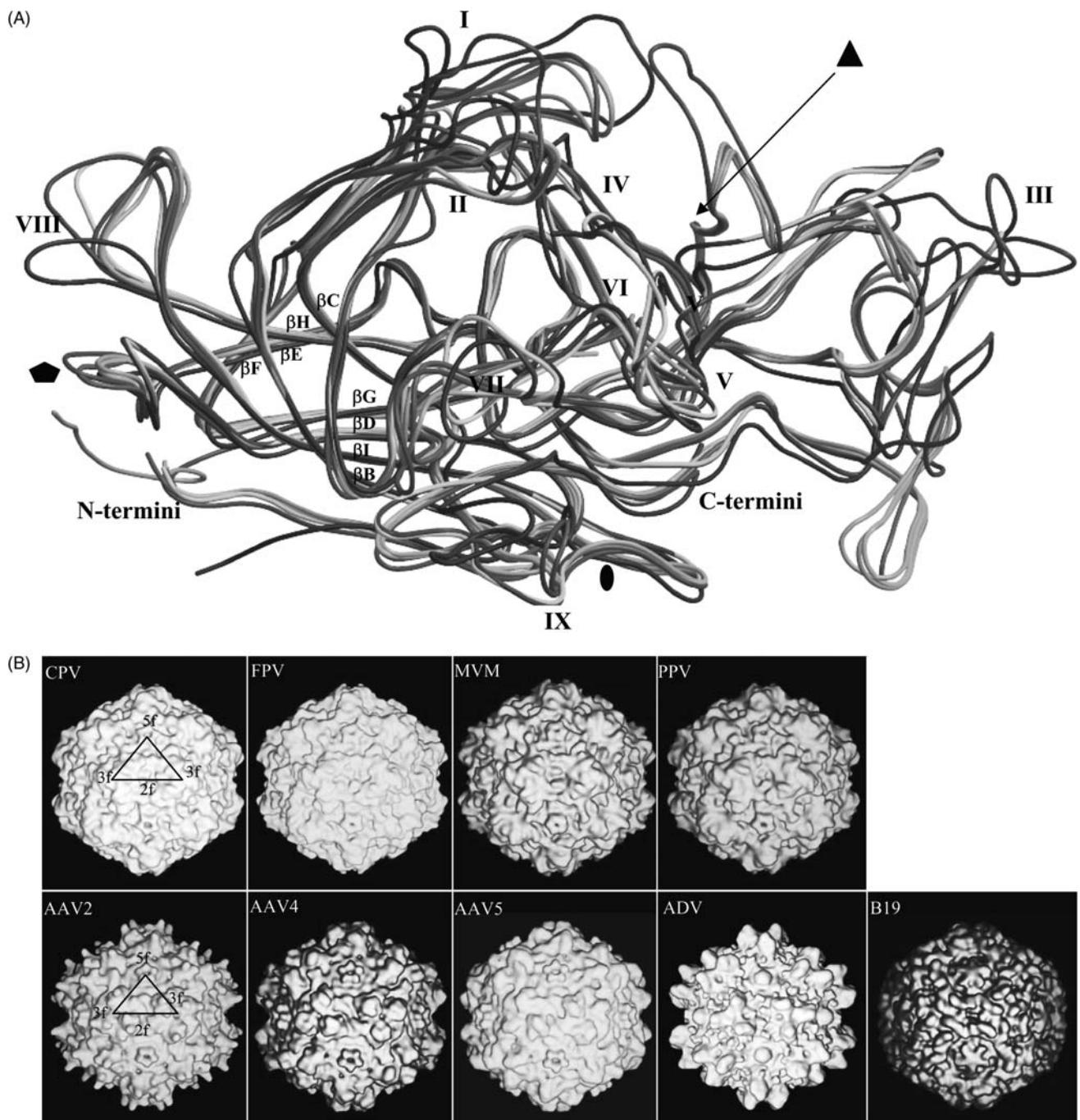
The unique region of VP1, the minor parvovirus capsid protein, in addition to the VP2 N-termini, have proven refractory to structural elucidation possibly because of the symmetry constraints imposed during structural determinations, as described in Chapter 9. However, the amino acid sequences of these capsid protein regions play essential functional roles during the viral life cycle, and will be discussed briefly here. The unique region of VP1 displays a phospholipase A<sub>2</sub> (PLA<sub>2</sub>) activity that is required for escape from late endosomes during viral trafficking to the nucleus after receptor mediated entry (Zádori *et al.*, 2001). In lieu of structural detail as part of the intact capsid, sequence analysis and model building exercises show that the PLA<sub>2</sub> domain within the VP1 N-terminus has a helical content consistent with known PLA<sub>2</sub> motifs (Zádori *et al.*, 2001). Despite low sequence homology to more conventional PLA<sub>2</sub> proteins, such as that in bee venom, the parvoviral PLA<sub>2</sub> sequences have the conserved catalytic and calcium binding sites required for this function (Zádori *et al.*, 2001). This unique VP1 region also contains basic amino acid clusters that function as nuclear localization signals (NLS), which direct nuclear import following entry and escape from the endosome (Vihinen-Ranta *et al.*, 2002). The PLA<sub>2</sub> and NLS functions are thought to be facilitated by the externalization of the VP1 unique peptide domains through pores at the icosahedral 5-fold axes (see Chapter 9) of the intact capsid. The exact mechanism of this externalization is still under intensive investigation but mutational studies of the MVM and AAV capsid proteins show that residues at the base and lining the 5-fold channel play essential roles in the PLA<sub>2</sub> function required for infectivity (Farr and Tattersall, 2004; Bleker *et al.*, 2005).

In parvoviruses that undergo VP2 to VP3 cleavage, the VP2 N-termini are also postulated to become externalized via the channels at the icosahedral 5-fold axes of their capsids (Tsao *et al.*, 1991; Xie and Chapman, 1996; Agbandje-McKenna *et al.*, 1998; Simpson *et al.*, 2002). Repulsion of phosphorylated VP2 N-terminal residues from the capsid interior following DNA packaging is thought to trigger this externalization, allowing their subsequent removal by cleavage of VP2 to VP3 (Maroto *et al.*, 2000). This process can be

simulated in empty particles by heating to <70°C, while the capsids remain intact (Hernando *et al.*, 2000). The VP2 N-termini are also reported to facilitate nuclear export of newly-assembled parvovirus virions through interactions with the exportin molecule CRM1 (Maroto *et al.*, 2004).

The residues in the N-terminus of VP1, required for its PLA<sub>2</sub> function, and at the VP2 to VP3 cleavage site cannot be accommodated through the 5-fold channel without structural rearrangement of the surrounding β-ribbons, and immunological studies support the idea that this pore may ‘breathe’ (Cotmore *et al.*, 1999). However, once the VP1/2 N-termini have passed through the 5-fold channel, a glycine-rich sequence that is conserved in most parvoviruses can be accommodated within the channel (Xie and Chapman, 1996; Agbandje-McKenna *et al.*, 1998). Interestingly, while still able to accommodate a glycine-rich sequence, these channels are narrower in the B19 structure (Kaufmann *et al.*, 2004), consistent with the observation that B19 does not undergo VP2 to VP3 cleavage and that the VP1 unique region is always located on the capsid surface (Rosenfeld *et al.*, 1992).

The structure of the polypeptide sequence that is common to all the capsid proteins, which corresponds to VP2, VP3, or VP4 in viruses from different genera, is observed in a T = 1 icosahedral arrangement. The overall structural topology of this protein is the same for all parvoviruses (Figure 10.1A), even for pairs of viruses where amino acid sequence identity is only ~20 percent, as is observed, for example, between B19 and MVM (Chapman and Rossmann, 1993) and B19 and AAV2 (Kaufmann *et al.*, 2004; Padron *et al.*, 2005). The structural similarities between these viruses are ~70 percent, illustrating the greater conservation of tertiary structure compared with primary sequence. A CPV structure-based comparison of members of the *Parvoviridae* showed that the residues making up a core 8-stranded β-barrel motif (Figure 10.1A) and those lining the inner capsid surface are the most conserved. Surface loop residues inserted between the β-strands are the most varied (Chapman and Rossmann, 1993), and give rise to three types of surface topology (Figure 10.1B) (Xie *et al.*, 2002; Padron *et al.*, 2005). The most pronounced surface topology differences occur close to the icosahedral 2-fold axes and the protrusions surrounding the icosahedral 3-fold axes (Figure 10.1). Group 1 contains members of the *Parvovirus* genus, for example, CPV, FPV, MVM, and PPV, distinguished by a single pinwheel protrusion at the icosahedral 3-fold axes formed by loops contributed from three monomers, and a wider 2-fold depression. The *Densovirus* (DNV) capsid adopts a second topology, appearing relatively spherical with no large surface protrusions. The third group contains the AMDV, B19, AAV2, AAV4, and AAV5 capsids, which have three distinct mounds at a distance of ~20–26 Å from the icosahedral 3-fold axes, each mound resulting from the interaction of two 3-fold related monomers. The depression at their 2-fold axis also appears to be slightly deeper, particularly in B19 (Figure 10.1B).



**Figure 10.1 Comparison of parvovirus structures.** (A) Superimposition of coil representations of the VP2/VP3 monomers of AAV2 (brown), AAV4 (magenta), B19 (blue), CPV (orange), FPV (green), MVM (red), PPV (pink) (pdb accession Nos. 1LP3, AAV4 coordinates are yet to be deposited, 1S58, 2CAS, 1C8E, 1MVM, and 1K3V), AAV5 (cyan) and ADV (grey) (pseudo-atomic model built into cryo-EM densities; McKenna et al., 1999, Walters et al., 2004). The most variable surface regions on the capsid are labeled (I–IX) as discussed by Padron et al., 2005 and Govindasamy et al., 2005. The eight-strands of the core  $\beta$ -barrel are labeled  $\beta\beta$ – $\beta\mathrm{I}$ . The approximate 2-fold (filled oval), 3-fold (filled triangle) and 5-fold (filled pentagon) axes are shown. This figure was generated using the program Bobscript (Esnouf, 1997).

(B) Low resolution surface maps of AAV2, AAV4, AAV5, ADV, B19, CPV, FPV, MVM, and PPV, at 13 Å resolution, colored as in (A). Viruses in the top panel and bottom panel form group I and group III, respectively in Padron et al., 2005. The surface map images were generated as previously described (Belnap et al., 1999) from atomic/pseudo-atomic coordinates as referenced in (A). The black triangles on the CPV and AAV2 capsid surfaces depict a viral asymmetric unit bound by a 2-fold (2f), two 3-folds (3f) and a 5-fold (5f) axis. See also Color Plate 10.1.

The observed differences in the capsid surface features of the parvoviruses might be indicative of evolutionary changes that allow them to recognize and infect different hosts and host cells, and to evade host immune surveillance, while conservation of the  $\beta$ -barrel motif suggests a need to conserve core structural domains that may be required for assembly. However, there do not appear to be any unifying phenotypic similarities between parvoviruses with similar capsid surface topologies (Padron *et al.*, 2005). Viruses with similar surface topologies:

- do not necessarily recognize the same receptor molecules;
- do not necessarily have similar tissue tropisms and/or pathogenic phenotypes, and
- are not antigenically cross-reactive.

However, the *Amdovirus*, *Dependovirus* and *Parvovirus* capsids, regardless of local surface topology, utilize analogous capsid regions for these functions.

The structural information from the VP2/VP3/VP4 common region enables functional domains to be mapped onto the capsid. Slight structural variations in the capsid surface are known to modulate host cell tissue tropism, pathogenic disparities and antigenic differences between highly homologous strains (Parrish, 1991; Chang *et al.*, 1992; Agbandje *et al.*, 1993, 1995; Strassheim *et al.*, 1994; Wikoff *et al.*, 1994; Parker and Parrish 1997; Agbandje-McKenna *et al.*, 1998; Simpson *et al.*, 2000, 2002; Govindasamy *et al.*, 2003; Hueffer *et al.*, 2003a,b; Llamas-Saiz *et al.*, 1996; López-Bueno *et al.*, 2003; Palermo *et al.*, 2003). Surface regions also control variations in receptor attachment specificity between members of the same genus (Xie *et al.*, 2002; Govindasamy *et al.*, 2003; Hueffer *et al.*, 2003a,b; Kern *et al.*, 2003; Opie *et al.*, 2003). In addition, efforts to locate VP amino acids required for assembly have identified residues at the interface between icosahedral symmetry axes that suggest a role for both conserved and variable regions in the building of the parvovirus capsid (Carreira *et al.*, 2004; Reguera *et al.*, 2004; Bleker *et al.*, 2005). The capsid determinants of receptor attachment, tissue tropism and pathogenicity, and antigenicity will be discussed in the following sections.

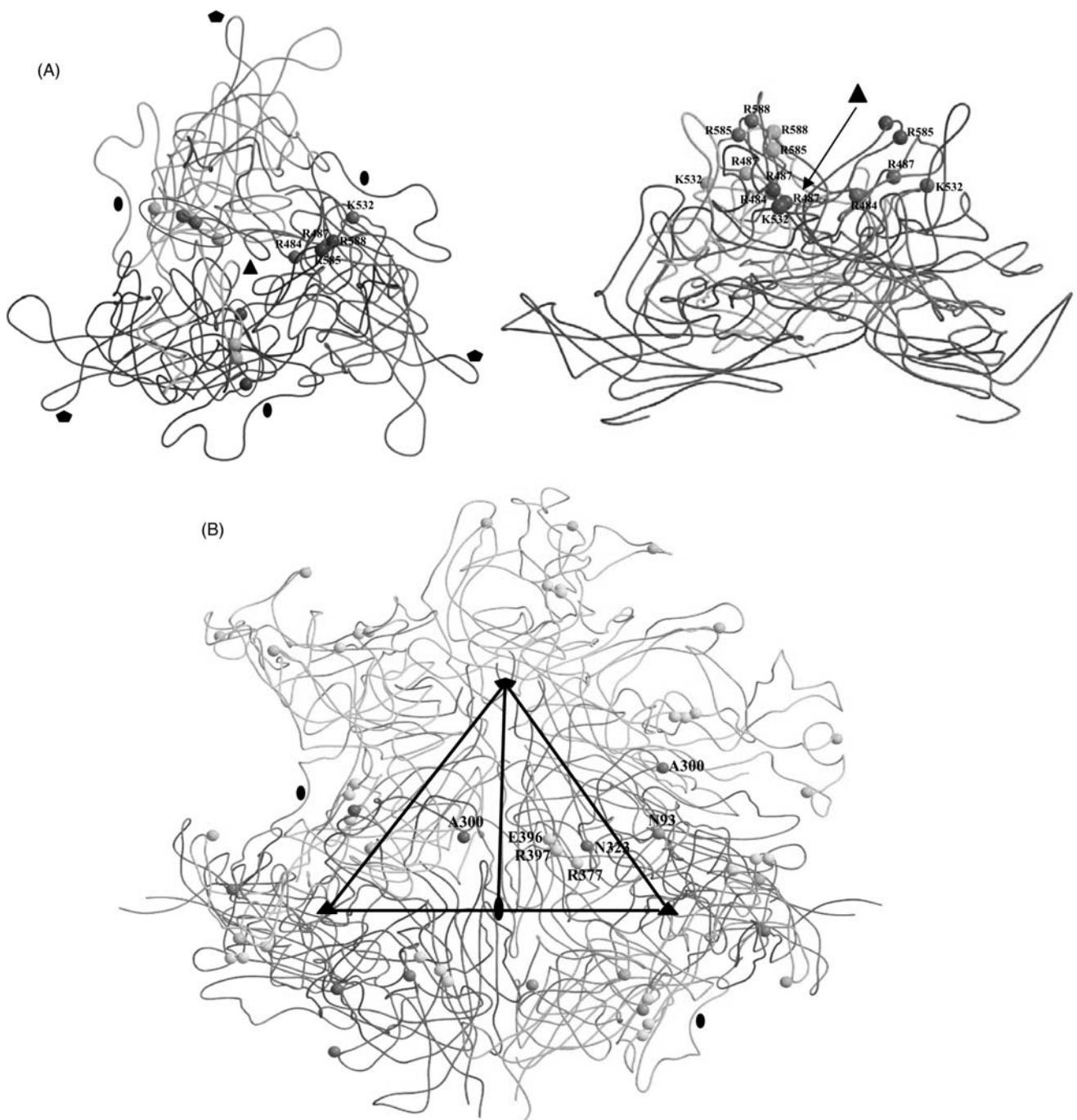
## RECEPTOR ATTACHMENT PHENOTYPES OF PARVOVIRUS CAPSIDS

Receptor-mediated attachment and entry are essential first steps in the parvoviral life cycle. Mutational and biochemical analysis, in combination with structural mapping, shows clearly that regardless of parvovirus genus or sequence homology, common regions of the capsid may be used to interact with carbohydrate moieties/receptor molecules during cell recognition and infection/transduction. However, these recognition sites are not restricted to a single feature or capsid region. The depression at the icosahedral 2-fold

axis, the wall between the 2- and 5-fold depressions, the base of the 3-fold mound, and the icosahedral 3-fold axis have all been mapped as possible cell surface recognition sites. For most of these viruses, the recognition site is only configured on assembled capsids, and spatially distant sites on the capsid may be involved in the recognition of one receptor molecule, as reported for CPV (see below; Govindasamy *et al.*, 2003; Hueffer *et al.*, 2003a,b).

Cell transduction phenotypes for members of the *Dependovirus* genus appear to be due to the ability of different AAV capsids to use different cell surface carbohydrates for cell binding and entry. Heparin sulfate has been identified as the carbohydrate component important for AAV2 cell binding and transduction (Kern *et al.*, 2003; Opie *et al.*, 2003), with human fibroblast growth factor 1 (Qing *et al.*, 1999) or integrin  $\alpha_v\beta_5$  acting as co-receptors (Summerford *et al.*, 1999). Amino acids R484, R487, K532, R585, and R588 are the basic residues in VP3 responsible for AAV2's interaction with heparin (Figure 10.2A). AAV1 does not contain critical residues R585 and R588 (Rabinowitz *et al.*, 2002), and does not bind heparin, although it is ~83 percent identical to AAV2. The carbohydrate moiety recognized by AAV1 for transduction is yet to be determined. AAV3 is ~87 percent identical to AAV2 and also lacks R585 and R588, but it binds heparin sulfate, albeit with lower affinity than AAV2 (Rabinowitz *et al.*, 2002). AAV4 and AAV5 are ~55 percent identical to AAV2 and to each other, and both use sialic acid rather than heparin sulfate for cellular transduction (Kaludov *et al.*, 2001). Platelet-derived growth factor receptor molecules, PDGFR $\alpha$  or PDGFR $\beta$ , are also required as co-receptors to mediate AAV5 transduction (Di Pasquale *et al.*, 2003). The carbohydrate molecules mediating cell binding by other distinct AAV serotypes, such as AAV7–11 and avian adeno-associated virus (AAAV), are still under investigation.

Residues contributing to the heparin binding sites of AAV2 are clustered from 3-fold related VP3 molecules at the base of the mounds surrounding the icosahedral 3-fold axes (Xie *et al.*, 2002; Kern *et al.*, 2003; Opie *et al.*, 2003) (Figure 10.2A). Comparison of a cryo-EM reconstructed image of AAV4, at 13 Å resolution, with AAV2 at the same resolution, showed local surface structure variations proximal to this basic patch (Padron *et al.*, 2005). More recently, the crystal structure of AAV4 became available (Govindasamy *et al.*, 2005). Three of the five AAV2 residues that form the basic patch, including the critical R585 and R588, are different in AAV4, and the topology of loops forming this region of the 3-fold mounds are drastically different. In addition AAV4 has a deeper 2-fold depression because the loop containing K532, which lies on the wall of the 2-fold depression at the base of the 3-fold mound in AAV2, adopts a different conformation (Govindasamy *et al.*, 2005; Padron *et al.*, 2005). Comparisons of the AAV2 and AAV4 structures with a pseudo-atomic model of AAV5 built into its cryo-EM reconstructed density show that the major surface variations between all three viruses involve residues



**Figure 10.2** Proposed receptor attachment sites on the AAV2 and CPV capsids. The coordinates were obtained as in Figure 10.1. (A) AAV2 VP3 trimer (monomers in brown, magenta and green) viewed down the icosahedral 3-fold axes (left-hand side) and rotated 90° (right-hand side). The C<sub>α</sub> positions of residues R484, R487, K532, R585, and R588 (labeled and in the blue oval) that are clustered on the AAV2 capsid from 3-fold symmetry-related monomers to form a basic patch required for heparin sulfate binding are shown as balls colored according to their monomers. The approximate icosahedral 2-, 3-, and 5-fold axes are shown as the filled oval, triangle, and pentagons, respectively. (B) CPV VP2 reference (in orange) bounded by its icosahedral 2-fold (magenta), 3-fold (dark green and light green) and 5-fold (cyan) related monomers. The residues that control CPV sialic acid binding (R377, E396, and R397) on the wall of the 2-fold axes are shown in yellow balls in all monomers. The residues implicated in Tfr binding (93, 300, and 323) are shown in balls colored according to their contributing monomers. The approximate icosahedral 2- (filled oval), 3- (filled triangle), and 5-fold (filled pentagon) axes are shown. See also Color Plate 10.2.

clustered at and around the 3-fold mounds, in the 2-fold depression, and on the floor of the depressions surrounding the icosahedral 5-fold axes. In addition, structural models generated for the other distinct AAV serotypes show a similar trend in surface variation (Padron *et al.*, 2005). There are no consensus sequences for sialic acid binding, but extrapolation from studies on CPV and MVM (see below) suggest that the icosahedral 2-fold axes may function as sialic acid binding regions. Thus the observed local surface loop variations between the AAV serotypes at the icosahedral 2- and 3-fold axes may control their abilities to recognize different carbohydrate components of cell surface receptors and play a role in their cellular transduction phenotypes.

B19, the best characterized member of the *Erythrovirus* genus, is known to have hemagglutinating activity (HA) that depends upon the non-sialylated glycolipid globoside, which is its infectious cellular receptor (Brown *et al.*, 1993). Cryo-EM and image reconstruction showed this receptor binding in a depression at the viral icosahedral 3-fold axes (Chipman *et al.*, 1996). This 3-fold attachment site is proximal to the heparin-binding regions identified in AAV2 (Xie *et al.*, 2002; Kern *et al.*, 2003; Opie *et al.*, 2003). Despite the low sequence homology between B19 and the AAVs, their capsid topology is remarkably similar (Figure 10.1, p. 127) (Kaufmann *et al.*, 2004; Padron *et al.*, 2005). Whether or not this surface similarity results in the use of comparable capsid regions for receptor molecule(s) recognition remains to be determined by mutational analysis and structural studies of virus:receptor complexes.

The most extensively characterized members of the *Parvovirus* genus with respect to cell surface receptor attachment phenotype are CPV and FPV. Mutational and erythrocyte binding analysis of these viruses showed that they display a pH-dependent phenotype for binding (Chang *et al.*, 1992; Barbis *et al.*, 1992), and identified three amino acids residues, R377, R396, and E396, on the wall of the icosahedral 2-fold dimple-like depression (Figure 10.2B) that were required for HA (Tresnan *et al.*, 1995). These studies also identified sialic acid as the carbohydrate component of the receptor engaged in this interaction (Tresnan *et al.*, 1995). However, CPV and FPV do not replicate in erythrocytes, and thus the observed attachment most likely does not play a physiologically relevant role in the viral life cycle. In addition, a non-hemagglutinating mutant of CPV is able to productively infect susceptible host cells, indicating that sialic acid binding may not be a requirement or a component of the infectious receptor interaction (Tresnan *et al.*, 1995).

The infectious cell surface receptors for CPV and FPV have been identified as the canine and feline transferrin receptors (CTfR and TfR), respectively (Parker *et al.*, 2001; Palermo *et al.*, 2003). Further analysis clearly showed that the canine TfR controls CPV host range, facilitating the binding of CPV, but not FPV, capsids to canine cells (Hueffer *et al.*, 2003a,b). Mutational, binding and structural studies of CPV mutants identified three distinct regions of the capsid, involving residues 93, 300, and 323, as

being responsible for CTfR binding (Govindasamy *et al.*, 2003; Hueffer *et al.*, 2003a,b). These loci, separated by up to 37 Å, are located on the capsid surface between the icosahedral 2-, 3- and 5-fold axes (Figure 10.2B) (Govindasamy *et al.*, 2003). Elucidating the nature of the contact(s) between these three capsid regions and the canine and feline TfR awaits the analysis of the structure of CPV and FPV with their respective receptor molecules.

The ability to bind cell surface sialic acid is a characteristic shared by several other parvoviruses, including bovine parvovirus (BPV), MVM, and PPV (Cotmore and Tattersall, 1987; Thacker and Johnson, 1998; López-Bueno *et al.*, 2005; Tijssen, personal communication). The BPV capsid is capable of binding to glycophorin A (Thacker and Johnson, 1998), a heavily sialylated protein found on erythrocyte membranes, although its infectious cell surface receptor is unknown. PPV's sialylated glycoprotein receptor is also yet to be identified. Similarly, the infectious receptor for MVM remains unknown but neuraminidase treatment of cells blocks attachment and inhibits infection (Cotmore and Tattersall, 1987; López-Bueno *et al.*, 2005), indicating that sialic acid is an essential carbohydrate component of its infectious cell surface receptor(s).

A crystallographic study of the prototype strain of MVM (MVMp) crystals soaked with sialic acid identified the icosahedral 2-fold as a possible carbohydrate binding site (López-Bueno *et al.*, 2005). This study represents the first visualization of a receptor component bound to a parvovirus capsid at high resolution. The binding site is proximal to the CPV residues 377, 396, and 397 (Figure 10.2B) that control its binding to sialic acid during erythrocyte hemagglutination (Tresnan *et al.*, 1995), as discussed above. The identification of this sialic acid binding site on MVMp and the mutations on CPV that inhibit sialic acid binding strongly point to the common use of the 2-fold depression as a binding site for this carbohydrate on the autonomous parvovirus capsid. The possible use of this binding site by members of the *Dependovirus* genus for sialic acid recognition remains to be assessed.

## TISSUE TROPISM AND PATHOGENICITY DETERMINANTS

Members of the *Dependovirus* genus are non-pathogenic. The role of their major capsid protein, VP3, in tissue transduction is discussed above in relation to the types of carbohydrate moieties recognized as components of receptor molecules during infection. The role of the major *Densovirus* coat protein, VP4, in tissue tropism and pathogenicity is under investigation, and is not discussed in this chapter. For the *Erythrovirus* genus, comparisons of the available B19 capsid amino acid sequences have identified the VP1 unique region as the most variable locus, and thus the relationship of structure to tropism and pathogenicity differences remains to be determined.

For members of the *Parvovirus* and *Amdovirus* genera, pronounced *in vitro* tissue tropism and *in vivo* pathogenicity disparities between highly homologous strains are well documented (Hahn *et al.*, 1977; Engers *et al.*, 1981; Spalhotz and Tattersall, 1983; Tattersall and Bratton, 1983; Kimsey *et al.*, 1986; Alexandersen, 1990; Brownstein *et al.*, 1991, 1992; Segovia *et al.*, 1991, 1995, 1999; Chang *et al.*, 1992; Bloom *et al.*, 1993, 1998; Bergeron *et al.*, 1996; Maxwell *et al.*, 1995; Ramírez *et al.*, 1996). Host range and pathogenicity differences between highly homologous viruses might occur at various stages of the viral life cycle, including cell receptor attachment, viral entry, uncoating, DNA replication or transcription. For these viruses, particularly with AMDV, CPV/FPV, MVM, PPV as models, molecular analysis, *in vitro* and *in vivo*, shows that VP2/VP3, their major coat protein, plays a crucial role in tissue tropism and the onset of an *in vivo* pathogenic outcome following cell infection (Gardner and Tattersall, 1988a,b; Ball-Goodrich *et al.*, 1991; Ball-Goodrich and Tattersall, 1992; Brownstein *et al.*, 1992; Chang *et al.*, 1992; Bloom *et al.*, 1993, 1998; Parrish *et al.*, 1988; Parrish, 1991; Truyen *et al.*, 1994, 1996; Maxwell *et al.*, 1995; Fox *et al.*, 1999).

Two strains, AMDV-G and AMDV-Utah, which are approximately 97 percent identical in their VP2 sequence, have provided the viral models for probing host range and pathogenicity determinants in AMDV, a virus that can cause chronic and persistent diseases in mink (Bloom *et al.*, 1994). AMDV-G is a tissue culture-adapted strain that is non-pathogenic, while AMDV-Utah is highly pathogenic *in vivo* but does not grow in tissue culture. CPV and FPV cause an enteric disease in puppies and kittens, respectively, but are >99 percent identical at the DNA level and differ by only 9 or 10 out of 584 amino acids in their VP2 sequence (Parrish and Carmichael, 1986; Parrish *et al.*, 1988; Parrish, 1991). CPV and FPV can both replicate in cat cells *in vitro*, but only CPV can replicate in dogs and dog cells, although there are CPV variants, with capsid mutations, that can replicate *in vivo* in cats. FPV tropism in dog cells is restricted to selected tissues (Truyen *et al.*, 1994, 1996). The prototype and immunosuppressive strains of MVM, MVM<sub>p</sub> and MVM<sub>i</sub> respectively, also display disparate tropisms and pathogenic phenotypes despite the fact that their VP2 molecules are approximately 97 percent identical (Ball-Goodrich *et al.*, 1991). *In vitro*, MVM<sub>p</sub> replicates in mouse fibroblast cell lines, while MVM<sub>i</sub> replicates in mouse T lymphocytes and hematopoietic cell lineages. *In vivo*, MVM<sub>p</sub> infection of adult and newborn mice is asymptomatic, while MVM<sub>i</sub> causes a lethal infection (Kimsey *et al.*, 1986; Brownstein *et al.*, 1991, 1992; Segovia *et al.*, 1991, 1995, 1999; Ramírez *et al.*, 1996). PPV is a causative agent of reproductive failure in pigs (Dunne *et al.*, 1965; Mengeling and Cutlip, 1976). Several PPV strains can be distinguished by pronounced differences in their tissue tropism and *in vivo* pathology, although their VP2 proteins are approximately 99 percent identical (Molitor and Joo, 1990; Bergeron *et al.*, 1996). NADL-2 is the attenuated vaccine strain that is non-pathogenic, but can be lethal if

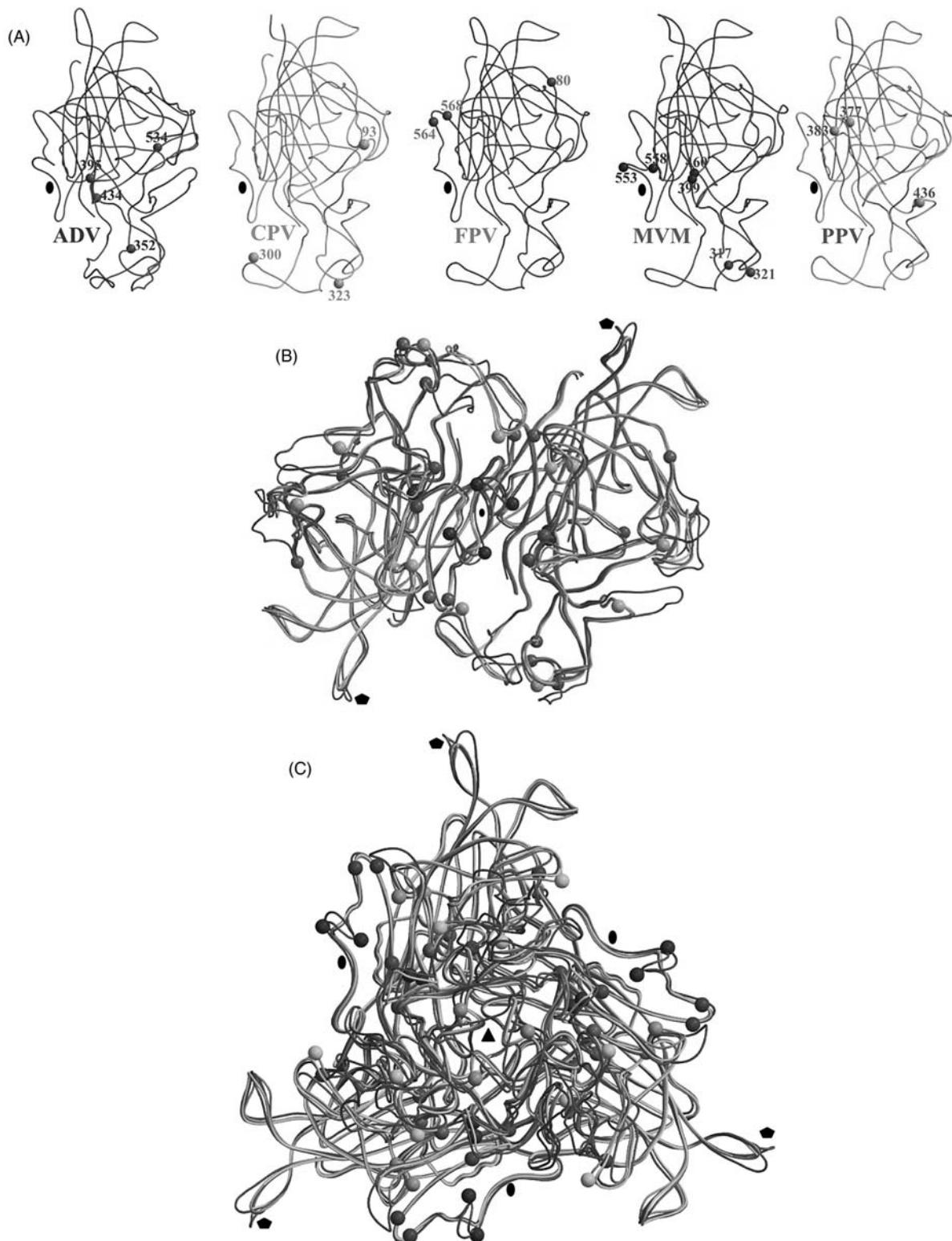
injected *in utero*, while Kresse, IAF-A54, IAF-76 and NADL-8 are virulent forms (Cutlip and Mengeling, 1975; Mengeling and Cutlip, 1975; Kresse *et al.*, 1985; Choi *et al.*, 1987).

For AMDV, CPV, FPV, MVM, and PPV, changes at three or fewer amino acid positions, at any one time, appear to govern tropism and the pathogenic outcome of infection, as determined by site-directed mutagenesis, forward mutations, and chimeric viruses (Gardner and Tattersall 1988b; Parrish *et al.*, 1988; Ball-Goodrich *et al.*, 1991; Parrish, 1991; Ball-Goodrich and Tattersall, 1992; Chang *et al.*, 1992; Horiuchi *et al.*, 1994; Bergeron *et al.*, 1996; Parker and Parrish, 1997; Bloom *et al.*, 1998; Truyen *et al.*, 1994, 1996; Fox *et al.*, 1999; Hueffer *et al.*, 2003a,b). The VP2/VP3 residues that control host range and pathogenicity differences include the following: AMDV – 352, 395, 434, 534; CPV/FPV – 93, 323, 80, 564, 568; MVM – 317, 321, 399, 460, 553, 558, and PPV – 378, 383, 436. The available pseudo-atomic model of AMDV built into a cryo-EM reconstructed image and atomic structures of CPV, FPV, MVM, and PPV capsids enable these residues to be mapped onto the respective capsid (Figure 10.3) (Agbandje *et al.*, 1993; Llamas-Saiz *et al.*, 1996; Xie *et al.*, 1996; Agbandje-McKenna *et al.*, 1998; McKenna *et al.*, 1999; Simpson *et al.*, 2000, 2002; Govindasamy *et al.*, 2003). Despite local capsid surface differences, the majority of these tropism and pathogenicity determinants are similarly located on the capsids of AMDV, CPV/FPV, MVM, and PPV, on or close to the capsid surface, in the depression at the icosahedral 2-fold axes or the ‘wall’ that surrounds it (Figure 10.3B) and on the shoulders of the 3-fold protrusions (Figure 10.3C). PPV residue 436 is located on the top of the icosahedral 3-fold protrusion (Figure 10.2C); however, more recent data suggests that it plays a very minor role in determining PPV pathogenicity (Peter Tijssen, personal communication).

In all the viruses, the determinant residues are located throughout the primary amino acid sequence, but become clustered on the assembled 3D capsid (Figure 10.3). For example, FPV tropism and pathogenicity determinant residues 80, 564, and 568 are brought together on the shoulder of the 3-fold protrusions by a 5-fold symmetry operation (Truyen *et al.*, 1994, 1996; Govindasamy *et al.*, 2003). In MVM, forward mutation residues 399, 460, 553, and 558, that confer fibrotropism to MVM<sub>i</sub> are clustered in the 2-fold depression (Agbandje-McKenna *et al.*, 1998). A 3-fold symmetry operation locates the allotropic determinant residues 317 and 321 (Ball-Goodrich *et al.*, 1992) proximal to the 2-fold depression on the wall immediately above residues 399 and 460 in the depression (Figure 10.3C).

## ROLE OF RECEPTORrecognition IN TISSUE TROPISM AND PATHOGENICITY

Cell binding and entry studies with CPV/FPV and MVM had shown that initial cell recognition/receptor attachment



**Figure 10.3** Tissue tropism and pathogenicity determinants for members of the Parvovirus genus. (A) Coil representations of the VP2/VP3 monomers of ADV, CPV, FPV, MVM and PPV (generated from coordinates and colored as in figure 10.1A) viewed approximately down the icosahedral 2-fold axes. The  $\text{C}\alpha$  positions of residues implicated in tropism and pathogenicity determination are shown as balls and colored accordingly to the monomers. In MVM, forward mutations conferring fibrotropism to MVM<sub>i</sub> are in blue. (B) and (C) Superimposition of the monomers in (A) viewed down the icosahedral 2-fold and 3-fold axes, respectively. The tropism and pathogenicity/virulence determinants are colored as in (A) and clustered mainly in and around the depression at the icosahedral 2-fold axes. The approximate icosahedral 2- (filled oval), 3- (filled triangle), and 5-fold (filled pentagon) are shown. See also Color Plate 10.3.

was not restricted in non-permissive cell lines and suggested that specific interactions with differentiation-dependent intracellular factor(s) in the permissive host cell, following initial entry, was required for replication to occur (Spalhotz and Tattersall, 1983; Gardner and Tattersall, 1988a). It was postulated that host range was controlled at this second interaction step (Spalhotz and Tattersall, 1983; Horiuchi *et al.*, 1992). Specifically, it was suggested that the block in restrictive cell lines occurred after entry and conversion of the genomic single-strand DNA to replicative form intermediates, but prior to viral genome transcription. However, structural co-localization of the critical *in vitro* tissue tropism and *in vivo* pathogenicity determinants with capsid surface residues that most likely control cell surface receptor recognition prompts re-evaluation of these earlier assumptions.

In the CPV/FPV system, residues 93 and 323 that are involved in differentiating the host range and *in vivo* pathogenicity of CPV and FPV (Parrish *et al.* 1988; Parrish, 1991; Chang *et al.*, 1992) form part of the predicted TfR binding site. This site also involves residues in the 300 region that, in new strains of CPV, support mutations implicated in the host range control of CPV capsid binding by canine TfR (Parker and Parrish, 1997; Hueffer *et al.*, 2003a). The 300 region is also proximal to capsid residues 80, 564, and 568 that control FPV host range (Truyen *et al.*, 1994, 1996). In the MVM/p system, residues 399, 460, 553, and 558, implicated in determining tissue tropism, are located in the vicinity of the sialic acid binding site recently identified in MVMp (López-Bueno *et al.*, 2005). Furthermore, an additional seven (of the 14) amino acids that vary between the VP2 sequence of MVMi and MVMp are clustered in this 2-fold region, with the allotropic determinants, 317 and 321, nearby on the shoulder of the protrusions at the icosahedral 3-fold axes that can also be considered as the wall of the 2-fold depression (Figure 10.3B and C) (Agbandje-McKenna *et al.*, 1998). For AMDV and PPV, tissue tropism and pathogenicity determinants are also located on or close to the capsid surface, in analogous regions to CPV/FPV and MVM. These observations point to a role for capsid surface interactions with primary cell surface receptor(s) in the differential tropism and pathogenicity of CPV and FPV, and of MVMi and MVMp, and such interactions may also modulate infection by AMDV and PPV strains (Agbandje-McKenna *et al.*, 1998; Simpson *et al.*, 2002; Govindasamy *et al.*, 2003; Hueffer *et al.*, 2003a,b; López-Bueno *et al.*, 2005).

## ANTIGENIC PROPERTIES OF PARVOVIRUS CAPSIDS

Antibody binding can neutralize virus at various stages in the infectious process, including receptor attachment, trafficking and uncoating (Smith and Moser, 1997). The elaborate loop regions between the strands of the viral  $\beta$ -barrel, which make up the protrusions at or surrounding the

icosahedral 3-fold axes, and form the walls between the depressions at the 2- and 5-fold axes, are the most immunogenic sites in the parvovirus capsid. For some members of the *Parvoviridae*, the available data suggest that neutralization can be mediated by more than one mechanism. Neutralizing epitopes have been mapped for members of the *Dependovirus*, *Erythrovirus* and *Parvovirus* genera, but not for the *Densovirus*. Many viruses, including members of the *Parvoviridae*, are known to sustain adaptive capsid mutations that enable them to escape antibody recognition and thus evade the immune response of their host. Members of the *Parvoviridae* for which escape mutants have been mapped include CPV and MVM. Significantly, the capsid surface structures of GmDNV and JcDNV are not decorated with protrusions (Simpson *et al.*, 1998; Bruemmer *et al.*, 2005), possibly because these viruses are not exposed to a humoral immune response in their host. According to this scenario, structural mutations and elaborations in the capsid surfaces that would allow them to evade an ongoing immune response have never been selected in the densoviruses, thus explaining their smoother architecture when compared with the vertebrate-infecting genera, which exhibit elaborate immunogenic loop decorations.

Antigenically, AAV2 is the best characterized member of the dependoviruses, since peptide mapping has been used to identify both linear and conformation-dependent epitopes on the capsid (Wistuba *et al.*, 1997; Wobus *et al.*, 2000). Three linear epitopes (A1, A69, and B1) and four conformational epitopes (A20, C24-B, C37-B, and D3) have been reported, although the residues for the C24-B site have not been mapped. For the linear epitopes, A1 recognizes VP1 only, while A69 recognizes a peptide in the VP1/VP2 common region, and B1 recognizes all three capsid proteins via an epitope at their C-termini (Wobus *et al.*, 2000). Antibodies to the conformation-dependent epitopes provide clear evidence that there are multiple mechanisms for parvoviral capsid neutralization. Thus, the C24-B and C37-B epitopes elicit antibodies that neutralize by inhibiting receptor attachment, while antibodies to the A20 site neutralize at a postattachment step, and D3 antibodies are non-neutralizing. Localization of the C37-B epitope (Figure 10.4A, residues 493–502, 601–610, VP1 numbering) close to the heparin binding site on the capsid is consistent with its function. Antibodies to the C37-B epitope specifically recognize AAV2, and not AAV1, 3, 4, or 5, in keeping with the observation that surface loops close to the heparin binding region are the most variable when the capsid structures of AAV2, AAV4, and AAV5 are compared (Govindasamy *et al.*, 2005; Padron *et al.*, 2005). The D3 epitope (Figure 10.4A, residues 474–483) is fairly conserved in the sequences of most AAV serotypes (Padron *et al.*, 2005), but D3 antibodies recognize serotypes AAV1, 3 and 5, not 4. This observation is consistent with these residues being close to structural differences between AAV2 and AAV4 at the mounds surrounding the icosahedral 3-fold axes (Govindasamy *et al.*, 2005; Padron *et al.*, 2005). AAV serotype reactivity to the A20 antibody

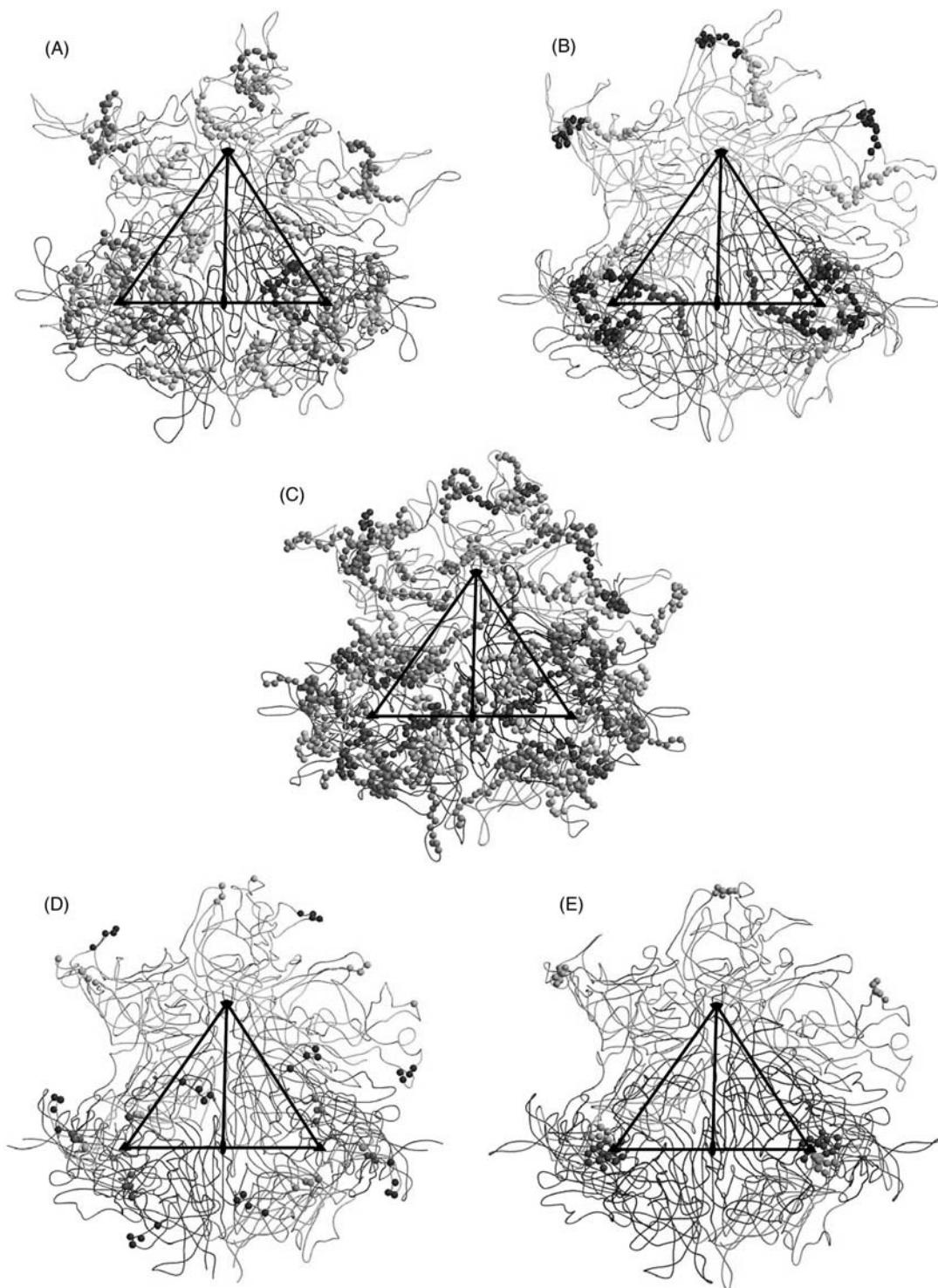
is limited to AAV2 and AAV3. This epitope (Figure 10.4A, residues 272–281, 369–378 and 566–575) maps to three different regions in the primary sequence (Wobus *et al.*, 2000) that come together in the wall between the 2-, 3-, and 5-fold axes in the AAV2 capsid structure (Xie *et al.*, 2002). Superimposition of the AAV4 and AAV5 VP3 coat protein structures, obtained by X-ray crystallography and cryo-EM and image reconstruction (Govindasamy *et al.*, 2005; Walters *et al.*, 2004; Padron *et al.*, 2005), onto the X-ray structure of AAV2 VP3, identified these regions as some of the most variable between the three viruses. The AAV4 capsid also has the unique phenotype of not being recognized by the B1 antibody, which is directed to a linear epitope (residues 726–733) at the extreme C-terminal end of the AAV VP3 protein (Wobus *et al.*, 2000). Comparison of the AAV2 and AAV4 VP3 crystal structures at the B1 epitope amino acids showed no major conformation differences, with the Cα atoms being superimposable, suggesting that the histidine residue in AAV4, where all other AAVs so far identified have an arginine or lysine, is involved in the capsid antibody interaction (Govindasamy *et al.*, 2005). However, considering that a single amino acid change on a viral surface is able to ablate antibody recognition (Llamas-Saiz *et al.*, 1996), it is perhaps not surprising that AAV4 and AAV5 are not recognized by A20 and that AAV4 is not recognized by B1.

Antigenic epitopes on the highly immunogenic AMDV capsid have been mapped with peptides (Bloom *et al.*, 1997, 2001; McKenna *et al.*, 1999) to capsid regions similar to those described for AAV and CPV/FPV (see below), located on the wall between the 2-, 3-, and 5-fold axes, and on the inner wall of its 3-fold mounds. Interestingly, binding of antibodies to the AMDV capsid tends to result in immune enhancement of infection (ADE), rather than neutralization, a phenomenon that is unique to AMDV in the *Parvoviridae* family and is an important component of its disease mechanism. However, *in vitro*, an antibody directed against VP2 residues 428–446 (Figure 10.4B, in the wall of the 2-fold depression), which is capable of eliciting antibody-dependent enhancement, antibody-dependent enhancement (ADE), is able to neutralize infectivity in CrFK cells. This capsid region includes residue 434 (Figure 10.3) that is implicated in AMDV host range and pathogenicity, as discussed above. Another well characterized AMDV antigenic epitope, residues 487–501 (Figure 10.4B), is capable of inducing ADE, but is not neutralizing. This epitope is located on the inner wall of the mounds at the icosahedral 3-fold axes. Thus if interactions between the capsid and its receptor molecule define the tissue tropism and pathogenicity of highly homologous strains, as suggested above, the neutralization of AMDV by an antibody to the 428–446 epitope could be due to steric hindrance of receptor attachment.

Neutralizing epitopes on the erythrovirus B19 capsid have been mapped to the VP1 unique region and to the VP1/VP2 overlapping sequence (Sato *et al.*, 1991a,b; Yoshimoto *et al.*, 1991; Brown *et al.*, 1992; Rosenfeld *et al.*, 1992; Saikawa *et al.*, 1993). Most of the VP1/VP2 neutralizing epitopes

are located on the loops that make up the protrusions on the B19 capsid surface (Figure 10.4C) (Sato *et al.*, 1991a,b; Yoshimoto *et al.*, 1991; Brown *et al.*, 1992; Kaufmann *et al.*, 2004). Antibodies that recognize VP2 residues 57–77, 345–365, and 446–466 are reported to inhibit HA by B19 (Brown *et al.*, 1993), while those generated for peptides from residues 253–515 (Figure 10.4C, epitope residues 253–272, 309–330, 325–346, 359–382, 449–468, and 491–515) are neutralizing. Mapping these residues onto the crystal structure of B19 (Kaufmann *et al.*, 2004) identifies residues 57–77, 253–272, 314–330, 325–346, and 359–382 on the wall between the 2- and 5-fold depressions and at the base of the 3-fold protrusions. The loop containing residues 309–313 is disordered in the B19 structure (Kaufmann *et al.*, 2004) but residues 314–330 structurally superimpose onto a portion of the AAV2 C37-B epitope (Figure 10.4C). Thus if the infectious receptor attachment site in B19 does co-localize with the heparin sulfate binding site of AAV2, as suggested (Kaufmann *et al.*, 2004), inhibition of receptor attachment would be a possible mechanism for neutralization by the antibody to this peptide. Residues 449–468 and 491–515 are located inside the assembled capsid, so neutralization by antibodies directed against these sites would have to occur after host cell entry or prior to capsid assembly.

Antigenic regions of the CPV and FPV capsids, mapped using peptide mapping and natural escape mutant analysis, also encompass the capsid surface ridges between the 2-fold and 5-fold depressions, the ‘shoulder’ of the 3-fold protrusions, regions close to the top of the 3-fold protrusions, residues that form the β-ribbons at the 5-fold axes and the VP1 unique region (Rimmelzwaan *et al.*, 1990; Lopez de Turiso *et al.*, 1991; Chang *et al.*, 1992; Cortés *et al.*, 1993; Langeveld *et al.*, 1993, 1994; Strassheim *et al.*, 1994). Two dominant immunogenic regions of the CPV/FPV capsid, referred to as epitope A and B (Figure 10.4D) and associated, in selected escape mutants, with mutations in VP2 residues 93, 222, 224, and 426, and 299, 300, 302, and 305, respectively, are located on the shoulder of the 3-fold protrusions and on the wall between the icosahedral 2- and 5-fold axes, respectively (Strassheim *et al.*, 1994; Agbandje *et al.*, 1995). Natural variants of CPV exist that are also resistant to monoclonal antibodies directed against epitope B. Cryo-EM and image reconstruction of CPV complexed with Fab fragments from a monoclonal antibody to epitope B identified an antibody footprint as previously mapped by sequence analysis of capsid neutralization escape mutations (Wikoff *et al.*, 1994). The capsid region surrounding and including epitopes A and B, show the greatest structural variation between CPV and FPV, and their host range and antigenic mutants (Agbandje *et al.*, 1993; Llamas-Saiz *et al.*, 1996; Simpson *et al.*, 2000; Govindasamy *et al.*, 2003). Residue 93 in epitope A and 299 and 300 in epitope B, plus a third capsid region containing residue 323 that is also immunogenic, are involved in host range determination for CPV and FPV and form the large footprint proposed as the TfR recognition site (Govindasamy *et al.*, 2003). Thus the mechanism



**Figure 10.4** Antigenic regions on parvovirus capsids. Antigenic regions on the AAV2 (A), ADV (B), B19 (C), CPV (D), and MVM (E) capsids are shown for the reference monomers (colored as in Figure 10.1) bounded by their icosahedral 2-fold (magenta), 3-fold (dark green and light green) and 5-fold (cyan) related monomers. For AAV2 (A), the C<sub>α</sub> positions for the A20 epitope (VP1 numbering residues 272–281, 369–378, and 566–575) is shown in greenish yellow, the C37-B (residues 493–502 and 601–610) in pink and D3 (residues 474–483) in orange. The ADV (B) epitope at VP2 residues 428–446 is shown colored according to the monomers and the 487–501 epitope is colored in purple. The B19 (C) VP2 epitopes are colored as follows: 57–77 (pink); 253–272 (orange); 314–330 (part of 309–330) (red); 359–382 (yellowish green); 449–468 and 491–515 (grey). In CPV (D) antigenic epitope A (residues 93, 222, 224, and 426) is colored according to the monomers and epitope B (residues 299, 300, 302, and 305) is in grey. In MVM (E) escape mutant residues are shown in balls colored according to the monomers. The approximate icosahedral 2- (filled oval), 3- (filled triangle), and 5-fold (filled pentagon) axes are shown in A–E for a viral asymmetric unit. See Color Plate 10.4.

for neutralization of viral infection by antibodies to these regions is likely the steric hindrance of receptor attachment, mediated by blocking essential residues that are required for specific interactions.

Escape mutants have also been reported for MVM, arising during long-term, passive, monoclonal antibody therapy in mice with severe immunodeficiency (SCID) (López-Bueno *et al.*, 2003). The mutant viruses that were able to escape neutralization, and which gave disease symptoms similar to the wild-type virus infection, contained single amino acid changes at VP2 amino acid positions 433–439, located in a surface loop at the very center of the protrusions at the icosahedral 3-fold axes (Figure 10.4E). Three such loops are located at the top of the 3-fold protrusions on the MVM capsid. The mechanism of neutralization by the antibodies to this epitope at the icosahedral 3-fold axes of MVM is unknown.

## SUMMARY

Despite the apparent simplicity of the  $T = 1$  parvovirus capsid, the proteins forming the protective coat around the parvovirus genome are capable of performing, or engaging the partners necessary for performing, the numerous interactions required for the propagation of progeny. While local capsid surface differences have produced three general parvovirus capsid topology groups, the functional regions co-localize to similar surface regions. The available 3D information for members of four main parvovirus genera, combined with the amenability of the parvoviral ssDNA genome to mutational analysis, has enabled the annotation and visualization of functional domains that play essential roles in the viral life cycle, particularly receptor attachment, *in vitro* tropism and *in vivo* pathogenicity, and antigenicity. A basic understanding of these phenotypes and the mapping of essential capsid sequences onto the capsid structure is a necessary step for infection and disease control, and will allow the genetic manipulation of parvovirus capsids for applications directed at developing foreign antigen and gene delivery systems.

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# SECTION C

## Viral Life Cycles: Molecular Interactions between Virus and Host Cell

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# The parvovirus life cycle: an introduction to molecular interactions important for infection

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MATTHEW D. WEITZMAN

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At every step of a virus life cycle there are numerous molecular interactions between the virus and its host cell that together determine the success of the infection. The smaller the virus the more dependent it is on cellular systems to ensure a productive infection. The parvovirus genome consists of terminal palindromic sequences flanking two genes that encode the non-structural replication proteins (Rep or NS) and the capsid proteins that make up the virus structure. Due to their limited coding capacity, parvoviruses have developed a plethora of ways in which these small viruses can recruit machinery from their environment to aid in their ability to produce progeny. There are interactions at the cell surface that determine attachment and entry of the virus capsid. There are interactions in the cytoplasm that control intracellular trafficking. And there are numerous nuclear interactions that regulate uncoating, transcription, replication, assembly and packaging of progeny virions. Although the genomes of the autonomous and dependoparvoviruses are relatively similar they have in some cases exploited quite different strategies to ensure successful infection. In this review I will introduce some of the molecular interactions that are described in further detail in the subsequent chapters of this book. Together they provide an in-depth look at the current state of knowledge for the various steps along the path of parvovirus infection.

## INTERACTIONS FOR ENTRY AND TRAFFICKING

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The parvovirus capsids are similar in terms of size and overall structure, and there are shared surface features (Chapman

and Rossmann, 1993; see Chapters 9 and 10). There are also molecular interactions with the host cell that are common among the different parvoviruses. Structural information combined with genetic data has been important for understanding the molecular interactions of the virus particles (Chapman and Rossmann, 1993). The parvovirus capsid proteins all contain a highly conserved  $\beta$ -barrel motif and the remainder of the protein consists of hypervariable regions connecting the eight strands of the  $\beta$ -barrel. Differences in surface topologies between the different parvovirus capsids are due to the highly variable sequences that form surface loops. Variation in the capsid structure on the surface exposures may be responsible for phenotypic differences in terms of usage of cellular receptors or antigenicity of virus particles. The use of recombinant vectors has been particularly informative for identifying individual molecular interactions along the route of virus entry and initiation of infection.

## Interactions that mediate virus entry

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The primary interaction for any virion during infection is with cell surface molecules that can act as attachment receptors to enable the early steps in virus infection. There may be many interactions at the cell surface and the relative abundance of viral receptors and co-receptors can act as a major determinant of viral tropism (see Chapters 12 and 13). The different serotypes of adeno-associated virus (AAV) seem to have adapted varied approaches by which to gain entry into host cells. The primary attachment site used by AAV2 is the ubiquitous heparan sulfate proteoglycan (Summerford and Samulski, 1998). AAV2 binding to cells can be completed by

soluble heparin (Summerford and Samulski, 1998) and this binding can also be used as a basis for column purification of virions (Clark *et al.*, 1999; Zolotukhin *et al.*, 1999). Other proteins have been implicated in AAV2 entry including human fibroblast growth factor receptor 1 (FGFR1) (Qing *et al.*, 1999), the  $\alpha\beta\gamma$  integrins (Summerford *et al.*, 1999), and an unknown 150 kDa glycoprotein (Mizukami *et al.*, 1996). For AAV4 and AAV5 sialic acid has been identified as a cellular attachment site, with different carbohydrate linkages determining specificity (Walters *et al.*, 2001). In addition, the platelet-derived growth factor (PDGF) receptors have been shown to play a role in capsid binding for AAV5 (Di Pasquale *et al.*, 2003). Sialic acid is also used by a number of the autonomous parvoviruses including Aleutian mink disease virus (AMDV), bovine parvovirus (BPV) and minute virus of mice (MVM) (Cotmore and Tattersall, 1987). Canine parvovirus (CPV) can bind to sialic acid but infectivity requires binding to additional cellular receptors (Barbis *et al.*, 1992; Hueffer *et al.*, 2003b). Other parvovirus attachments include the erythrocyte P antigen globoside that is used by B19 (Brown *et al.*, 1993) and the transferrin receptors that are used for binding and entry by CPV and Feline panleukopenia virus (FPV) (Parker *et al.*, 2001).

A common feature of the capsid interactions across the parvovirus family appears to be the involvement of raised structural elements around the 3-fold axis of symmetry in receptor attachment. The binding sites for heparan sulfate on the AAV2 capsid have been mapped to a patch of basic residues on the inner surfaces of the protrusions of the 3-fold axis of symmetry (Kern *et al.*, 2003; Opie *et al.*, 2003). Small changes in the capsid structure can drastically alter cell tropism and host range. This is highlighted by the autonomous parvoviruses CPV and FPV (Parker *et al.*, 2001; Hueffer *et al.*, 2003a, b), as well as two different strains of MVM (Gardiner and Tattersall, 1988; Ball-Goodrich *et al.*, 1991). Cross-packaging vectors that use the same recombinant AAV (rAAV) vector genome in different capsids have shown that the tropism can vary among different tissues *in vivo* (Rabinowitz *et al.*, 2002). Comparisons between the structures of two of the AAV capsids (AAV2 and AAV5) suggests that it is surface variation that determines the differences in cell surface attachment, tissue tropism, and antigenicity between these closely related serotypes (Xie *et al.*, 2002; Walters *et al.*, 2004). The patches of residues used by AAV2 for heparan sulfate binding are absent from AAV5, but the residues involved in binding to its sialic acid attachment site remain to be determined. Although much of the human population possess antibodies to AAV capsids, the epitopes may vary between serotypes and not all neutralizing antibodies will cross-react (Chirmule *et al.*, 1999; Moskalenko *et al.*, 2000; Peden *et al.*, 2004).

## Interactions for intracellular trafficking

The role of cellular functions in intracellular trafficking of parvovirus capsids has mainly been investigated using drugs

that interfere with specific pathways. Although the attachment sites vary among different parvoviruses, it is thought that they all enter by receptor-mediated endocytosis. Entry of AAV2 and CPV virions into the cell occurs by endocytosis through clathrin-coated pits and is inhibited by overexpression of a dominant interfering mutant of dynamin (Duan *et al.*, 1999; Bartlett *et al.*, 2000; Parker and Parrish, 2000). Drugs that disrupt the endosomal pH lead to blocks in infection for AAV2 infection (Bartlett *et al.*, 2000; Douar *et al.*, 2001), MVM (Ros *et al.*, 2002), and CPV (Basak and Turner, 1992), suggesting a role for acidification in the infection process. MVM particles are routed into late endosomes (Ros *et al.*, 2002). Active transport mechanisms move virions through the cytoplasm and microtubules have been shown to play a role in trafficking for CPV (Vihinen-Ranta *et al.*, 1998) and AAV (Sanlioglu *et al.*, 2000b). After endosomal release, by unknown mechanisms, it appears that there is an interaction with the proteasome. In the case of AAV it is suggested that capsids are ubiquitinated and that proteasome inhibitors enhance transduction by preventing degradation (Douar *et al.*, 2001; Yan *et al.*, 2002). In contrast, it has been shown that these same inhibitors block infection by MVM by interfering with nuclear translocation of incoming virions (Ros *et al.*, 2002; Ros and Kempf, 2004). These discrepancies may result from differences in cell lines and experimental approaches, or could reflect alternative trafficking pathways determined by structural differences. The cell surface proteins used for entry may also play a role in determining the intracellular trafficking pathways used by the virus. Whether binding to cellular receptors plays a role in establishing an intracellular environment conducive to viral infection, via activation of specific signaling pathways, remains to be determined for most parvoviruses. Cell binding by AAV2 leads to activation of a Rac1 and phosphatidylinositol-3 (PI3) kinase cascade, which may direct viral movement along the cytoskeletal network to the nucleus (Sanlioglu *et al.*, 2000a). It is interesting to note that in a study of alterations to gene expression levels induced during AAV infection, the empty capsid produced most of the changes observed for the wild-type virus (Stilwell and Samulski, 2004). It should be noted that there may be multiple routes for intracellular trafficking and the pathways may vary between different cell types (which may explain the discrepancies reported between different studies).

Viruses that replicate in the nucleus have developed a number of strategies to deliver their genetic load into the nuclear compartment (Kasamatsu and Nakanishi, 1998). The details of nuclear transport are still unclear for the parvoviruses but presumably there are interactions of capsids with cellular components that contribute to translocation into the nucleus and uncoating of the viral genome. Accumulation of particles in a perinuclear localization has been observed for AAV2 (Weitzman *et al.*, 1996b; Bartlett *et al.*, 2000). In addition it has recently been suggested that the adenovirus capsid can facilitate trafficking of the AAV capsid into the nucleus (Xiao *et al.*, 2002). In contrast,

a powerful technique for single virus tracing visualized individual virus particles in live cells and suggested a rapid trafficking and nuclear accumulation of virions (Seisenberger *et al.*, 2001). The VP1 protein is required for infectivity of AAV2 (Hermonat *et al.*, 1984) and therefore the unique N-terminus of this capsid protein must contain an activity important for infection. Within this region is a conserved phospholipase A2 (PLA<sub>2</sub>) motif (Zadoni *et al.*, 2001; Girod *et al.*, 2002) that provides an activity that is required for a step in the virus life cycle following perinuclear accumulation of virions but prior to the onset of early gene expression. Modifications to the capsid during the trafficking process may also result in exposure of sequences that aid transport through the nuclear membrane (Cotmore *et al.*, 1999). Once inside the nucleus the single-stranded genome is released. Although this process is not understood, it has been suggested that the AAV capsid can also determine the rate of uncoating of the virus by cellular factors (Thomas *et al.*, 2004). This may be a contributing factor towards the different transduction rates observed for rAAV derived from various AAV serotypes (Gao *et al.*, 2002; Rabinowitz *et al.*, 2002).

## INTRANUCLEAR INTERACTIONS OF VIRAL DNA AND PROTEINS

Many viruses induce the cell to enter an appropriate stage of the cell cycle that will facilitate viral replication. The limited coding capacity of parvovirus genomes precludes the expression of genes designated exclusively for this function. Although some parvoviral proteins do affect cell-cycle progression, they do not induce the S-phase entry that would be most conducive to viral DNA synthesis. In the case of the non-autonomous AAVs, co-infection with a helper virus can stimulate S-phase entry and thus contribute to the helper activities. The autonomous parvoviruses wait for the host cell to go into S-phase in order to replicate. This may result in an initial latent period in which viral genomes may be protected within packaged particles (Tullis *et al.*, 1994; Thomas *et al.*, 2004). The parvovirus genome consists of a single-stranded DNA molecule with palindromic repeats at the termini (see Chapter 7). The self-complementary sequences in the termini can fold back on themselves to form hairpin structures that contain the viral replication origins and provide primers for DNA synthesis. The two termini are different for most autonomous parvoviruses, whereas in the AAV genome they are direct inverted repeats. Entry into S-phase of the cell cycle is thought to promote the conversion of single-stranded genomes into duplex molecules, which can efficiently serve as templates for transcription and subsequent replication. The first gene to be transcribed encodes the non-structural proteins that are required for replication (referred to as NS1 and NS2 for the autonomous parvoviruses and Rep40, Rep52, Rep68, and Rep78 for AAV). In depth discussions of

the mechanisms of parvovirus replication and the functions of the replication proteins can be found in Chapters 14 and 15.

## Interactions with helper viruses

Productive AAV infection requires helper functions that can be supplied by either co-infection with a helper virus or by DNA damaging agents (Muzyczka and Berns, 2001). Helper viruses include adenovirus (Ad), herpes simplex virus (HSV), and human papillomavirus (HPV). Other treatments known to induce AAV replication are UV light, hydroxyurea, chemical carcinogens and metabolic inhibitors of cellular DNA replication. AAV has therefore developed ways to replicate under a diverse set of conditions, suggesting that in each case the helper induces changes in the cellular environment that facilitate AAV replication. The exact way in which the various treatments enable AAV replication is not completely clear.

The ability to replicate AAV in extracts from uninfected cells suggests that cellular factors are sufficient for replication (Ward *et al.*, 1994; Ni *et al.*, 1998). This implies that helper viruses induce modifications to these proteins or alterations in their cellular localization that facilitate AAV replication. Viral infections are subject to spatial regulation with the formation of specific nuclear structures where viral transcription and replication occur. One of the shared features of the helper viruses may be the establishment of viral replication centers within the nucleus of the infected cell and the redistribution of cellular factors to aid AAV replication. Analysis of cellular compartmentalization during parvovirus infections has revealed temporal and spatial regulation of DNA replication, capsid assembly and packaging (Hunter and Samulski, 1992; Weitzman *et al.*, 1996a; Wistuba *et al.*, 1997). At early stages of infection the Rep proteins are detected at punctate replication foci in the nucleus, where they co-localize with viral DNA. At late stages Rep proteins become distributed throughout the nucleoplasm. The Cap proteins transiently appear in the nucleoli and at later stages are found in the nucleoplasm, where they co-localize with Rep.

Both adenovirus and herpes simplex virus (HSV) establish centers of replication where viral and cellular replication factors accumulate (Knipe, 1989; Wilcock and Lane, 1991; Bosher *et al.*, 1992; Pombo *et al.*, 1994). The viral Rep protein has been shown to co-localize in the nuclei of co-infected cells to sites where the single-stranded DNA-binding proteins of both helper virus reside (ICP8 for HSV-1 and Ad-DBP for adenovirus) (Weitzman *et al.*, 1996a; Heilbronn *et al.*, 2003; Stracker *et al.*, 2004). Specific replication centers/bodies have also been observed for autonomous parvoviruses such as MVM (Bashir *et al.*, 2001), AMDV (Oleksiewicz *et al.*, 1996) and H1 (Cziepluch *et al.*, 2000). Cellular proteins involved with DNA synthesis and cell-cycle control are also found accumulated at these sites (Bashir *et al.*, 2001). As parvovirus replication is tightly coupled to the proliferative

state of the host cell, even autonomous parvovirus replication can be enhanced by co-infection with adenovirus, and this is also accompanied by relocalization into adenovirus replication centers (Fox *et al.*, 1990).

In addition to the formation of replication centers, there are other defined steps in the virus life cycle that have been shown to be enhanced by adenovirus helper proteins. The E1a protein can activate AAV promoters by relieving repression of the p5 promoter by YY1 (Shi *et al.*, 1991; Lewis *et al.*, 1995), and by recruiting the p300 histone acetyltransferase (Lee *et al.*, 1995). A complex of E1b55K and E4orf6 proteins aids AAV replication and may indirectly facilitate cytoplasmic accumulation of AAV messenger RNAs (mRNAs) (Samulski and Shenk, 1988). The virus associated RNA of adenovirus, VAI, stimulates translation of adenoviral RNAs and it appears to do the same for AAV (West *et al.*, 1987). The product of the E2a gene is a single-strand DNA-binding protein (Ad-DBP) that is directly required for increasing the processivity of the polymerase during AAV replication (Ward *et al.*, 1998). In addition this protein may have effects on the transcription of AAV promoters (Chang and Shenk, 1990). Cellular proteins also play indirect roles in enabling replication by regulating transcription of the *rep* gene. Cellular transcription factors implicated in activation of AAV2 genes in the presence of helper virus include Sp1, YY1, and MLTF (Chang *et al.*, 1989; Shi *et al.*, 1991; Pereira and Muzyczka, 1997).

The set of helper functions supplied by HSV are a very different group of proteins from those used by adenovirus. The minimal HSV proteins that support AAV replication in cell culture are the helicase–primase complex of UL5, UL8, and UL52: together with the UL29 gene product ICP8 (Weindler and Heilbronn, 1991). As these proteins can induce AAV replication in the absence of a viral polymerase, they must facilitate the use of the cellular replication machinery. Expression of these four HSV proteins alone results in the formation of a complex at discrete intranuclear sites, and the AAV Rep protein is found co-localized at these centers (Stracker *et al.*, 2004). Similar to the Ad-DBP protein (Ward *et al.*, 1998), the ICP8 protein alone enhances AAV replication in an *in vitro* assay (Stracker *et al.*, 2004). The enzymatic functions of the helicase–primase complex do not appear to be required and their role may be merely to ensure that the ICP8 protein (and possibly other cellular factors) are in the correct intranuclear location (Ward *et al.*, 1998). *In vitro* studies using a reconstituted system of purified HSV replication proteins have shown that the HSV polymerase UL30 can also be used to replicate AAV (Ward *et al.*, 2001). The helicase–primase complex was again not required for UL30-dependent DNA synthesis on the AAV template in this *in vitro* system (Ward *et al.*, 2001).

**replication.** The non-structural replication proteins (NS and Rep) of the parvoviruses are the key viral proteins that recognize the replication origins within the terminal repeats (see Chapters 14 and 15). These proteins share many features characteristic of proteins involved in rolling-circle DNA replication. The larger two Rep proteins of AAV (Rep68 and Rep78) bind specifically to double-stranded DNA containing a repeating motif of GCTC (McCarty *et al.*, 1994a, b; Weitzman *et al.*, 1994). This sequence has been termed the Rep recognition sequence (RRS), the Rep-binding element (RBE) or the Rep-binding site (RBS). An adenosine triphosphate (ATP)-dependent DNA helicase activity of the Rep proteins unwinds the stem of the terminal repeat to generate a single-stranded region that is cleaved by an endonuclease reaction at a sequence called the terminal resolution site (TRS). The protein then becomes linked to the 5' phosphate at the nick through a tyrosine at residue 156. The Rep protein works as an oligomer and multiple regions are required for multimerization and enzymatic activities (Weitzman *et al.*, 1996a; Smith *et al.*, 1997). It is the N-terminus that contain the specific DNA-binding domain and the nuclease motif (Owens *et al.*, 1993; Cathomen *et al.*, 2000; Davis *et al.*, 2000; Hickman *et al.*, 2002; Yoon-Robarts and Linden, 2003; Hickman *et al.*, 2004).

In addition to proteins of the helper viruses, there are many cellular factors that interact with the viral genome or viral proteins to regulate AAV replication. Cellular proteins involved in AAV replication have been identified through biochemical methods and genetic screens. Several *in vitro* replication assays have been established, which can replicate AAV templates using cell extracts and purified proteins (Ni *et al.*, 1998; Ward *et al.*, 1998). AAV replication has been reconstituted *in vitro* using purified replication factor C (RFC), replication protein A (RPA), and proliferating cell nuclear antigen (PCNA), as well as a fraction containing polymerase activity and other unidentified factors (Ni *et al.*, 1998). Sensitivity to polymerase inhibitors as well as the requirement for PCNA, suggest that either polymerase delta or eta are being used by AAV in this system. The involvement of any of these proteins in AAV replication *in vivo* has yet to be established, although RPA is also found at viral replication centers (Stracker *et al.*, 2004). One common feature of the different conditions under which AAV replicates, is the enhancing role of a single-stranded DNA-binding protein: Ad-DBP from adenovirus, ICP8 from HSV and RPA from the cell. These three proteins are also found at viral replication centers, interact with the Rep protein and enhance its activities (Stracker *et al.*, 2004).

Cellular proteins that bind the viral terminal repeats may participate in regulating aspects of the AAV life cycle. The first cellular protein reported to bind the AAV inverted terminal repeat (ITR) was a single-stranded DNA-binding protein that recognizes the D-sequence (ssD-BP) and was subsequently identified as the chaperone-associated protein FKBP52 (Qing *et al.*, 1997, 2001). It is unclear which functions of this protein are relevant to its role in the AAV

## Interactions for replication

In order to replicate their genetic material, viruses must recruit and assemble replication factors at an origin of

life cycle but it is suggested that phosphorylated FKBP52 binds to the D-sequence and blocks second-strand synthesis (Qing *et al.*, 2001). The phosphorylation status of the protein correlates with rAAV transduction efficiency and treatments that enhance transduction, such as E4orf6 expression and hydroxyurea, lead to dephosphorylation of the protein (Qing *et al.*, 1997, 1998). Binding of replication proteins to the terminal repeats may also mediate association of the replicating genome with the cellular nuclear matrix, and this may be important for transcription/replication (Bodnar *et al.*, 1989). There is also evidence to suggest that the ITR can act as both an enhancer and an initiator for transcription (Flotte *et al.*, 1993; Pereira *et al.*, 1997; Haberman *et al.*, 2000), implying that it is recognized by cellular proteins that regulate transcription.

Genetic screens provide powerful approaches to identifying roles for cellular factors in regulating virus infections. A one-hybrid screen designed to identify cellular proteins that recognize the Rep-binding sequence within the AAV ITR found that the cellular zinc-finger 5 protein (ZF5) is a potent negative regulator of AAV2 replication (Cathomen *et al.*, 2001). Ectopic expression of ZF5 led to an ITR-dependent repression of the p5 promoter and dramatic reduction in both AAV2 replication and production of AAV2 vectors. A screen of a phage display cDNA library identified a heterogeneous nuclear ribonucleoprotein A/B-related protein that binds to single-stranded DNA in the genome of FPV and can modify virus replication (Wang and Parrish, 1999).

Genetic two-hybrid screens in yeast have also been used to isolate cellular polypeptides that interact with the Rep proteins. The positive coactivator 4 (PC4) protein has been identified as a Rep-interacting protein in two independent yeast 2-hybrid screens (Muramatsu *et al.*, 1998; Weger *et al.*, 1999). Over-expression of PC4 downregulates AAV promoters in the absence of a helper virus but enhances AAV replication in the presence of adenovirus. Rep binding to PC4 may contribute to the inhibitory effects of Rep on transcription and replication of other viruses. Rep may modulate PC4 interactions to block its negative effects on polymerase delta or RNA processing. Some cellular proteins can bind to Rep and enhance its activities. For example the high mobility group protein 1 (HMG1) stimulates Rep binding, *trs* nicking, ATPase activity and repression of the p5 promoter (Costello *et al.*, 1997). Other proteins that interact with Rep include the importin alpha receptor that mediates nuclear import (Cassell and Weitzman, 2004), the Sp1 factor that mediates transcriptional activation (Pereira and Muzychka, 1997) and PKA/PrKX that may be involved in inhibition of adenovirus (Di Pasquale and Chiorini, 2003).

The replication process for the autonomous parvovirus genome is slightly different from that of AAV (see Chapter 14 for details). It occurs by a modified rolling-hairpin model and in most cases only one of the two strands is packaged (as opposed to AAV where both are packaged with similar efficiencies). The non-structural protein NS1

shares many of the enzymatic activities of the two large AAV Rep proteins, including specific DNA-binding, helicase, endonuclease, and ATPase functions (Cotmore and Tattersall, 1988; Wilson *et al.*, 1991; Nuesch *et al.*, 1992; Cotmore *et al.*, 1995), and it is absolutely required for replication. The 3' end serves as the primer for DNA synthesis, and a ligation step takes place at the 5' end to yield a molecule with covalently joined ends. A duplex dimer molecule is then generated and the dimer bridge junction is ultimately resolved to yield monomers for progeny and feedback intermediates. Purified NS1 binds the sequence (ACCA)<sub>2-3</sub> (Cotmore *et al.*, 1995), but its ability to cut a TRS-like sequence in the dimer bridge is modulated by a cellular factor, parvovirus initiation factor (PIF), which stabilizes its binding (Christensen *et al.*, 1997a, b). This distinguishes MVM replication from AAV, where cellular factors are not required for nicking at the TRS by purified Rep. In addition to PIF, DNA synthesis also requires PCNA, RPA and a fraction containing polymerase delta and eta (Christensen *et al.*, 1997a). Like the AAV Rep protein, the NS1 protein also interacts with RPA, and this coordinates progression of the replication fork (Christensen and Tattersall, 2002). Cellular transcription factors also recognize the left terminal hairpin of the MVM genome (Burnett and Tattersall, 2003).

## Interactions that mediate integration and persistence

One intriguing aspect of the AAV life cycle is the observation that, in the absence of productive replication, AAV infection can result in a latent infection (Berns *et al.*, 1975; Laughlin *et al.*, 1986; see Chapters 16 and 17 for detailed reviews). The viral DNA becomes integrated into the cellular genome in tandem arrays of several genome equivalents in a head-to-tail configuration (Cheung *et al.*, 1980; Laughlin *et al.*, 1986). Although integration was initially thought to occur at random, it is now clear that the wild-type virus is capable of site-specific integration into a region of the host genome on human chromosome 19 (Kotin *et al.*, 1990; Samulski *et al.*, 1991; Kotin *et al.*, 1992). Integration is targeted to this locus (termed AAVS1) about 70 percent of the time and occurs within a region of several hundred nucleotides.

The first evidence that suggested a role for Rep proteins in targeting integration came from the observation that vectors lacking the *rep* gene no longer integrated in a site-specific manner (Walsh *et al.*, 1992). Supplying the large Rep proteins (Rep68/78) in *trans* was sufficient to target integration of a DNA sequence flanked by AAV viral ITRs (Balague *et al.*, 1997; Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Recchia *et al.*, 1999). The basis for Rep-mediated targeting of integration was revealed by the discovery of Rep binding and nicking sites within the integration locus (Weitzman *et al.*, 1994; Urcelay *et al.*, 1995). The Rep protein creates a complex between the viral ITR and the

human sequence, tethering the DNA molecules for recombination (Weitzman *et al.*, 1994). Mutations that affect the DNA binding or nicking activities of Rep also impair targeted integration (Urabe *et al.*, 1999). The DNA requirements have been defined using an episomal recombination assay or plasmid transfection (Linden *et al.*, 1996; Young and Samulski, 2001). The ITR is the only viral genetic element required in *cis*, but a functional nicking site may not be essential for site-specific integration (Young and Samulski, 2001). The minimal requirements within the integration locus span the Rep-binding element, the nicking motif, and a spacer region (Linden *et al.*, 1996; Meneses *et al.*, 2000). In addition, there may be contributing effects from the status of the chromatin around the AAVS1 locus (Lamartina *et al.*, 2000). AAV can remain stably integrated, with minimal expression of Rep proteins, until activated by infection with a helper virus. The mechanism by which the integrated genome is rescued to allow productive replication remains unclear. Various models have been proposed that involve the ITRs, a replicative mechanism, and/or cellular nucleases (Samulski *et al.*, 1983; Gottlieb and Muzyczka, 1988; Wang *et al.*, 1996; Young and Samulski, 2001; Ward *et al.*, 2003; see Chapter 17).

The integration capabilities of serotypes other than AAV2 have not yet been extensively examined. A similar sequence has been identified in the monkey genome and targeted integration by the Rep protein of AAV4 has been demonstrated (Amiss *et al.*, 2003). The AAVS1 locus is also conserved in the mouse genome (see Chapter 16), suggesting that murine model systems may be useful for studying the genetics and cellular distribution of targeted integration *in vivo*. The site-specific integration of AAV2 suggests that the requirements for targeting by parvoviral replication proteins are:

- a binding site within the viral genome;
- a similar binding site within the target site; and
- an appropriately located nicking site (Weitzman *et al.*, 1994; Linden *et al.*, 1996; Young and Samulski, 2001).

Although AAV sequences have been detected in human specimens, it should be noted that, as yet, there have been no reports of integration into chromosome 19 in human tissue samples, even after analysis with sensitive polymerase chain reaction (PCR) assays (R. Clark, personal communication). Recently AAV sequences have been shown to be widely distributed in multiple tissues from humans (Gao *et al.*, 2004) and non-human primates (Gao *et al.*, 2003). Site-specific integration was not examined but these endogenous viral genomes appear to exist in a variety of molecular forms. Integration of autonomous parvovirus genomes has also not been reported, but an artificial target generated by placing NS1 binding and nicking sites into an episome was sufficient to direct integration (Corsini *et al.*, 1997).

Vectors based on AAV generally do not retain the *rep* gene, and therefore lack the targeted integration attributable to the wild-type virus. Long-term gene expression from these vectors demonstrates that the vector DNA can

persist for long periods of time (Vincent-Lacaze *et al.*, 1999; Nakai *et al.*, 2001). In some cases the vector DNA may be integrated into the host chromosomes (Omori *et al.*, 1999; Nakai *et al.*, 2003), although the cellular recombination pathway used for rAAV integration is still unclear (Yang *et al.*, 1997). The frequency of integration *in vivo* may be very low and instead there is evidence suggesting extrachromosomal persistence of vector genomes (Nakai *et al.*, 2001; Schnepp *et al.*, 2003). AAV sequences can be detected in high molecular weight DNA molecules that are thought to represent circles and concatemers of the input genome (Duan *et al.*, 1998; Vincent-Lacaze *et al.*, 1999). Recombination between vector genomes has been exploited as a way of increasing the potential coding capacity of rAAV through dual vector approaches (Duan *et al.*, 2000a; Nakai *et al.*, 2000; Sun *et al.*, 2000).

## Interactions with the DNA repair machinery

The terminal repeats of parvoviruses form unusual DNA structures, and it is likely that these are recognized by cellular factors. Eukaryotic cells possess complex machinery that monitors the host genome for DNA damage and breaks, leading to activation of signaling checkpoints that enable repair to proceed (Zhou and Elledge, 2000). It is possible that this same machinery recognizes extrachromosomal viral genetic material and may play a role in processing of viral genomes. This has recently been demonstrated for adenovirus (Stracker *et al.*, 2002), where the virus prevents signaling and processing of the viral genome by degrading the cellular Mre11/Rad50/Nbs1 repair complex that functions as a sensor of DNA damage (Carson *et al.*, 2003). There is also growing evidence to suggest links between parvovirus genomes and the cellular DNA damage apparatus.

In response to DNA damage an intricate network of proteins execute a cellular response consisting of signal transduction cascades activated by the PI3-like kinases ataxia-telangiectasia mutated (ATM) and ATM-Rad3 related (ATR) (Shiloh 2003). The AAV ITRs have been suggested to elicit a cellular DNA damage response involving ATM (Raj *et al.*, 2001). Mammalian cells possess two main pathways for repair of DNA double-strand breaks (DSBs): homologous recombination and non-homologous end-joining (NHEJ). The cellular proteins Ku86 and Rad52 are factors involved in repair of DSBs, and they have been suggested to bind directly to the ITR of rAAV (Zentilin *et al.*, 2001). The DNA-PKcs and Ku proteins are predominantly involved in the non-homologous end-joining pathway of DSB repair, while Rad52 is part of the machinery responsible for homologous recombination. The relative binding of these factors may determine the pathway used for processing of the AAV genome. The cellular DNA damage response may limit AAV infection, as transduction by vectors is higher in cells deficient for the ATM kinase (Sanlioglu *et al.*, 2000a; Zentilin *et al.*, 2001).

AAV may also benefit from interactions of its helper virus with the cellular DNA repair machinery. For example the adenovirus E1b55K and E4orf6 proteins can enhance transduction from a rAAV vector by overcoming barriers to second-strand synthesis of the genome (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). The mechanism by which this occurs is undefined, but because E1b55K/E4orf6 degrade the Mre11/Rad50/Nbs1 complex (Stracker *et al.*, 2002; Carson *et al.*, 2003), it seems plausible that this could contribute to the helper function for AAV. Indeed, evidence suggests that degradation of Mre11 correlates with increased rAAV transduction and replication (unpublished observations), although the molecular mechanism is still under investigation. Cellular repair factors have been observed at replication centers for adenovirus and HSV (Stracker *et al.*, 2002; Carson *et al.*, 2003; Wilkinson and Weller, 2004), and it will be interesting to determine whether these factors are found at parvovirus replication centers and how they influence virus infection. It is possible that the cellular repair machinery may represent an obstacle to AAV replication, acting as a cellular defense to the virus. This may also apply to autonomous parvoviruses, as MVM replication has been shown to be more efficient in cells defective for DSB repair (Tauer *et al.*, 1996).

The role of specific cellular repair proteins in processing parvovirus genomes remains to be determined. During transduction with rAAV vectors there is evidence for the formation of large concatemers and circularized forms of the vector genome (Duan *et al.*, 1998; Vincent-Lacaze *et al.*, 1999; Yang *et al.*, 1999; Nakai *et al.*, 2000, 2001; Sun *et al.*, 2000). Examination of the junctions between viral genomes in these concatemers revealed variable orientations and heterogeneity, suggesting that some processing and recombination between viral ITRs occurs. While it is unclear which cellular factors are involved in this process, studies in SCID mice suggest that repair proteins may influence the episomal structure of rAAV genomes and circle formation (Song *et al.*, 2001; Duan *et al.*, 2003).

Integration of an AAV genome into the host chromosomes can occur for both wild-type AAV infection under non-permissive conditions (Kotin *et al.*, 1990; Samulski *et al.*, 1991) and during rAAV transduction (Yang *et al.*, 1997; Miller *et al.*, 2002; Nakai *et al.*, 2003). It is currently unclear whether circularization or concatemer formation are required, but tandem repeats of AAV genomes have been described in integrated DNA (Cheung *et al.*, 1980; Yang *et al.*, 1997). There is only very limited homology between the AAV genome and integration sites but genomic sequences flanking integrated AAV have been shown to possess small inversions and deletions (Yang *et al.*, 1997; Miller *et al.*, 2002; Nakai *et al.*, 2003). These observations suggest that integration occurs via a cellular pathway of NHEJ or illegitimate recombination (Russell, 2003). Agents that cause DNA damage can increase integration rates, suggesting that vector integration can occur at damaged sites (Russell *et al.*, 1995; Sanlioglu *et al.*, 2000a). While the viral

structures and *cis*-acting sequences involved in AAV integration are well described, the host proteins mediating this process have not been identified.

## INTERACTIONS FOR VIRION ASSEMBLY AND PACKAGING

A single *cap* gene produces the structural proteins that form the parvovirus virion. Three capsid proteins are generated with a shared C-terminus and variable N-termini. Molecular interactions between capsid proteins are required for assembly of the virion and these are described in detail in Chapter 21. Sixty capsid proteins come together to form the stable icosahedral virus particle with  $T = 1$  structural symmetry. Subunits are held together by intertwining loops at the 3- and 5-fold axes of symmetry that interact with neighboring capsid proteins. For some parvoviruses (CPV and MVM) there is structural evidence showing that the N-terminus of the VP2 protein runs through a cylindrical channel along each 5-fold axis and protrudes from the virion (Tsao *et al.*, 1991; Agbandje-McKenna *et al.*, 1998) but this is not seen for empty MVM (Cotmore *et al.*, 1999). In the case of AAV2 it is still not clear where the N-terminal regions of VP1 and VP2 are located in the assembled virus particle. Structural studies of empty capsids suggest that they are buried inside the particle (Kronenberg *et al.*, 2001), and this is supported by chimeric particles in which a peptide tag fused to the N-terminus could not be recognized in assembled virions (Hoque *et al.*, 1999). However, these observations are in contrast to studies with capsids that have packaged DNA, in which case foreign epitopes inserted into the N-terminus of VP1 and VP2 can be detected on the surface of the virus particle (Wu *et al.*, 2000; Shi *et al.*, 2001).

For many of the autonomous parvoviruses it has been shown that the major capsid protein is sufficient to assemble a virus particle. In the case of AAV2, capsids can be formed by VP3 together with either VP1 or VP2 (Ruffing *et al.*, 1992; Warrington *et al.*, 2004). Expression of VP3 alone results in protein detectable in both the nucleus and cytoplasm (Ruffing *et al.*, 1992; Hoque *et al.*, 1999). The N-terminus of VP2 contains a stretch of basic amino acids that can function as a nuclear localization sequence (NLS) and co-expression of either VP1 or VP2 results in nuclear accumulation of VP3 (Ruffing *et al.*, 1992; Hoque *et al.*, 1999). Addition of an NLS to the N-terminus of VP3 is sufficient for formation of virus-like particles, suggesting that the major role of VP2 is translocation of VP3 into the nucleus for capsid assembly (Hoque *et al.*, 1999). Mutational analysis suggests that the  $\beta$ -barrel structure is required for assembly of virus particles but additional residues may also fulfill important functions (Wu *et al.*, 2000; Reguera *et al.*, 2004). An intermediate structure for capsid assembly may be a trimer of capsid proteins that is generated in the cytoplasm and a resulting  $\beta$ -stranded motif may then drive

capsid protein oligomers into the nucleus for viral assembly (Lombardo *et al.*, 2000). Recent reports show that chimeric capsids can be generated by mixing capsid proteins from different AAV serotypes, and these hybrids can impart interesting altered phenotypes, which combine attributes of each individual capsid (Hauck *et al.*, 2003; Rabinowitz *et al.*, 2004).

**A model for the possible pathway of parvovirus assembly and packaging is proposed in Chapter 21.** Capsid assembly takes place in the nucleus and in the absence of Rep, capsids accumulate in nucleoli (Wistuba *et al.*, 1997). Rep proteins alter the subnuclear capsid distribution and co-localize with Cap proteins at discrete sites in the nucleoplasm (Hunter and Samulski, 1992; Wistuba *et al.*, 1997). Therefore it appears that, at least in the case of AAV, the non-structural proteins (and possibly helper virus functions) play a role in assembly and packaging. It is generally accepted that ssDNA progeny genomes are strand-displaced and associate with preformed empty capsids (Myers and Carter, 1980; Yuan and Parrish, 2001). Capsid mutants have been reported that produce empty virus particles, suggesting that a region of the protein may be involved in packaging (Wu *et al.*, 2000; Shi *et al.*, 2001). Empty capsids can assemble in the absence of viral DNA (Ruffing *et al.*, 1992). Rep proteins can be found complexed to the capsids during assembly and may bring the genome to the packaging complex (Dubielzig *et al.*, 1999). The Rep40 and Rep52 proteins are not required for replication but are essential for the accumulation of single-stranded progeny in the cell (Chejanovsky and Carter, 1989). These small Rep proteins possess helicase activity (Smith and Kotin, 1998) which is required to translocate viral genomes into preformed empty capsids in a preferred direction (King *et al.*, 2001). There is also evidence suggesting that non-structural parvovirus protein NS1 of MVM (Cotmore and Tattersall, 1989) and Rep78 of AAV2 (Prasad and Trempe, 1995; Prasad *et al.*, 1997) are associated with the termini of packaged DNA. Residues within the D sequence of the AAV2 ITR have been suggested to contain the elements required for packaging of progeny genomes (Wang *et al.*, 1997). There is evidence that the capsids of autonomous parvoviruses can bind directly to the viral ITRs (Willwand and Hirt, 1991) and may therefore also play a role in encapsidation.

**Presumably there are interactions of virus capsids with cellular proteins that could facilitate assembly, disassembly, and packaging but none has yet been identified.** Virus particles and packaging have been reported in baculovirus systems (Ruffing *et al.*, 1992; Urabe *et al.*, 2002) and therefore all the non-viral requirements for assembly/packaging are also present in insect cells. The nuclear localization of these events implies that cellular import factors will interact with the NLSs in the capsid proteins, but these are yet to be defined. A cellular extract can facilitate assembly of AAV2 capsids from purified proteins *in vitro* (Steinbach *et al.*, 1997), suggesting that there are cellular co-factors for assembly. One such factor might be the nucleolin protein

that can interact specifically with capsids (Qiu and Brown, 1999). *In vitro* systems for packaging the viral DNA may aid in studying the mechanism of parvovirus encapsidation and the factors required (Zhou and Muzyczka, 1998).

## SUMMARY

The parvoviruses present interesting model systems for studying virus-host interactions. Determining the molecular interactions that take place during infection will be important for understanding the life cycle and how it can lead to the pathologic phenotypes of the diseases caused by autonomous parvoviruses such as B19 and MVM (see Chapters in Section 3). Although our understanding of the natural biology of AAVs is somewhat limited, these viruses have been shown to act as efficient delivery systems (see Chapters in Section 4) and this has spurred on studies into their infection cycle and interactions with the host. Viral gene therapy vectors take advantage of the innate ability of viruses to transfer genetic material to the nucleus of the host cell for endogenous gene expression. A thorough understanding of the molecular interactions that take place during transduction will aid the development and optimization of these promising gene transfer vectors. Recombinant viral vectors have in turn provided powerful tools for studying the molecular interactions that characterize the parvovirus life cycle. Our understanding of the basic biology has resulted in significant improvements for rAAV vectors, e.g. production in the absence of adenovirus by helper plasmids (Xiao *et al.*, 1998; Grimm and Kleinschmidt, 1999), purification by affinity chromatography (Zolotukhin *et al.*, 2002), and increasing transgene capacity by using recombination between vectors (Duan *et al.*, 2000b; Nakai *et al.*, 2000; Sun *et al.*, 2000). Identifying endogenous cellular factors that recognize and regulate the parvovirus genome will ultimately lead to more efficient transduction from these vectors.

Structural studies of parvovirus capsids are now in a position to guide genetic engineering of viral vectors. Structure-based analysis, together with genetic modification, has revealed sites amenable to incorporation of foreign peptides (Girod *et al.*, 1999; Rabinowitz *et al.*, 1999; Wu *et al.*, 2000). This has enabled the development of vectors to target rAAV to alternative cellular receptors by incorporation of targeting ligands (reviewed in Rabinowitz and Samulski, 2000; Buning *et al.*, 2003; and Chapter 40). These have included an RGD peptide to target integrin receptors (Girod *et al.*, 1999; Shi and Bartlett, 2003), an NGR peptide to target CD13 receptor (Grifman *et al.*, 2001), and peptides that target the human luteinizing hormone receptor (Shi *et al.*, 2001) and unknown receptors on endothelial cells (Nicklin *et al.*, 2001). Retargeting efforts will benefit greatly from further understanding of virus-host interactions, including those at the cell surface and

others involved in the intracellular events. Structural information may also aid genetic modification to avoid the impact of immune-responses to AAV vectors (Huttner *et al.*, 2003).

Unraveling the intracellular trafficking pathways, will suggest ways in which transduction can be improved, e.g. by the use of proteasomal inhibitors with rAAV vectors (Duan *et al.*, 2000b). Understanding interactions with the genome and identification of endogenous cellular regulators of the AAV life cycle is also important for ensuring efficient long-term transduction. It will be crucial to determine the consequences of introducing foreign genetic material into the host cell, whether it be damage responses to ITRs or genetic rearrangements resulting from integration into the host genome. It will be interesting to understand how AAV mediates site-specific integration and the role of cellular factors in maintaining viral latency. Although the concept of targeted integration may be appealing for gene therapy vectors, a lot more needs to be understood about the mechanism used by the wild-type virus and the potentially deleterious effects of Rep expression.

Every step of the virus life cycle is governed by molecular interactions between the virus and the host. The simplicity of the parvoviruses has required them to develop ways of exploiting the cellular machinery to ensure productive infection and coexistence with the host. There is still much to be gleaned from further probing the details of these interactions and they are guaranteed to continue to yield insights into fundamental cellular processes.

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# Cell infection processes of autonomous parvoviruses

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MAIJA VIHINEN-RANTA AND COLIN R. PARRISH

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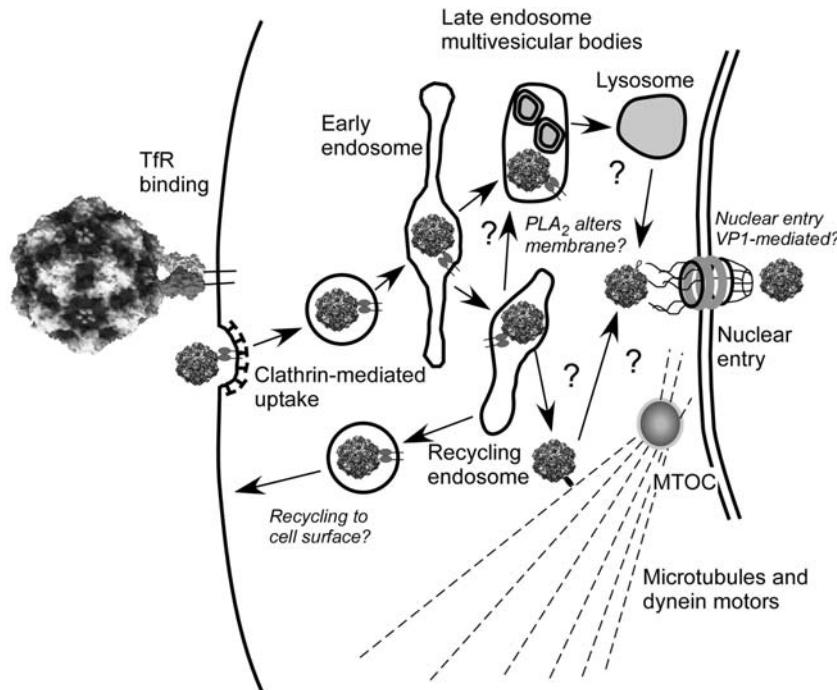
Like most animal viruses, parvoviruses have developed a variety of strategies for infecting cells, which initiate with binding to cell surface receptors, proceed through uptake into the cell through receptor-mediated endocytosis, capsid release into the cytoplasm and transport to the nuclear pore, uncoating or release of the viral genome (Greber, 2002; Sieczkarski and Whittaker, 2002; Pelkmans and Helenius, 2003). Infection by parvoviruses is a multistep process that involves a variety of viral and cellular components, and since replication is in the nucleus, specific mechanisms are required to transport the genome and other components both to the nuclear pore and into the nucleus (Whittaker and Helenius, 1998; Whittaker, 2003; Whittaker *et al.*, 2000). The initial recognition of cells involves attachment to one or more cell surface receptors. Parvoviruses then take advantage of endosomal trafficking within the cell, so that vesicles containing the virus particles are transported by microtubule-dependent motors through the cytoplasm. After release from the endosome into the cytoplasm the parvovirus capsid may then use additional cytoskeleton-driven transport of the capsid or of viral components to the vicinity of the nucleus, in a process that is also mediated either by microtubules or cytoskeletal motors (Sodeik, 2000; Mabit *et al.*, 2002; Suikkanen *et al.*, 2003a). Finally, translocation into the nucleus is an active process that transports the viral DNA and the more-or-less intact capsid into the nucleus for replication. A diagrammatic summary of our current understanding of the infection pathway of autonomous parvoviruses is shown in Figure 12.1.

## CAPSID STRUCTURE AND ITS RELATIONSHIP TO CELL INFECTION

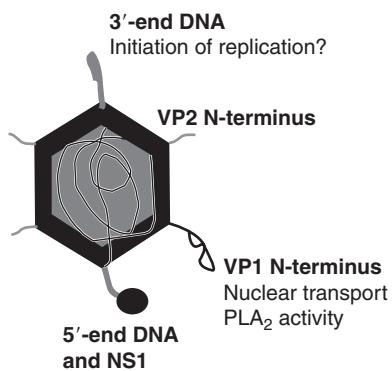
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The capsid is the key to successful infection of cells and, although we have had high resolution structures of the capsids of several parvoviruses for a number of years, we do not understand in detail how it mediates cell infection, nor the role of the various features of the capsids in these processes. Although the capsid is very stable in the environment, being able to resist temperatures of up to 65°C, flexibility is required during infection to allow genome release and to reveal protein structures that are normally found within the capsid. Defining the details of the capsid processes required for infection is still a key challenge to understanding the infection of cells by parvoviruses. A summary of the variable structures of the capsid of the autonomous parvoviruses is shown in Figure 12.2.

The ~26 nm diameter parvovirus capsid is assembled from 60 copies of the structural proteins, which for the autonomous viruses come in two or more overlapping forms. VP1 and VP2 of minute virus of mice (MVM) and canine parvovirus (CPV) are translated from alternatively spliced mRNAs, while VP3 is formed by the cleavage of a peptide from the N-terminus of VP2, which is exposed outside the full (DNA containing) capsids (Tattersall *et al.*, 1977; Tullis *et al.*, 1992; Weichert *et al.*, 1998). In other autonomous viruses the various capsid proteins are translated from the same message through the use of alternative initiation codons. VP1 contains the complete sequence of



**Figure 12.1** The uptake and endosomal trafficking pathways within the cell, and the likely route that is taken during cell infection by autonomous parvovirus capsids. The example of canine parvovirus is used as an illustration. The virus binds the transferrin receptor on the surface of cells and enters through clathrin-mediated endocytosis. It appears that the virus remains in association with the capsid in the endosomal system of the cell for a period before it leaves the endosome by an as-yet undefined process to enter the cytoplasm. Transport within the cytoplasm appears to involve the microtubular cytoskeleton, and the particle appears to enter the nucleus in a process that may involve targeting through the nuclear pore that is mediated by the N-terminal sequences of the VP1 protein exposed outside the capsid.



**Figure 12.2** A cartoon showing structures that may be exposed on the outside of parvovirus capsids under some conditions. The N-termini of several VP2 molecules are exposed on the outside of DNA-containing capsids, although the specific function of that sequence is not known and it can be cleaved off by proteases without affecting virus viability. The N-terminus of VP1 is essential for infectivity of the capsid. That may be exposed after various treatments of the capsids and within the cell after uptake, where it appears to be involved in nuclear trafficking of the capsids during infection. It also modifies membranes through a phospholipase A<sub>2</sub> activity, which may regulate endosomal release. About 24 bases of the 5' end of the viral DNA is exposed on the outside of newly synthesized DNA containing capsids, and that is covalently attached to the non-structural protein 1 (NS1). The function of that sequence is not known, but it may be removed by nuclease treatment without affecting the capsid infectivity. The 3' end of the viral DNA can be exposed on the outside of the capsid after some treatments, and inside the cells it may be involved in the initiation of viral replication.

VP2 as well as a unique N-terminal sequence of 140 to 230 residues in different viruses. That sequence is necessary for viral infectivity, but not for capsid formation or DNA packaging (Tullis *et al.*, 1992; Vihinen-Ranta *et al.*, 2002). For most of the autonomous viruses the VP1 unique region cannot be detected on the outside of the capsids under normal conditions, but for human parvovirus B19 it appears that the 227 residue VP1 unique sequence is found outside the assembled capsid (Dorsch *et al.*, 2002). At least two functions of that sequence have been identified that may contribute to cell infectivity. There are a number of basic amino acid-containing sequences, at least some of which act as nuclear localization sequences (Lombardo *et al.*, 2002; Vihinen-Ranta *et al.*, 2002), which may act both during infection and after protein synthesis. The VP1 unique region also contains a phospholipase A<sub>2</sub> (PLA<sub>2</sub>) enzyme motif, which is active when released from the capsid (Zadori *et al.*, 2001; Suikkanen *et al.*, 2003b; Canaan *et al.*, 2004). When that sequence is expressed from bacteria, the unique region of porcine parvovirus (PPV) showed about 10<sup>3</sup>-fold higher PLA<sub>2</sub> activity than that of adeno-associated virus type-2 or human parvovirus B19. These enzymes were all calcium dependent, and largely insensitive to inhibitors of other secreted PLA<sub>2</sub> enzymes (Canaan *et al.*, 2004). It is likely that the PLA<sub>2</sub> enzyme would modify the lipid components of the endosome, which may allow more efficient viral release.

The atomic structures of CPV, feline parvovirus (FPV), MVM, B19, porcine parvovirus (PPV), and *Galleria mellonella* densovirus virus capsids have all been determined, and show that the capsid surfaces have a variety of

structural features. For the viruses from vertebrate hosts, these features include raised regions at or around the 3-fold axis of symmetry, a depressed region or dimple spanning the 2-fold axis, and a cylinder at the 5-fold axis of symmetry with a ~45 Å long by ~9 Å diameter channel that runs from the inside to the outside of the capsid (Tsao *et al.*, 1991; Agbandje-McKenna *et al.*, 1998; Simpson *et al.*, 1998, 2002). The structure of an insect parvovirus shows a relatively smooth surface compared with the viruses of vertebrates (Simpson *et al.*, 1998), perhaps because the lack of an antibody immune response in insects results in less selection of the surface structure of the capsid. The host ranges and tissue tropisms of CPV and FPV, and of MVM variants are controlled by a small number of sequences on the capsid surface (Ball-Goodrich and Tattersall, 1992; Chang *et al.*, 1992; Tsao *et al.*, 1992; Agbandje *et al.*, 1993; Agbandje-McKenna *et al.*, 1998; Xie *et al.*, 2002; Hueffer *et al.*, 2003a). In FPV and CPV those residues control binding of the capsids to transferrin receptors (TfR) (Hueffer *et al.*, 2003a).

The variability and flexibility in the capsid structure are still not well defined, but they are likely important for viral infectivity and capsid functions. Besides the externalization of VP1 and VP2 N-terminal sequences, flexibility in the surface loops was seen in CPV and FPV capsids when their structures were determined at different pHs, and the removal of two or three Ca<sup>++</sup> ions from the CPV or FPV capsids also altered the structure of adjacent loops (Simpson *et al.*, 2000). However the specific function of that Ca<sup>++</sup> is not known, and Ca<sup>++</sup>-binding sites were not present in the capsids of PPV or MVM.

## CELL RECEPTOR BINDING

Both carbohydrate and protein receptors have been reported for different parvoviruses, although the identities of many of those receptors are still not known. Human parvovirus B19 replicates only in human erythroid progenitor cells, and cell binding and infection requires the erythrocyte P antigen (globoside) (Brown *et al.*, 1993). However, a number of cells that express globoside on their surfaces are not susceptible to infection. This may be partly due to an intracellular block to transcription of the viral messages in non-erythroid cells (Brunstein and Astell, 1997; Liu *et al.*, 1992), but it is also likely that there is another protein-based receptor for B19. The α5β1 integrin has been shown to be a co-receptor for infection of erythroid progenitor cells (Weigel-Kelley *et al.*, 2001, 2003), but other receptors are also likely to be involved.

The transferrin receptor (TfR) is used by CPV and FPV for cell infection, and differences between the feline and canine TfR control the specific binding of those viruses to cells (Hueffer *et al.*, 2003b; Parker *et al.*, 2001). Studies using capsid and TfR mutants show that specific contacts are made between the apical domain of the TfR and the top and side

of the threefold spike of the capsid (Hueffer *et al.*, 2003a; Palermo *et al.*, 2003). They also show that the TfR acts as more than a simple tether dragging the capsid into the cell, since the precise interactions are important for successful cell infection – some changes in either the virus or the receptor allow capsid binding and cell uptake, but without cell infection resulting (Hueffer *et al.*, 2003a; Palermo *et al.*, 2003). The canine host range of CPV is controlled by the ability of the virus to bind surface receptors on canine cells, as both CPV and FPV capsids could bind, enter and infect feline cells, while only CPV was able to bind canine cells. The specific cell susceptibility was controlled by the TfR, since feline TfR expressed on Chinese hamster ovary (CHO)-derived TRVB cells allowed efficient binding of CPV and FPV, leading to infection, while canine TfR bound CPV but not FPV capsids, and allowed infection by CPV only (Hueffer *et al.*, 2003b).

CPV and FPV also bind to sialic acids on some erythrocytes and host cells, which appears to be specific for the N-glycolyl neuraminic acid (NeuGC) that is found on erythrocytes and cells of most cats, monkeys, and horses, but not on cells of most dogs (Barbis *et al.*, 1992; Basak *et al.*, 1994). The hemagglutination was temperature and pH dependent, and was controlled by residues adjacent to a depressed region (the dimple) on the capsid (Barbis *et al.*, 1992; Tresnan *et al.*, 1995). Mutations within a loop between VP2 residues 359–377 or of VP2 residue 323 make the sialic acid binding dependent on low pH (pH < 6.5), or can prevent sialic acid binding (Barbis *et al.*, 1992; Simpson *et al.*, 2000; Tresnan *et al.*, 1995). However, sialic acid binding does not mediate infection of cells in tissue culture since non-sialic acid-binding mutants infect the same range of cells as wild-type viruses, and treatment of cells with sialidases does not affect the efficiency of subsequent infection. FPV capsids bind sialic acids on feline cells most efficiently at pH 6.5 and 4°C. CPV binds sialic acid on feline cells at pHs > 7.0, but that binding at least partially inhibits infection as mutants, which do not bind the sialic acid are selected by passage of CPV in cat cells (Barbis *et al.*, 1992).

MVM capsids also bind sialic acids and hemmaglutinate mouse erythrocytes, and cell infection can be blocked by neuraminidase treatment of the cells prior to virus binding (Cotmore and Tattersall, 1989). The precise type of sialic acid or the specificity of binding has not been described, and it is also not known whether there are additional protein receptors that are required for cell infection by MVM (Agbandje-McKenna *et al.*, 1998).

## ENDOCYTOSIS AND ENDOSOMAL RELEASE

All parvoviruses appear to require receptor-mediated endocytosis for cell infection. CPV and MVM are taken up rapidly into cells, most likely by clathrin-mediated endocytosis as CPV entry was inhibited by over expression of the Lys44Ala (K44A) dominant interfering mutant of dynamin-2, and

the virus capsids were seen to be associated with clathrin-coated pits and vesicles at short times after cell uptake (Linser *et al.*, 1979; Parker and Parrish, 2000). However, clathrin-mediated uptake is not specifically required for CPV infection, as mutations in the cytoplasmic domain of the feline TfR that altered its ability to interact with the adapter protein 2 (AP2) prevented clathrin-mediated uptake into cells, but still allowed efficient virus infection (Hueffer *et al.*, 2004). However, replacing the transmembrane and cytoplasmic sequences of the feline TfR with equivalent sequences from the influenza neuramindase caused the receptor to localize to detergent-insoluble lipid rafts in the cell membrane, and also prevented the receptor from mediating cell infection by CPV (Hueffer *et al.*, 2004). The reason for this block to infection by receptor chimeras is not known, but could be related to capsid entry into an alternative endocytic pathway, or into endosomes from which the virus is unable to penetrate into the cytoplasm.

Infection by parvoviruses is readily inhibited by treatment of cells with lysosomotropic agents including NH<sub>4</sub>Cl and baflomycin A1, indicating that low endosomal pH is required for capsid infection or for endosomal trafficking (Basak and Turner, 1992; Parker and Parrish, 2000; Suikkanen *et al.*, 2003b). Shortly after uptake CPV capsids are seen to co-localize with transferrin in perinuclear endosomes (Parker and Parrish, 2000; Suikkanen *et al.*, 2002). After uptake capsids were detected in perinuclear endosomes for several hours by antibody staining (Parker and Parrish, 2000; Suikkanen *et al.*, 2002; Weichert *et al.*, 1998), while fluorescence *in situ* hybridization (FISH) studies showed that CPV DNA co-localized with the capsids in perinuclear compartments for at least 8 hours (Suikkanen *et al.*, 2002). The infectious process also appears to be slow. Thus, antibodies against the cytoplasmic tail of the TfR reduced virus infection when injected into cells 4 hours after virus inoculation, showing that some of the infecting capsids remain associated with the TfR in endocytic compartments for several hours after uptake (Parker and Parrish, 2000). In addition, CPV infection of cells could be blocked by injecting anticapsid antibodies into the cytoplasm as much as 4 or more hours after virus uptake, indicating that the infectious process leading to DNA release is also a slow process (Vihinen-Ranta *et al.*, 2000, 2002).

MVM capsids also require uptake through the endosomal system as infection can be blocked by baflomycin A1 or chloroquin for several hours after uptake from the cell surface (Ros *et al.*, 2002).

The mechanism of escape from endocytic vesicles into the cytosol is still unknown, although there does not appear to be wholesale lysis of the endosomal vesicle as there is little transport of alpha-sarcin or high molecular weight-labeled dextrans into the cytoplasm, while low molecular weight dextrans could enter the cytoplasm of cells when delivered with CPV capsids (Parker and Parrish, 2000; Suikkanen *et al.*, 2003b). The difference in permeability to alpha-sarcin and the low molecular weight dextrans

has not been explained, but may be due to the differences in the assays used to detect the entry of those compounds. The PLA<sub>2</sub> activity of the VP1-unique region of most parvoviruses is essential for infection, and this may act to alter the membrane structure of vesicles containing virus capsids, and this may enhance release of the capsids into the cytoplasm (Canaan *et al.*, 2004; Dorsch *et al.*, 2002; Suikkanen *et al.*, 2003b; Zadori *et al.*, 2001).

## TRANSPORT WITHIN THE CYTOPLASM

The capsids are thought to be released into the cytoplasm from a vesicle in a perinuclear location, and further processing and trafficking events are likely required in the cytoplasm before transport to the nucleus. Infection of cells by MVM is affected by the activity of the proteasome, since infection can be reduced by some proteasome inhibitors, including inhibitors of the chymotrypsin-like activity (N-tosyl-L-phenylalanine chloromethyl ketone and aclarubin), but not by inhibitors of the trypsin-like activity (Ros *et al.*, 2002). Mechanisms involved in that activity are not clear, but there is no clear evidence for ubiquitination of the capsid or direct proteolytic digestion of the capsids (Ros and Kempf, 2004).

Active transport mechanisms are also likely to be required for the particles or the viral genome to reach the nuclear pore. The cytoplasm contains a lattice-like mesh of molecules, which restrict the diffusion of macromolecular complexes (Luby-Phelps, 2000; Seksek *et al.*, 1997). When CPV capsids were injected into the cytoplasm they rapidly moved to a perinuclear location, but they only entered the nucleus 3–6 hours later (Vihinen-Ranta *et al.*, 2000). Both transport of the injected capsids to the perinuclear region and their nuclear transport were prevented by treatment of the injected cells with nocodazole, which depolymerizes microtubules, and transport was also blocked by injection of an antibody against the intermediate chain of dynein, the microtubule-based minus-end-directed motor protein (Suikkanen *et al.*, 2002, 2003a; Vihinen-Ranta *et al.*, 2000). Capsids were seen to be associated with tubulin and apparent dynein structures *in vitro* by electronmicroscopy, and viral capsids could also be co-precipitated from infected cells along with the intermediate chain of dynein. These results suggest that microtubules and dynein move CPV capsids within the cytoplasm, facilitating both their transport to the nucleus and infection (Suikkanen *et al.*, 2003a).

## NUCLEAR TRANSPORT

Transport of the capsid and/or viral DNA into the nucleus is an important step in infection that occurs principally through the nuclear pore complex (NPC). Although small

macromolecules can diffuse freely through the nuclear pore, transport of larger molecules is specific. It requires adenosine triphosphate (ATP) and soluble cytosolic factors, including alpha and beta importins and Ran-1, and is mediated by nuclear localization signals (NLSs) (Gorlich and Kutay, 1999; Nigg, 1997). Parvovirus capsids appear able to pass through the NPC intact, since CPV capsids injected into the cytoplasm of cells entered the nucleus over a period of 2 or more hours and could then be recognized by antibodies against intact capsids (Vihinen-Ranta *et al.*, 2000; Suikkanen *et al.*, 2003a). One reason for the slow nuclear entry of capsids is that the capsid structure must be modified to allow nuclear targeting by exposing nuclear localization sequences (NLSs). Within the CPV and MVM VP2 sequences there are two regions that contain clusters of arginines or lysines that might be classical NLSs, while the VP1 unique region contains several such sequences. A VP1 sequence (PAKRARRGYK) between residues 4 and 13 functions for nuclear transport when conjugated to BSA (Vihinen-Ranta *et al.*, 1997). That N-terminal unique sequence was detected on capsids between 1 and 4 hours after infection, and its exposure appears required for infectivity, since antibodies specific for the VP1 unique region blocked infection when injected into cells before virus inoculation (Vihinen-Ranta *et al.*, 2002). In addition, changes in that basic N-terminal VP1 sequence reduced the relative infectivity of the capsids (Vihinen-Ranta *et al.*, 2002). MVM capsids have NLS sequences in both VP1 and VP2, with two NLS sequences mapped near the VP1 N-terminus. An intact VP1 unique region that could mediate efficient nuclear transport was required for efficient cell infection by MVM capsids, and infection was reduced significantly by mutations in the basic sequences in the VP1 N-terminal sequence (Lombardo *et al.*, 2002; Tullis *et al.*, 1993). An internal basic sequence (KGKLTMRRAKLR) is active in a conformation-dependent manner when present in a trimer of the VP2 proteins (Lombardo *et al.*, 2000, 2002).

## VIRAL DNA RELEASE FROM THE CAPSID AND INITIATION OF REPLICATION

The mechanisms by which the viral DNA genome is released from the capsid for replication are not yet understood, but some suggestions have been made. Full capsids of MVM and CPV have between 20 and 30 nucleotides of the 5'-end of the viral genome exposed on the outside of the capsid, and the NS1 protein is covalently attached to the 5' end of that DNA in newly produced capsids (Cotmore and Tattersall, 1988; Wang and Parrish, 1999). That DNA is thought to pass through a pore at the 5-fold axis of the capsid (Farr and Tattersall, 2004; Xie *et al.*, 2002; Xie and Chapman, 1996). The 3' end of the viral DNA can also be exposed outside the capsid after treatments which change the capsid structure but which do not cause capsid

disintegration, and the 3' terminal hairpin can also act as a template for the DNA polymerase *in vitro* (Cotmore *et al.*, 1999; Vihinen-Ranta *et al.*, 2002). This suggests a mechanism where this extracapsid DNA exposed within the nucleus could be used as a template for initiating DNA replication by the host cell DNA polymerase, where the DNA would be removed without disassembly of the stable capsid. However, this has not been shown, and other mechanisms for release of the DNA from the capsid, including capsid disassembly, are also possible.

## CONCLUSIONS

The processes of cell infection by parvoviruses show many features seen for other viruses that replicate in the nucleus. It is clear that the small and stable parvovirus particles must bind specifically to their receptors, and that subtle changes in the capsids likely occur during the endosomal, cytoplasmic, and nuclear trafficking steps involved in infection. The high particle to infectious unit ratio of these viruses seen in tissue culture cells indicates that the process is inefficient, but that it can differ between different types of cells from the same host, and the infection of the natural cell targets in animals may be more efficient. Our ability to detect small changes in the virus and to follow small numbers of particles into the cell should allow us to define more specifically the intracellular trafficking and other events involved in cell infection.

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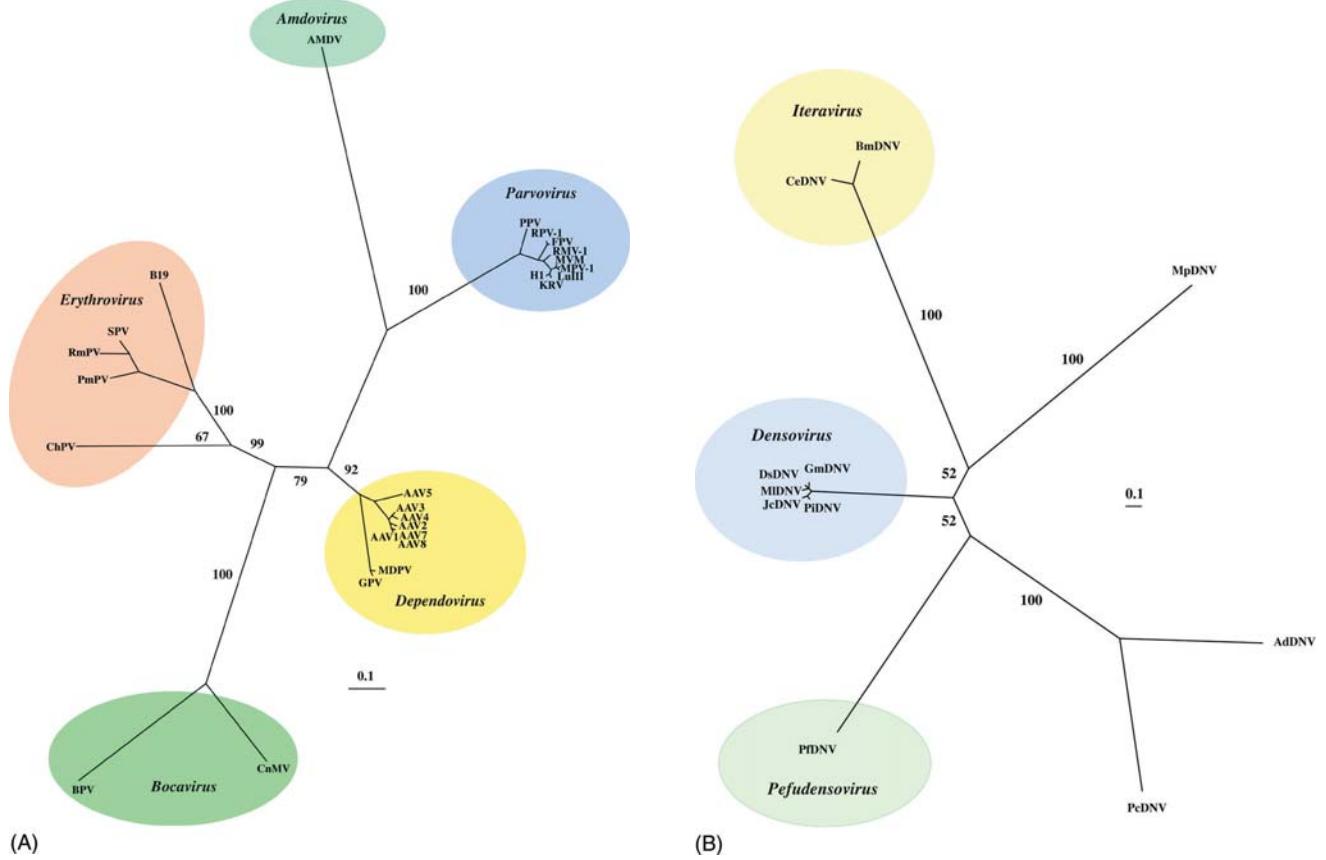
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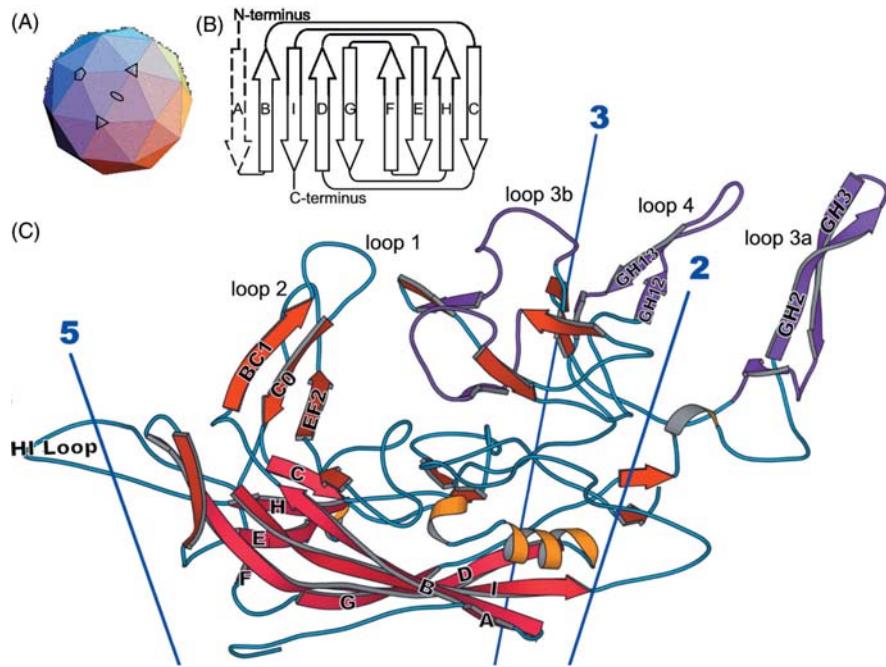
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# Colour plates

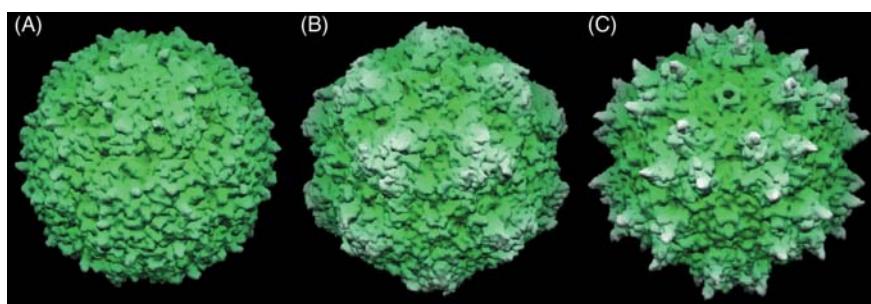
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**Plate 1.1** Panel A: Phylogenetic relationship between the non-structural genes of members of the subfamily Parvovirinae. Panel B: Phylogenetic relationship between the non-structural genes of members of the Densovirus and Iteravirus genera of the subfamily Densovirinae. The genus Brevidensovirus is not shown because of its great evolutionary distance from the other Densovirinae members. Phylogenetic trees were constructed, by Z. Zadori and P. Tijssen, using the programs included in the PHYLIP package (Felsenstein, 2004). For bootstrap analysis, the SEQBOOT program was run first. For distance matrix analysis, the aligned sequences were processed first with the PROTDIST (using Dayhoff's PAM 001 scoring matrix) and then with the FITCH program (global rearrangements). The most probable tree was calculated with the CONSENSE program, and the resulting trees visualized using TREEVIEW. (Redrawn from original trees kindly provided by Z. Zadori and P. Tijssen).

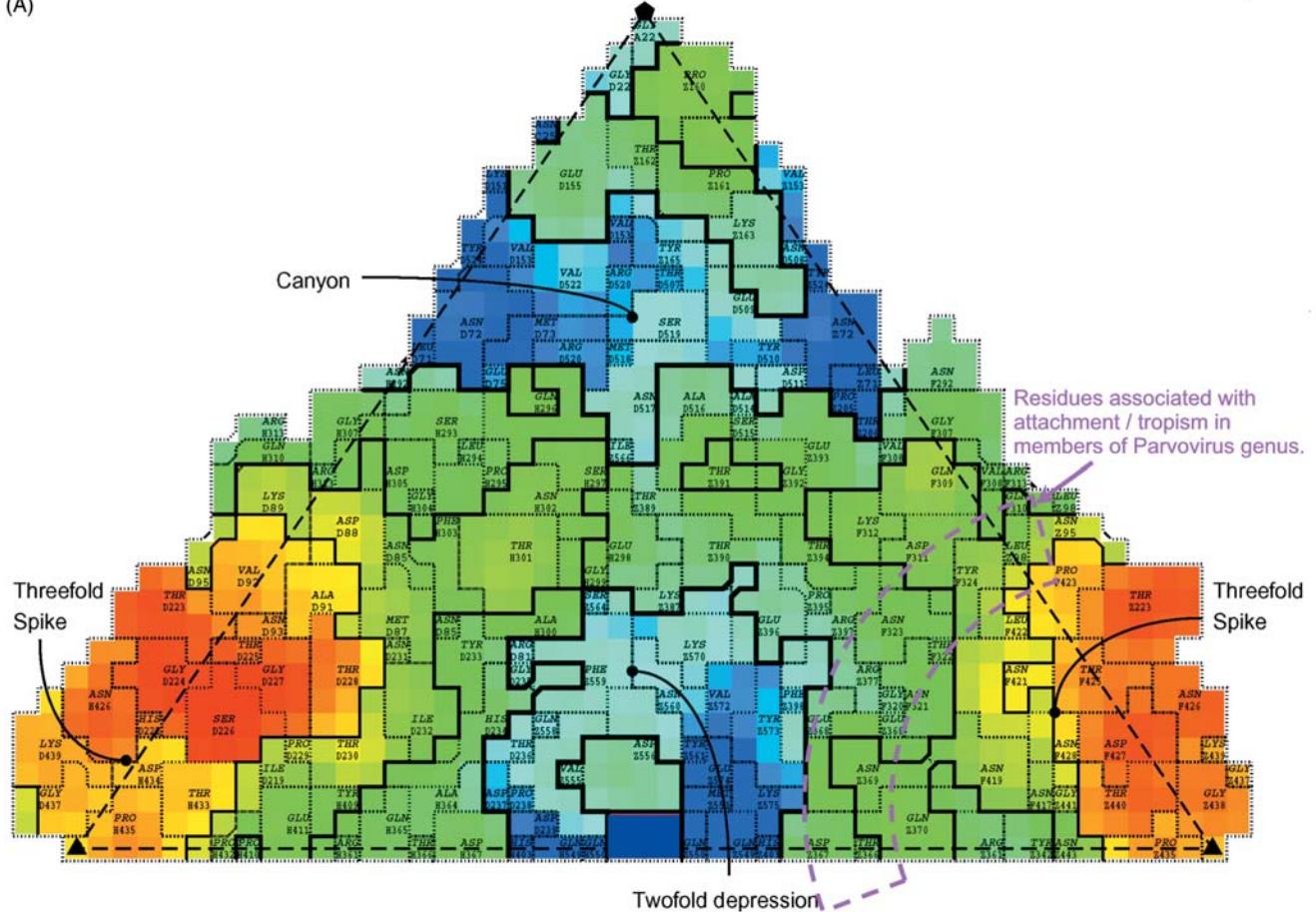


**Plate 9.1** The architecture of a parvovirus. (A) Parvoviruses are  $T = 1$  icosahedral viruses, meaning that their near spherical capsid is comprised of 60 copies of the capsid protein. The surface can be divided into an icosadeltahedron of 60 equal triangles, each designating one protein. Of course the capsid protein is not triangular, so the surface triangle is comprised of a set of unique atoms from each of several neighboring subunits that combined would complete one triangle. The icosadeltahedron has vertices where either 5-fold or 3-fold axes pass towards the center of the virus, examples shown as a pentagon and triangles respectively. Midway between each neighboring pair of 3-fold axes passes a 2-fold (ellipse). Successive application of the rotational symmetries can generate an entire capsid from a single subunit. (B) The topology of each subunit is a viral jellyroll  $\beta$ -barrel. The connectivity of the strands (arrows) is indicated in a schematic representation that is not supposed to indicate the length or structure of the intervening loops. Strand A is dashed, because it is present in parvo- and a number of other viruses, but not all. One can imagine the nine strands as consisting of two antiparallel sheets of five and four strands respectively. There is a discontinuity in the hydrogen-bonding between strands F and G as the second sheet is folded over the first. The schematic is simplified by omitting embellishing secondary structures from the loops. (C) The 3D fold of the AAV-2 subunit. The  $\beta$ -sheets that are highly conserved among parvoviruses are shown in fuschia at the bottom with strands labeled as in (B). The loops between the strands are very different from the loops of other viruses, and even differ significantly between the different genera of parvovirus. The loops are particularly convoluted in parvoviruses with the GH loop accounting for  $\sim 250$  residues. Most of these loops have extended chain structure, but there are additional secondary structural elements (red, orange, and purple), but these are not fully conserved, even among parvoviruses, and account for their unique properties in terms of antigenicity and association with cellular receptors. Loop secondary structures are labeled by the two letters of the bounding barrel strands and a number that increases towards the C-terminus. Other secondary structures are labeled with a single letter/number if they are nearly contiguous with a barrel strand. Thus CO immediately precedes strand C. ([C] is reproduced with permission from Xie et al., 2002, copyright of National Academy of Sciences, USA, 2002.)



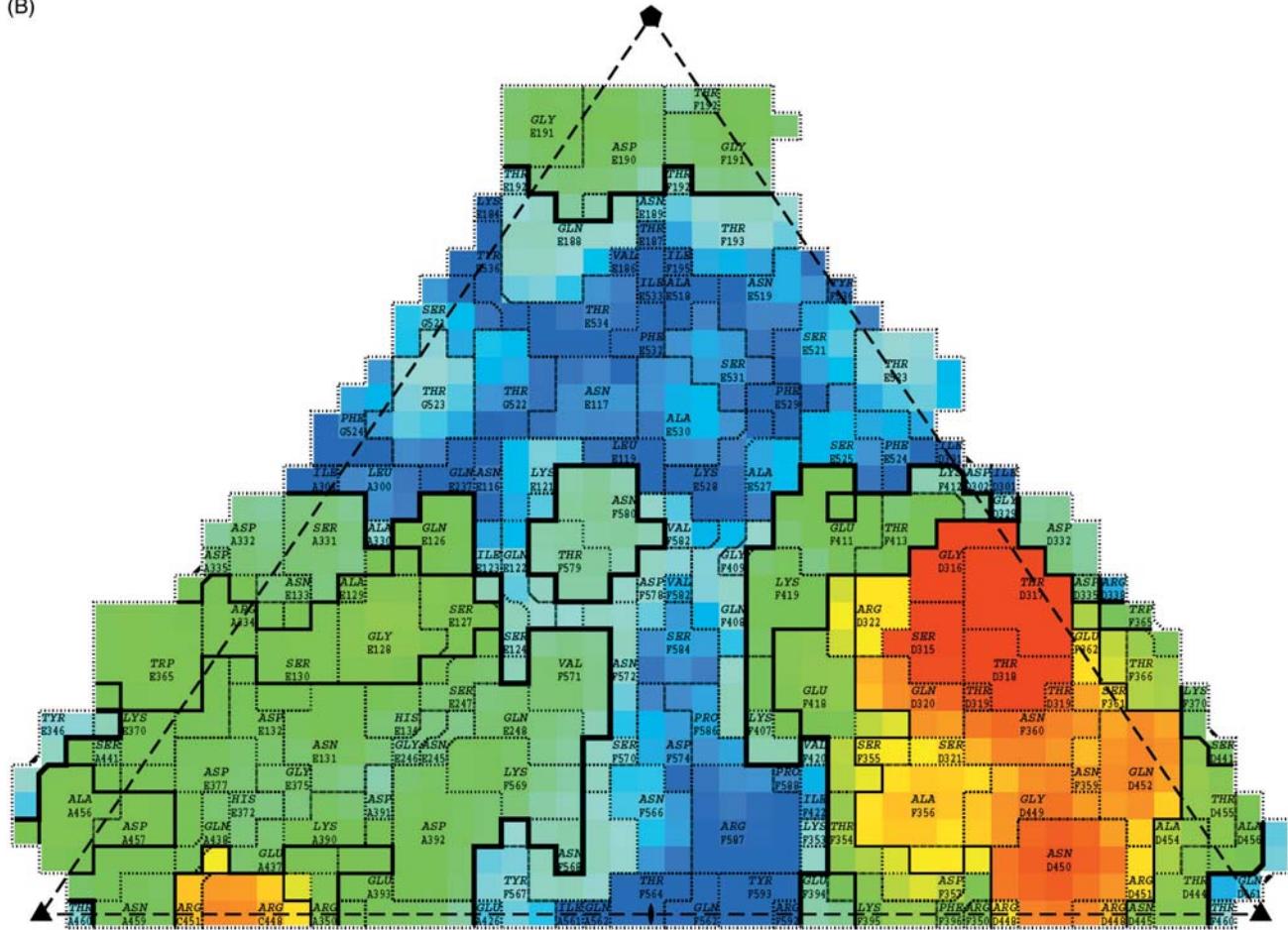
**Plate 9.3** Surface topology of parvoviruses (A) Densovirus; (B) Parvovirus; (C) Dependovirus and likely Erythrovirus. GRASP (Nicholls, 1992) surface representations calculated from the atomic structures are shown to scale for representatives of parvoviral genera (A) (insect) densovirus GmDNV (Simpson et al., 1998); CPV (Tsao et al., 1991) as a representative of genus Parvovirus – MVM (Agbandje-McKenna et al., 1998) and others would be very similar; and AAV-2 (Xie et al., 2002). B19, the Erythrovirus of known structure (Kaufmann et al., 2004) has an unseen disordered surface loop whose absence would distort a surface representation, but, in all other respects, its structure is most homologous to AAV-2 (Kaufmann et al., 2004), so it likely shares similar surface topology. The view is down a 2-fold axis (like Plate 10.2) with 3-fold axes left and right of center, and a 5-fold above center.

(A)

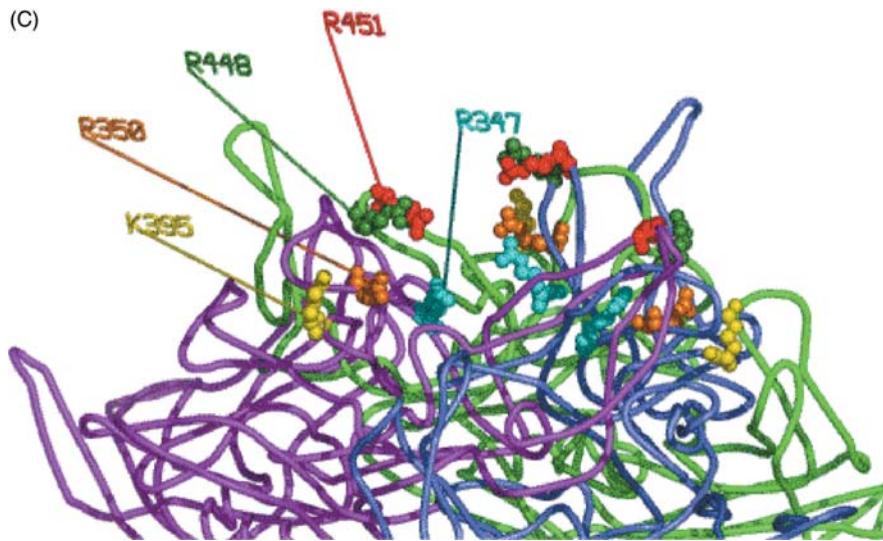


**Plate 9.4** Surface topologies receptor-attachment of (A) CPV (Tsao et al., 1991) and (B-D) AAV-2 (Xie et al., 2002). (A and B) show Roadmap schematics (Chapman, 1993) of one of the 60 icosahedral faces, bounded by the 5-fold (top) and two 3-folds (left and right), and viewed parallel to the 2-fold (bottom center). The solvent-accessible surface is colored like a topographical map with lower regions blue, elevated regions red. Surface residues are numbered with letter prefixes designating the subunit. (Different letters are used for CPV and AAV-2.) The region highlighted in (A) as associated with cell attachment and cell tropism represents a compilation from related parvoviruses aligned to the CPV surface. They include residues implicated in the cell tropism of MVM (Ball-Goodrich et al., 1991), PPV (Vasudevacharya and Compans, 1992), ADV (Bloom et al., 1988), and in the transferrin receptor-binding, cell specificity and hemagglutination of CPV and FPV (Parrish et al., 1988a; Parrish et al., 1988b; Barbis et al., 1992; Llamas-Saiz et al., 1996; Govindasamy et al., 2003; Hueffer et al., 2003). (C) Residues implicated by mutagenesis in the binding of AAV-2 to analogs of the heparan sulfate proteoglycan receptor and in cell entry (Kern et al., 2003; Opie et al., 2003) are shown (VP2 numbering) on a trimer from the AAV-2 structure (Xie et al., 2002) in which the backbones of subunits are in different colors. (D) The locations of these residues are mapped to a Roadmap surface of the region surrounding a 3-fold projected onto a plane. The region enclosing the implicated residues is shown with dashed blue lines, and lies between pairs of spikes, in the valley and on the side of one of the protrusions. These regions are repeated by the 3-fold symmetry, and one could imagine a heparan polysaccharide chain passing through one valley, over the 3-fold and out through another.

(B)



(C)



**Plate 9.4 (continued)**

(D)

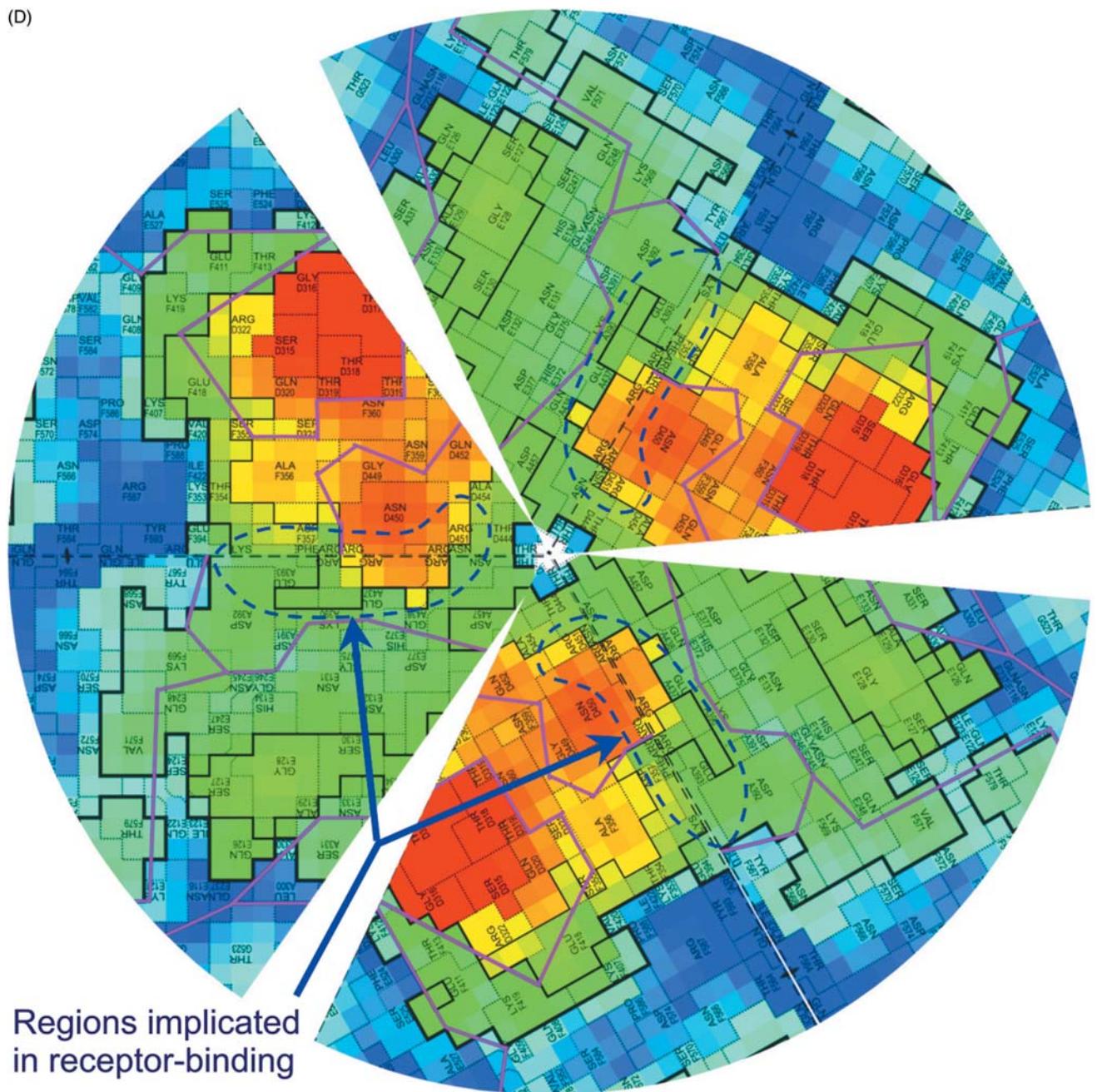
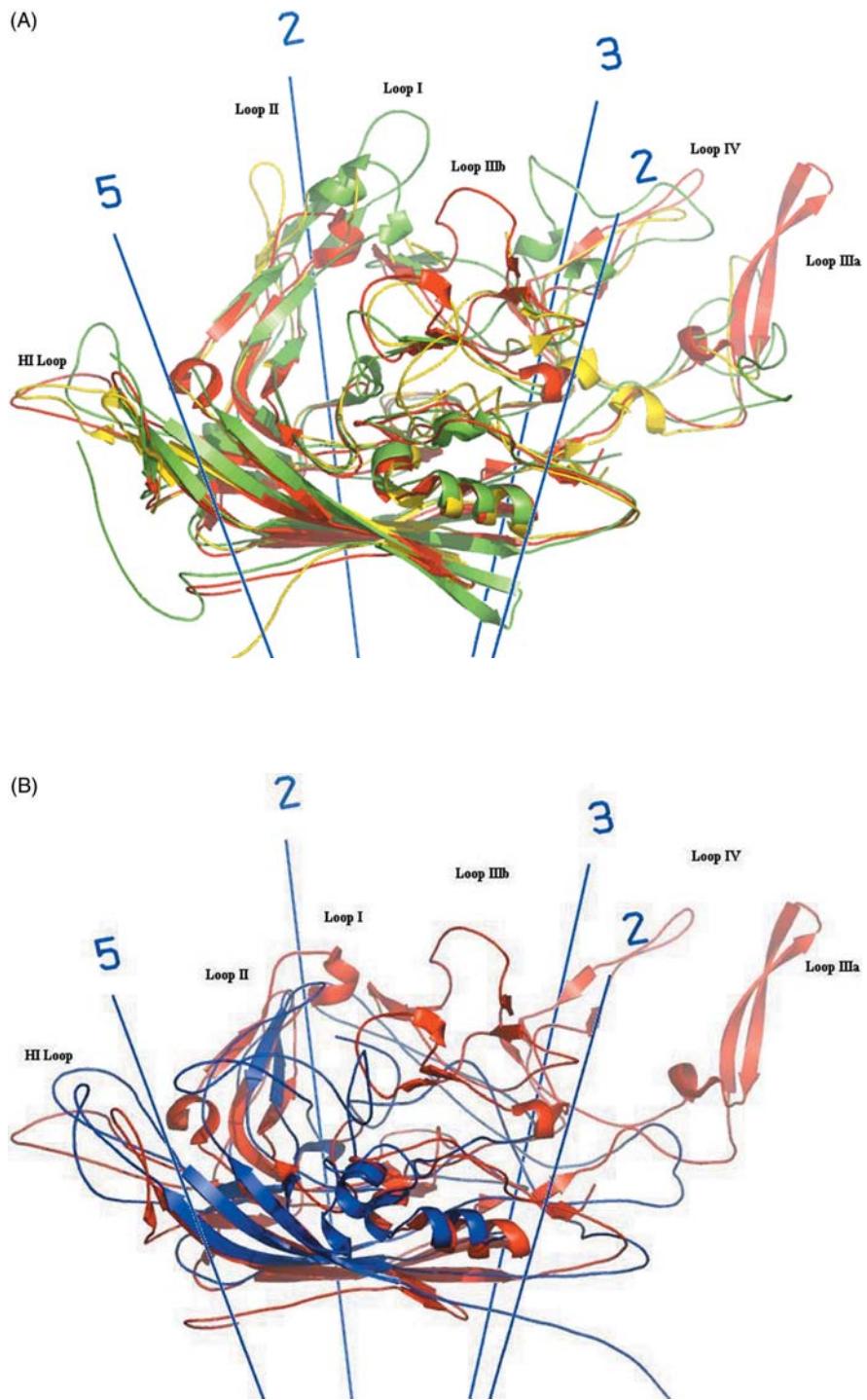
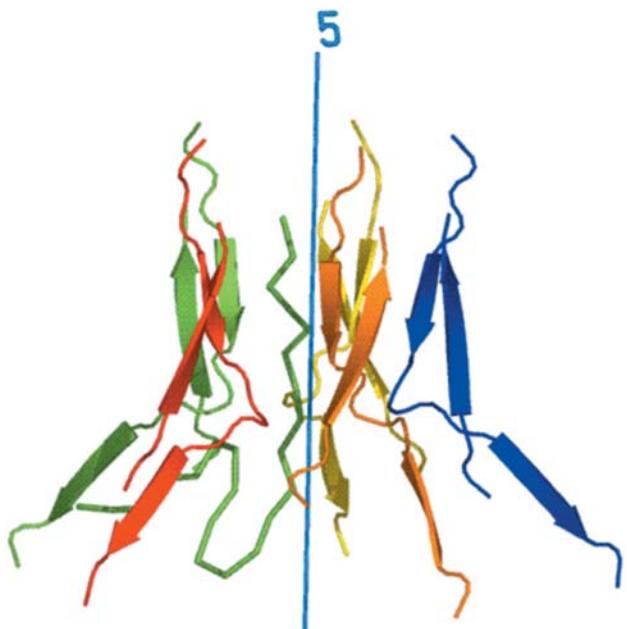


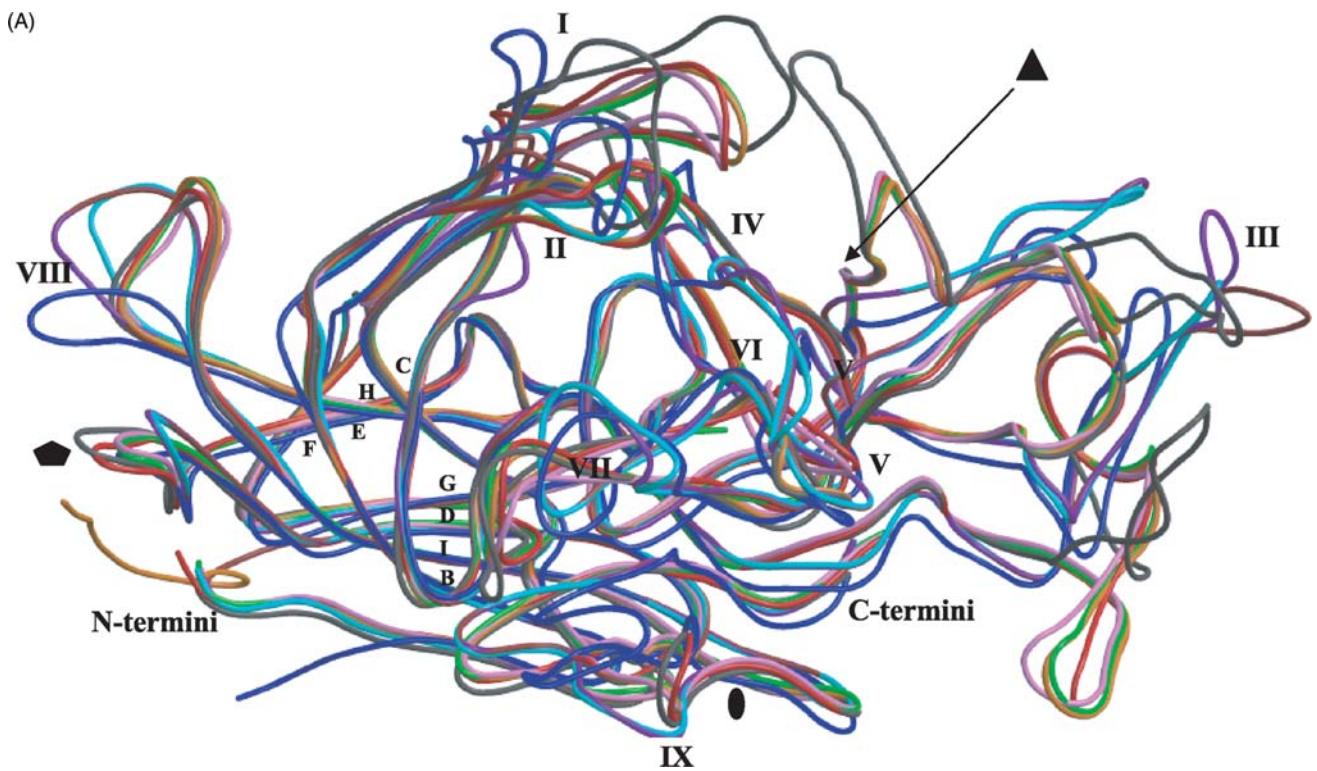
Plate 9.4 (continued)



**Plate 9.5** Superimposition of the subunit structures of: (A) CPV (green, Tsao et al., 1991), AAV-2 (red, Xie et al., 2002), and B19 (yellow, Kaufmann et al., 2004), and (B) DNV (blue, Simpson et al., 1998) and AAV-2 (red). Strands of the  $\beta$ -barrel superimpose well, as do the loops of AAV-2 and B19. The biggest differences are in loops 3 and 4, between CPV, DNV and a group including both AAV-2 and B19. DNV superimposes less well than the other parvoviruses (B).



**Plate 9.6** The 5-fold pore of CPV (Xie and Chapman, 1996). The  $\beta$ -ribbon of the DE loop is shown along with its symmetry equivalents (in different colors) surrounding the 5-fold. The tight turns of the ribbons on the external viral surface (top) are disordered and can not be modeled, but are visible in AAV-2. Up through the center, along the 5-fold, runs the glycine-rich sequence from one of the five neighboring subunits (green), so that up to one of five capsid protein N-termini can be on the external surface (top). Residues are numbered according to VP2, though it is not known whether it is VP1, 2 or 3 that is visualized crystallographically.



**Plate 10.1** Comparison of parvovirus structures. (A) Superimposition of coil representations of the VP2/VP3 monomers of AAV2 (brown), AAV4 (magenta), B19 (blue), CPV (orange), FPV (green), MVM (red), PPV (pink) (pdb accession Nos. 1LP3, AAV4 coordinates are yet to be deposited, 1S58, 2CAS, 1C8E, 1MVM, and 1K3V), AAV5 (cyan) and ADV (grey) (pseudo-atomic model built into cryo-EM densities; McKenna et al., 1999, Walters et al., 2004). The most variable surface regions on the capsid are labeled (I-IX) as discussed by Padron et al., 2005 and Govindasamy et al., 2005. The eight-strands of the core  $\beta$ -barrel are labeled  $\beta$ B- $\beta$ I. The approximate 2-fold (filled oval), 3-fold (filled triangle) and 5-fold (filled pentagon) axes are shown. This plate was generated using the program Bobscript (Esnouf, 1997). (B) Low resolution surface maps of AAV2, AAV4, AAV5, ADV, B19, CPV, FPV, MVM, and PPV, at 13 Å resolution, colored as in (A). Viruses in the top panel and bottom panel form group I and group III, respectively in Padron et al., 2005. The surface map images were generated as previously described (Belnap et al., 1999) from atomic/pseudo-atomic coordinates as referenced in (A). The black triangles on the CPV and AAV2 capsid surfaces depict a viral asymmetric unit bound by a 2-fold (2f), two 3-folds (3f) and a 5-fold (5f) axis.

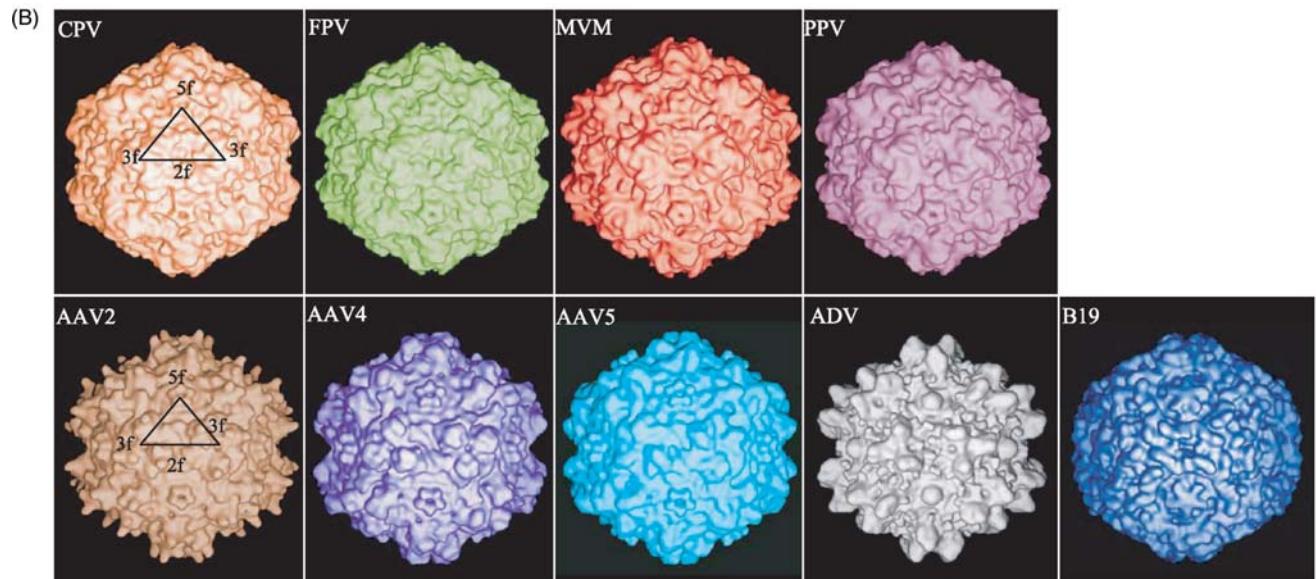


Plate 10.1 (continued)



Plate 10.2 Proposed receptor attachment sites on the AAV2 and CPV capsids. The coordinates were obtained as in Plate 10.1. (A) AAV2 VP3 trimer (monomers in brown, magenta and green) viewed down the icosahedral 3-fold axes (left-hand side) and rotated 90° (right-hand side). The C<sub>x</sub> positions of residues R484, R487, K532, R585, and R588 (labeled and in the blue oval) that are clustered on the AAV2 capsid from 3-fold symmetry-related monomers to form a basic patch required for heparin sulfate binding are shown as balls colored according to their monomers. The approximate icosahedral 2-, 3-, and 5-fold axes are shown as the filled oval, triangle, and pentagons, respectively. (B) CPV VP2 reference (in orange) bounded by its icosahedral 2-fold (magenta), 3-fold (dark green and light green) and 5-fold (cyan) related monomers. The residues that control CPV sialic acid binding (R377, E396, and R397) on the wall of the 2-fold axes are shown in yellow balls in all monomers. The residues implicated in Tfr binding (93, 300, and 323) are shown in balls colored according to their contributing monomers. The approximate icosahedral 2- (filled oval), 3- (filled triangle), and 5-fold (filled pentagon) axes are shown.

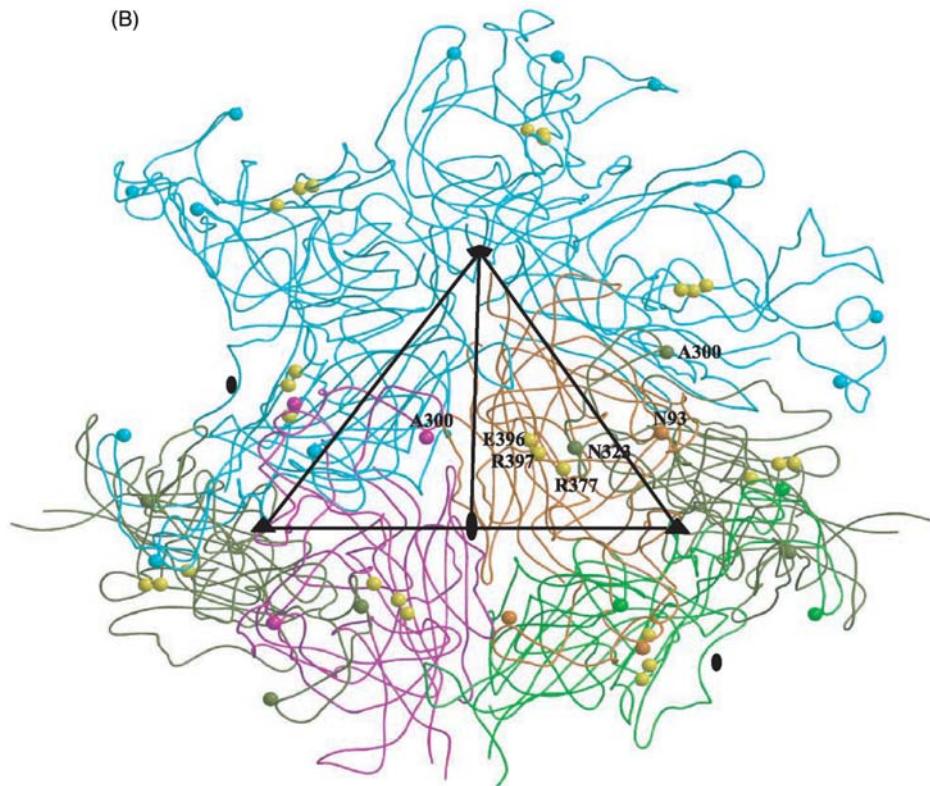


Plate 10.2 (continued)

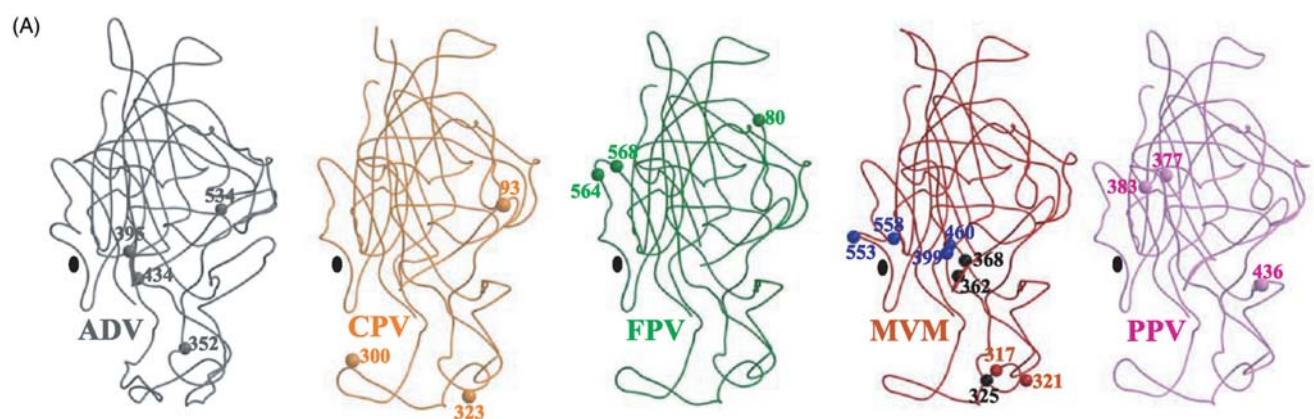
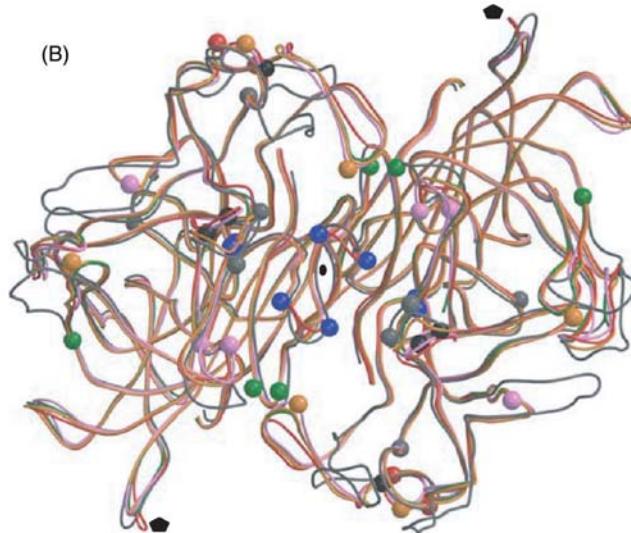


Plate 10.3 Tissue tropism and pathogenicity determinants for members of the parvovirus genus. (A) Coil representations of the VP2/VP3 monomers of ADV, CPV, FPV, MVM and PPV (generated from coordinates and colored as in plate 10.1A) viewed approximately down the icosahedral 2-fold axes. The C<sub>α</sub> positions of residues implicated in tropism and pathogenicity determination are shown as balls and colored accordingly to the monomers. In MVM, forward mutations conferring fibrotropism to MVM<sub>i</sub> are in blue. (B) and (C) Superimposition of the monomers in (A) viewed down the icosahedral 2-fold and 3-fold axes, respectively. The tropism and pathogenicity/virulence determinants are colored as in (A) and clustered mainly in and around the depression at the icosahedral 2-fold axes. The approximate icosahedral 2- (filled oval), 3- (filled triangle), and 5-fold (filled pentagon) are shown.

(B)



(C)

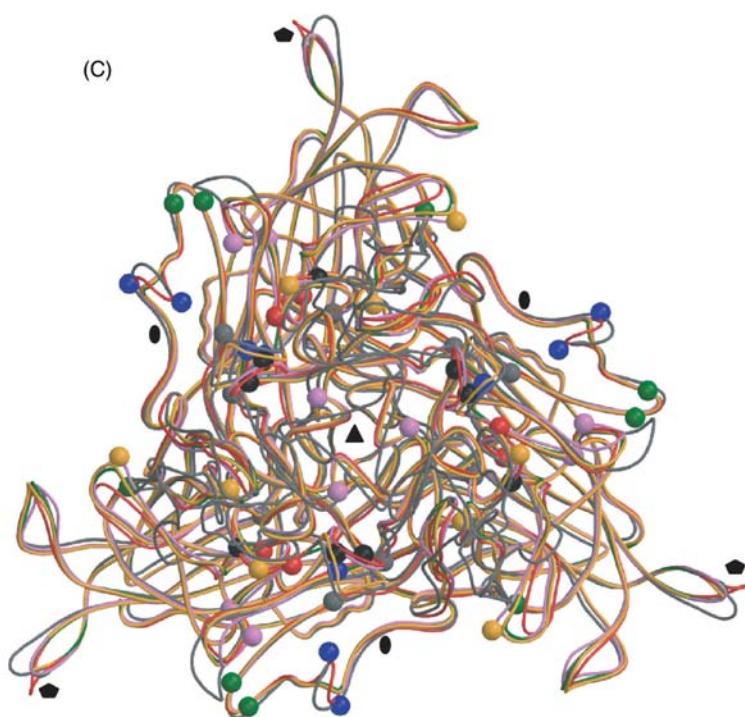
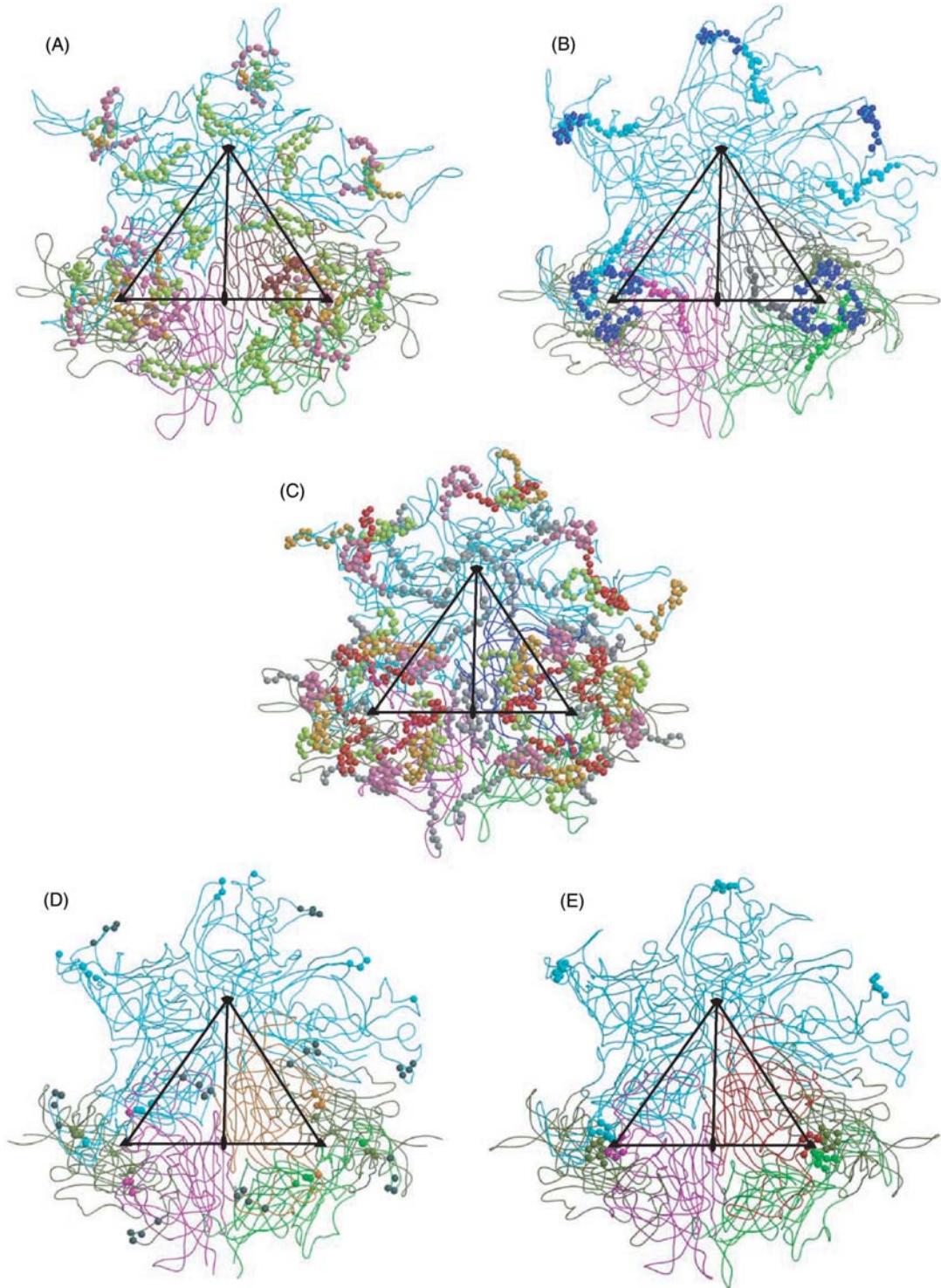
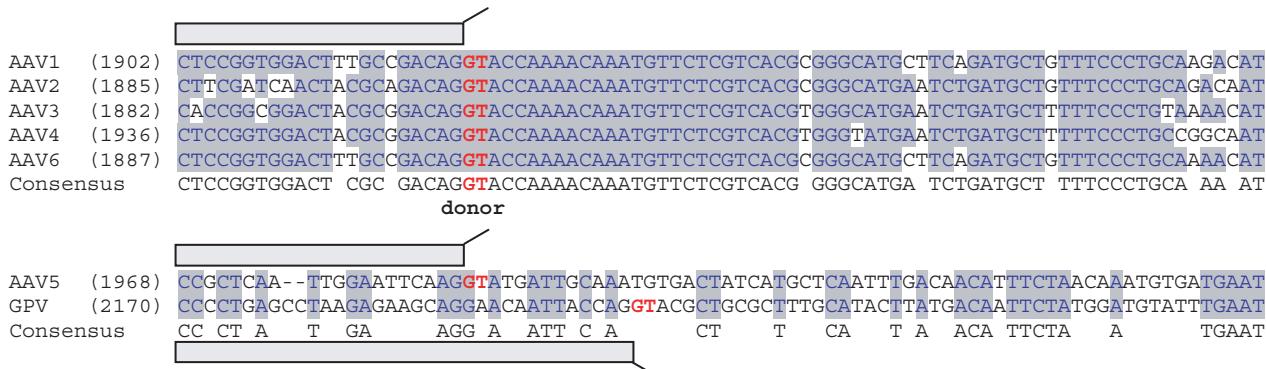


Plate 10.2 (continued)

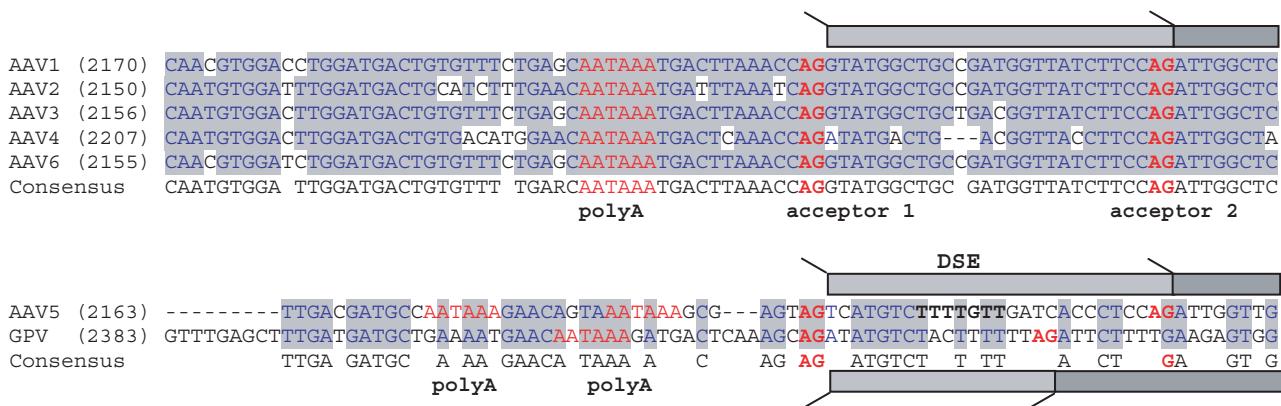


**Plate 10.4** Antigenic regions on parvovirus capsids. Antigenic regions on the AAV2 (A), ADV (B), B19 (C), CPV (D), and MVM (E) capsids are shown for the reference monomers (colored as in Plate 10.1) bounded by their icosahedral 2-fold (magenta), 3-fold (dark green and light green) and 5-fold (cyan) related monomers. For AAV2 (A), the  $C\alpha$  positions for the A20 epitope (VP1 numbering residues 272–281, 369–378, and 566–575) is shown in greenish yellow, the C37-B (residues 493–502 and 601–610) in pink and D3 (residues 474–483) in orange. The ADV (B) epitope at VP2 residues 428–446 is shown colored according to the monomers and the 487–501 epitope is colored in purple. The B19 (C) VP2 epitopes are colored as follows: 57–77 (pink); 253–272 (orange); 314–330 (part of 309–330) (red); 359–382 (yellowish green); 449–468 and 491–515 (grey). In CPV (D) antigenic epitope A (residues 93, 222, 224, and 426) is colored according to the monomers and epitope B (residues 299, 300, 302, and 305) is in grey. In MVM (E) escape mutant residues are shown in balls colored according to the monomers. The approximate icosahedral 2- (filled oval), 3- (filled triangle), and 5-fold (filled pentagon) axes are shown in A–E for a viral asymmetric unit.

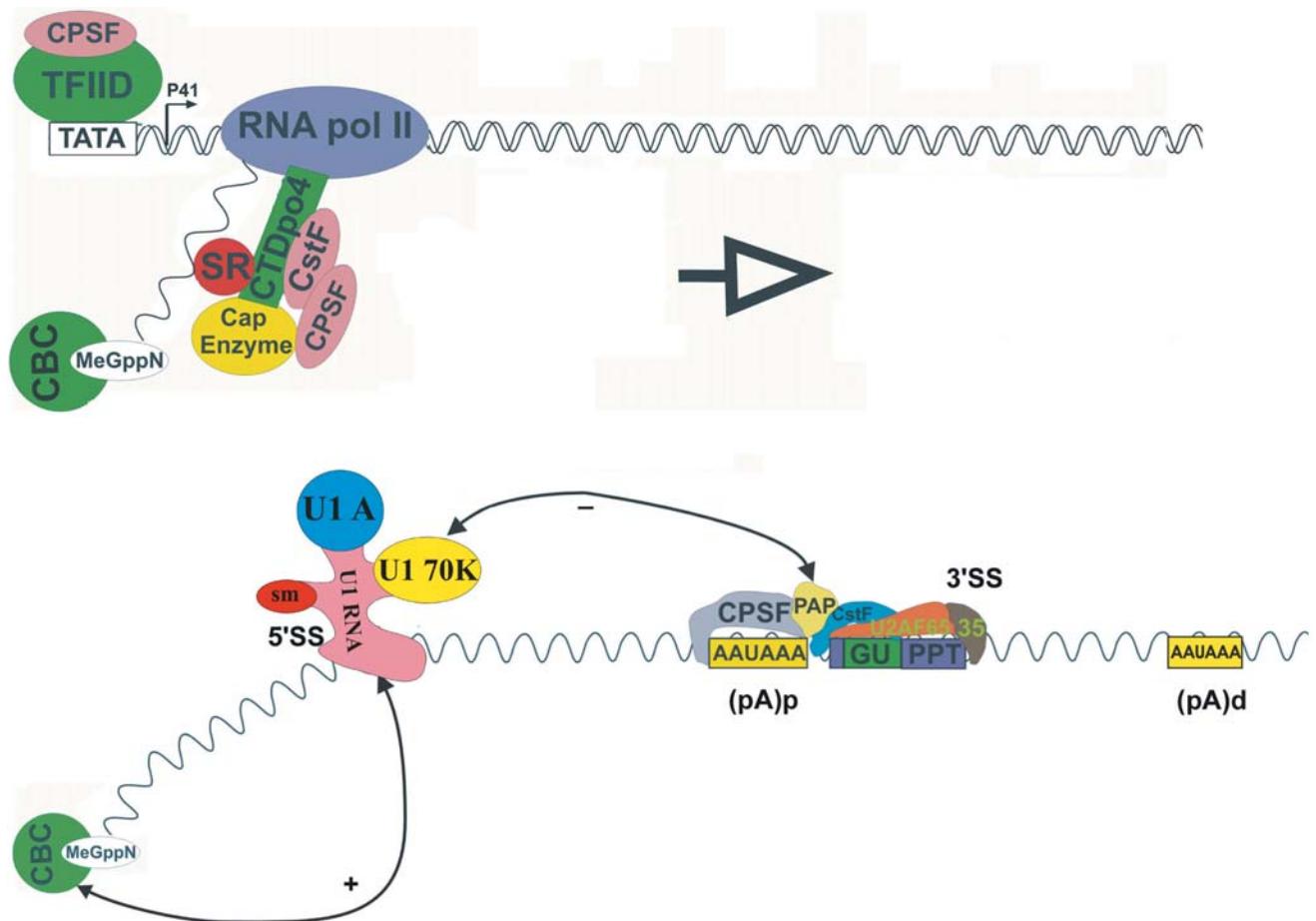
### AAV DONOR SITES:



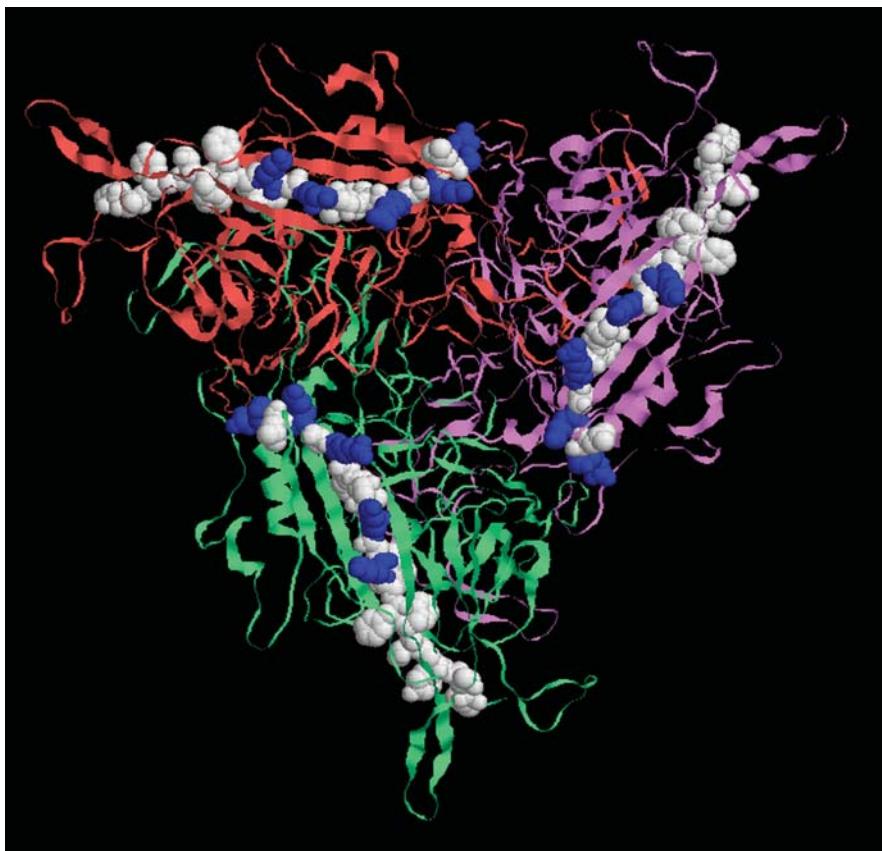
### AAV ACCEPTOR AND POLYADENYLATION SITES:



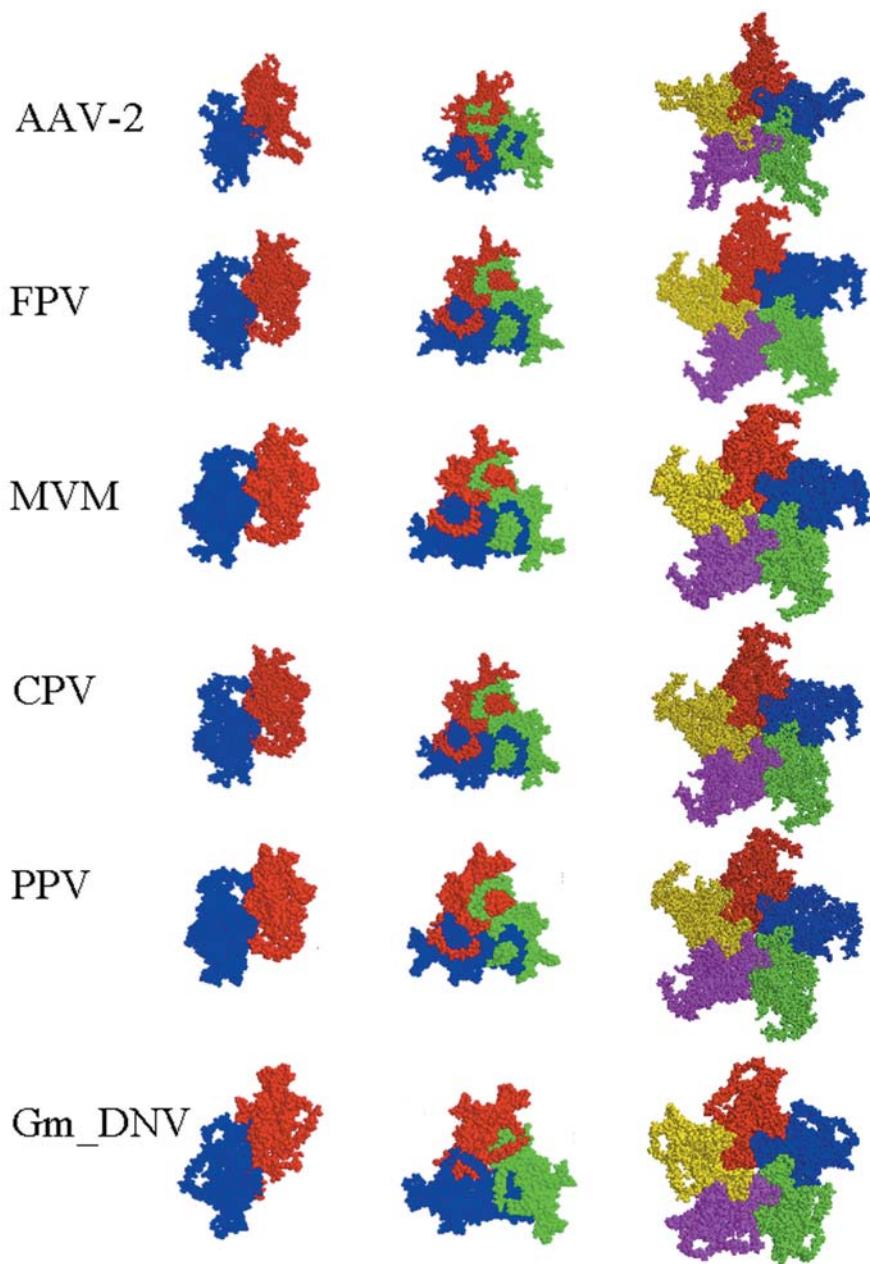
**Plate 18.6** Nucleotide sequence of the AAV and GPV 5' donor and 3' acceptor sites. The nucleotide sequence of the 5' splice donor region, and the 3' splice acceptor and polyadenylation site region are aligned for AAV1, AAV2, AAV3, AAV4, AAV6, and compared with AAV5 and GPV. A consensus sequence is also presented. The intron bordering nucleotides and potential polyadenylation signals are shown in red. The downstream element (DSE) required for AAV5 polyadenylation at (pA)<sub>n</sub> is also shown.



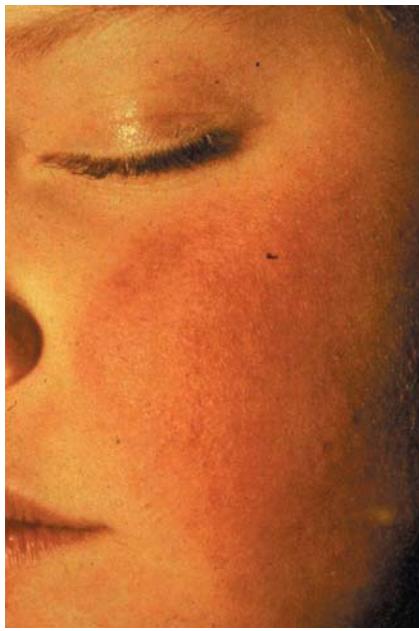
**Plate 18.8** A model proposing a possible explanation for the distance dependence of AAV5 polyadenylation at (pA)p. The top part of the diagram depicts elongating RNA polymerase, with the capping enzymes, polyadenylation factors CstF and CPSF, and SR proteins bound to the RNA pol II CTD. The polyadenylation factor CPSF is shown associated with TATA-bound TFIID, and the cap-binding complex (CBC) is shown associated with the cap structure (MeGppN) on the 5' end of nascent RNA. The bottom part of the diagram suggests how the U1 snRNP (and perhaps the U1 70 K or U1 A protein) may exert its negative effects on polyadenylation at (pA)p, by interacting with the polyadenylation factors associated with the AAUAAA. Also shown is the binding of the splicing factors U2AF(65 + 35) with the adjacent 3' splice site. Stabilization of U1 snRNP to the intron 5' splice site, which facilitates splicing and simultaneously inhibition of (pA)p may occur through interaction with the cap binding complex associated with the 5' cap site as shown, and may be more effective in this regard when the RNA is initiated closer to the donor site (i.e. RNA generated from the P41 promoter), than when the RNA is initiated at a distance (i.e. RNA generated from the P7 promoter). See text for further explanation.



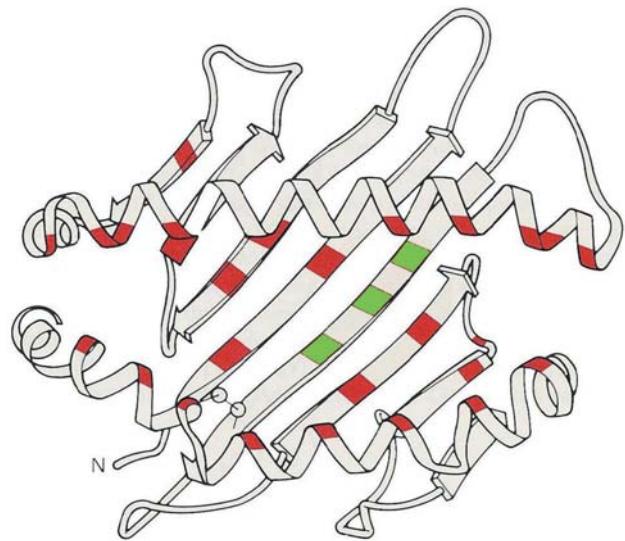
**Plate 20.3** Structure of the NLM of MVM displayed in a VP2 trimer. The plate shows a trimer of VP subunits of the MVMi capsid viewed down an icosahedral three-fold axis from the interior of the particle. The  $\beta$ -strand I containing the mapped nuclear localization motif (NLM) is shown as space-filling model colored white, with some of the basic residues important for transport in blue, and the rest of the protein in ribbon showing the  $\beta$ -barrel fold. Each subunit in the trimer has been drawn in a different color. The plate was generated with the program RasMol (Sayle and Milner-White, 1995) from available coordinates (PDB, Accession No. 1MVM).



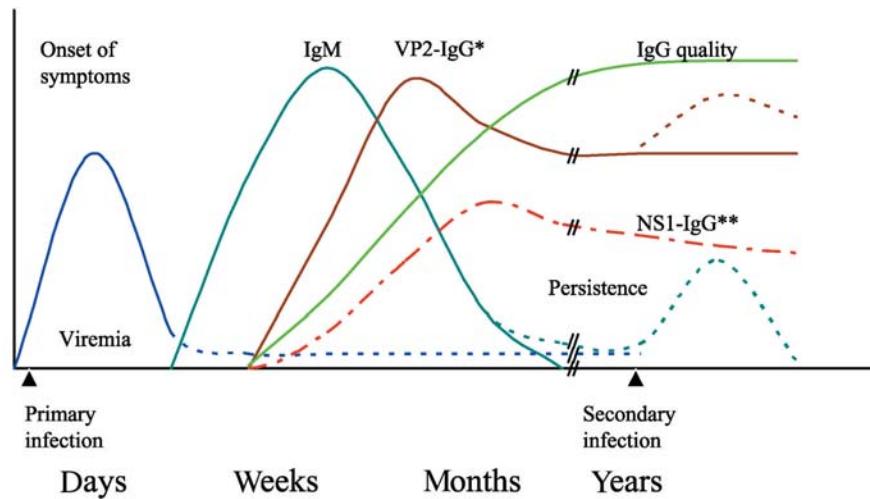
**Plate 21.1** *Visualization of parvovirus capsid protein interactions.* Capsid protein subunits (indicated by different colors) of adeno-associated virus type 2 (AAV2), feline parvovirus (FPV), minute virus of mice (MVM), canine parvovirus (CPV), porcine parvovirus (PPV) and *Galleria mellveolla* densovirus (Gm\_DNV) are viewed from outside at the 2-fold (first row), 3-fold (second row) and 5-fold (third row) symmetry axes. Note intertwining interactions at the 3-fold and 5-fold axes, which are not present at the 2-fold axes. Quantification of the interacting surface areas is shown in Table 21.1. (The images were generated and kindly provided by W.von der Lieth.)



**Plate 22.1** The rash of erythema infectiosum (from Susanne Modrow).



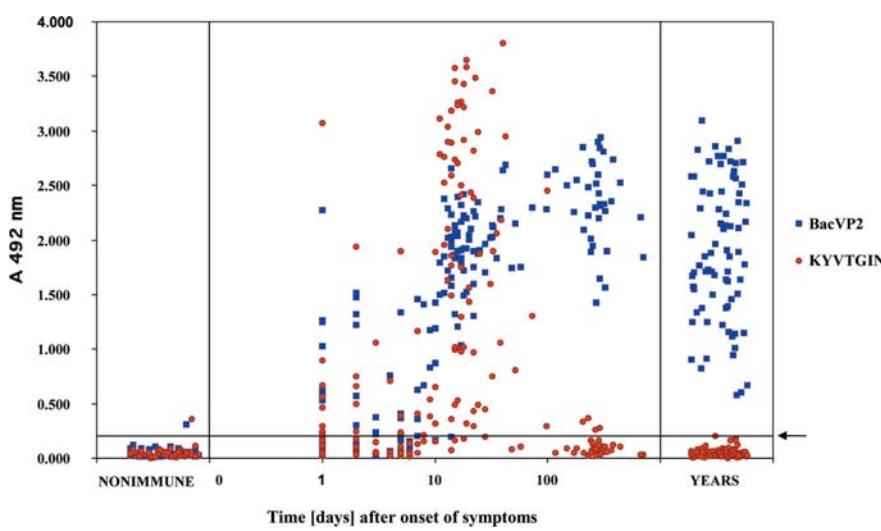
**Plate 22.5** Predicted location of the polymorphic amino acid residues of the MHC class II molecules mapped onto the outline structure of a class I molecule. Residues that may be important in parvovirus B19 infection are coloured green (Adapted from Brown et al., 1988, with permission.)



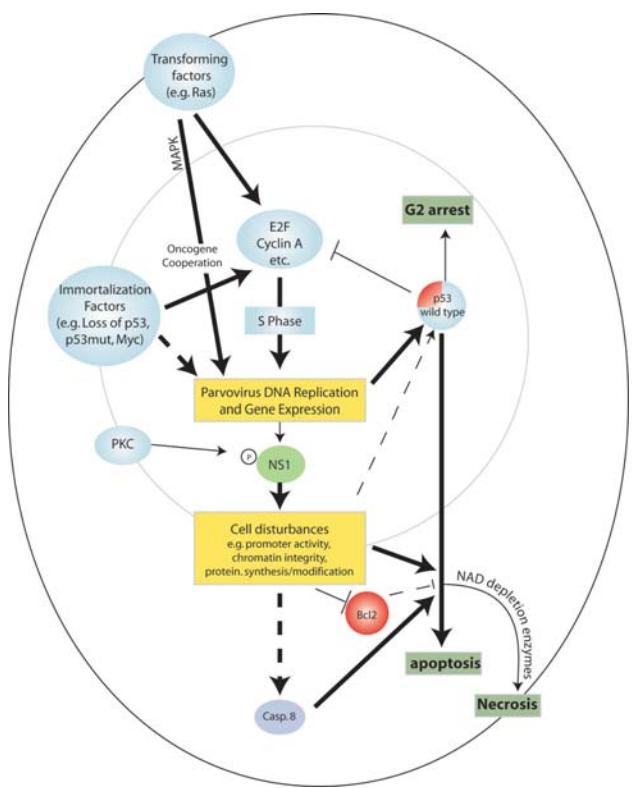
\* Conformational-VP2-IgG level

\*\* Prevalence of NS1-IgG

**Plate 24.1** B-cell response in erythrovirus infection.

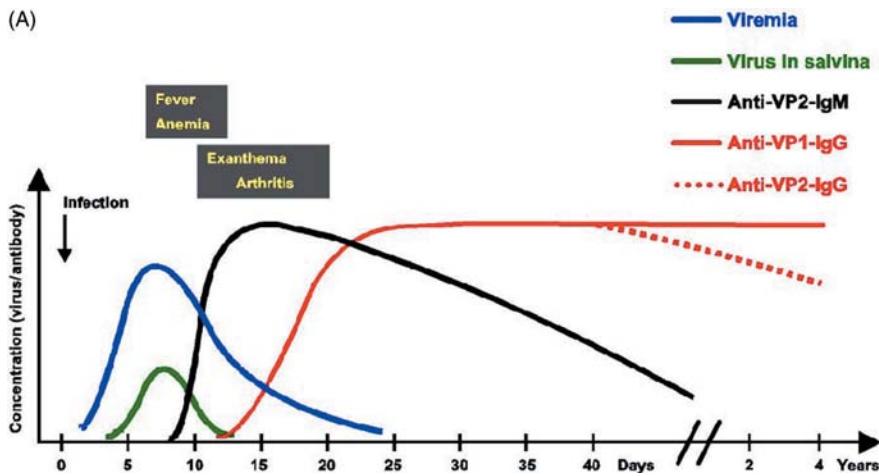


**Plate 24.2** IgG binding to VP2 capsid versus KYVTGIN epitope before and after B19 infection.

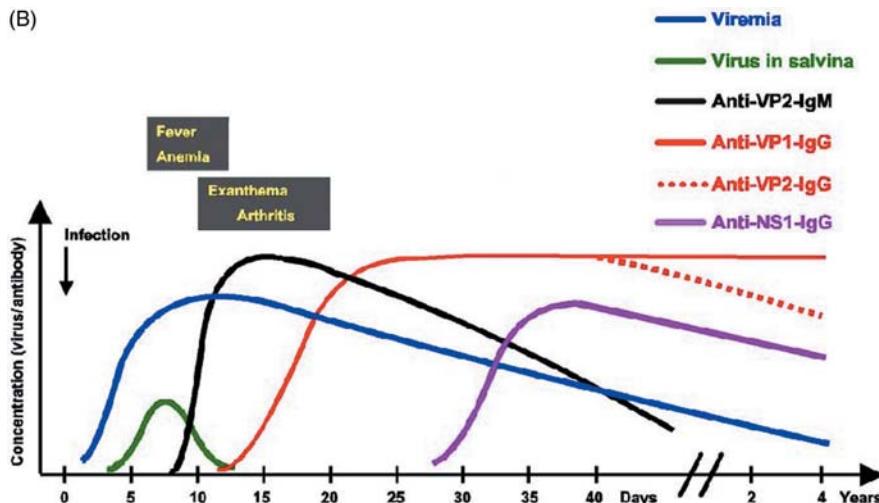


**Plate 25.1** Known positive (blue) or negative (red) cellular regulators of parvovirus DNA replication, gene expression, and cytopathic effects. The parvoviral NS1 protein is regulated through phosphorylation (*P*) and is essential for parvovirus-induced cell killing, yet its mode(s) of action remain(s) poorly understood. MAPK, mitogen activated protein kinase signaling pathway. →, stimulation; —, inhibition; broken line, hypothetical reaction.

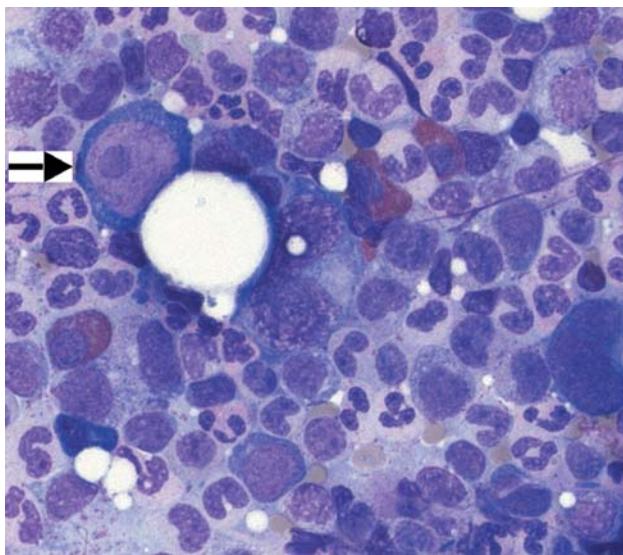
(A)



(B)



**Plate 27.3** (A) Serological parameters found in acute and past parvovirus B19 infections associated with virus elimination from the peripheral blood. (B) Serological parameters found in persistent parvovirus B19 infections.



**Plate 27.4** Bone marrow smear of a renal transplant patient with pure red cell aplasia (PRCA) from persistent parvovirus B19 infection, genotype 2. The bone marrow is depleted of all erythroid elements except for a few giant pro-erythroblasts with intranuclear inclusions (indicated by the arrow). The granulopoiesis is not affected and the morphology of megakaryocytes remains unchanged (kindly provided by L. Liefeld, Charité, Humboldt University, Berlin Germany).



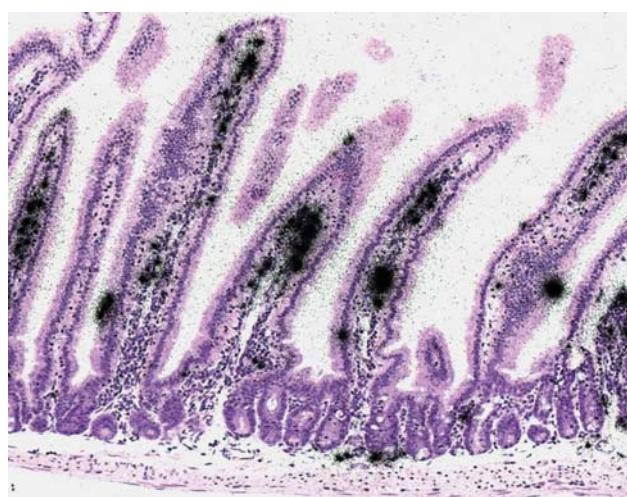
**Plate 27.7** Swelling of the knee joint in a patient with acute B19 infection and arthritis (kindly provided by Dr H.W. Lehmann, Clinic for Pediatrics, University of Giessen).



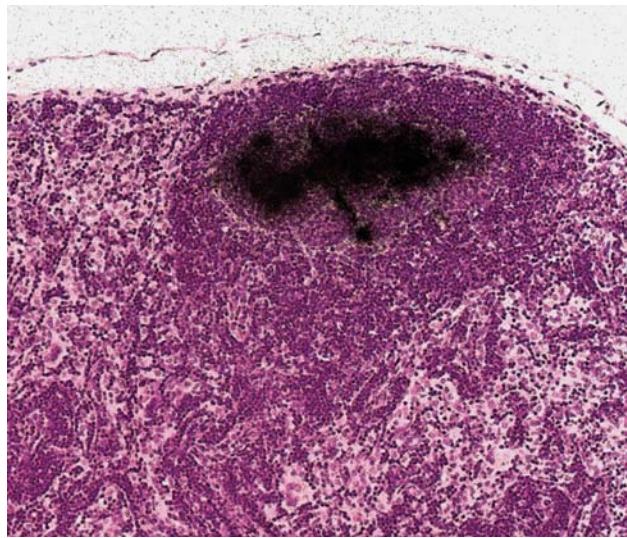
**Plate 27.8** Papular purpuric 'gloves and socks' syndrome (PPGSS) associated with acute parvovirus B19 infection (kindly provided by Dr Földer-Holst, Clinic for Dermatology, Venerology und Allergology, Universitätsklinik Schleswig-Holstein, Campus Kiel).



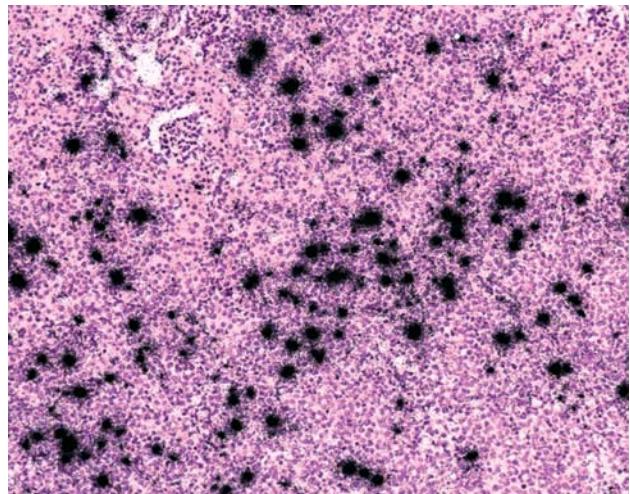
**Plate 27.6** (A, C) Facial rash of two different children acutely infected with parvovirus B19 as symptoms associated with erythema infectiosum. (B) Rash symptoms with an erythematous maculopapular pattern in the form of ringlets in the arm and shoulders of patient A with acute parvovirus B19 infection.



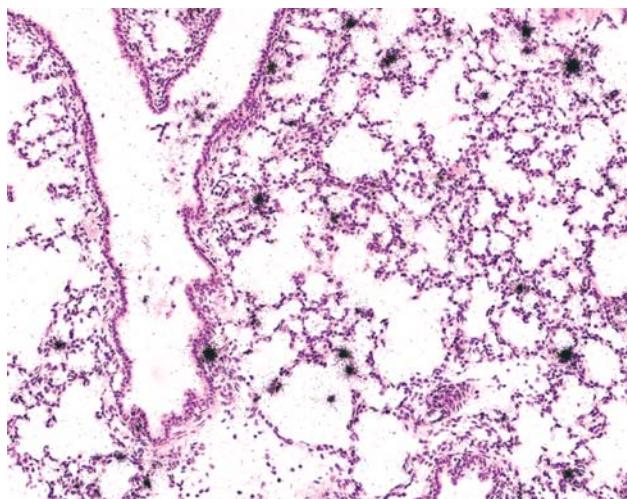
**Plate 28.1** Small intestine was collected from an adult mouse PID 7 after oronasal inoculation with MPV and analyzed by *in situ* hybridization with a random-primed  $^{32}\text{P}$  probe. Intestinal epithelium contains several positive cells (black cells), but most positive cells are in the lamina propria.



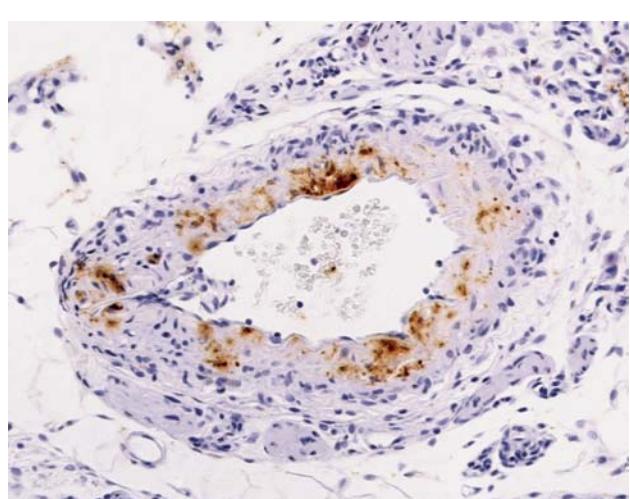
**Plate 28.2** Mesenteric lymph node was collected from an adult mouse 9 weeks after oronasal inoculation with MPV and analyzed by *in situ* hybridization with a random-primed  $^{32}\text{P}$  probe. Signal is localized to a germinal center.



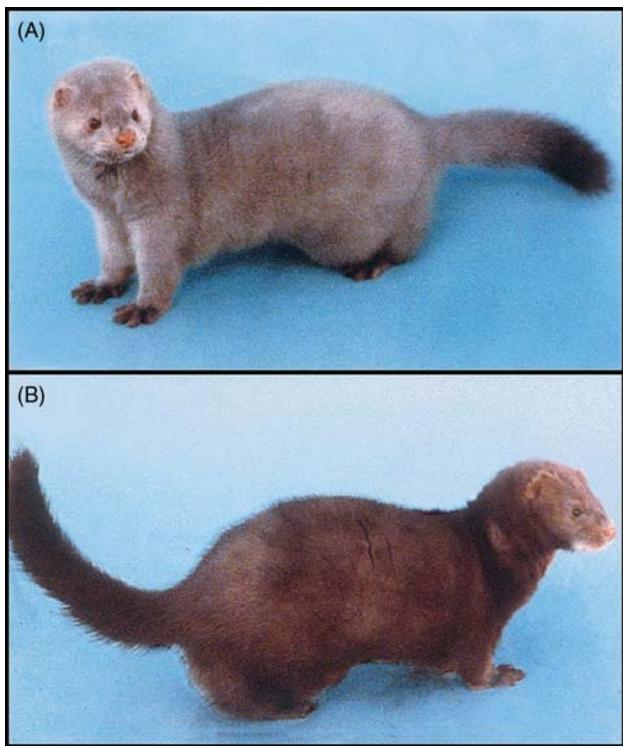
**Plate 28.4** At PID 8 after oronasal inoculation of RV in 6-day-old euthymic rats, kidney was collected and analyzed by *in situ* hybridization with a  $^{35}\text{S}$ -labeled riboprobe to detect viral genomic and replicative DNA. Tubular epithelium in the renal cortex contains numerous positive cells (black cells).



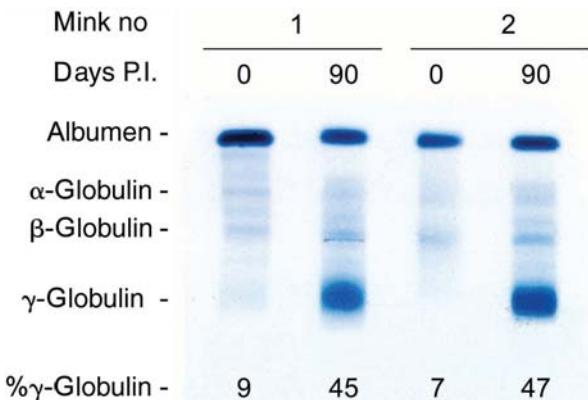
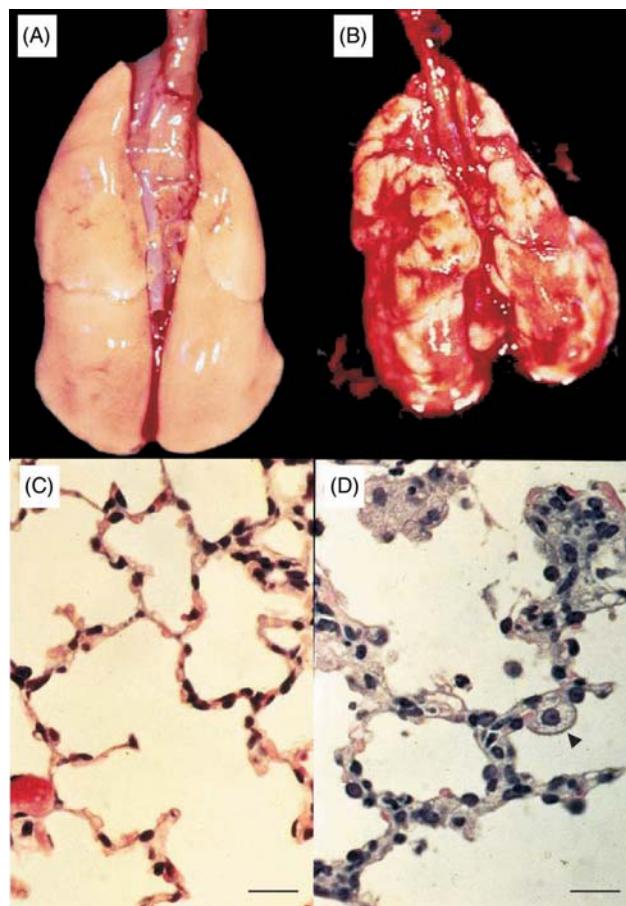
**Plate 28.3** At PID 8 after oronasal inoculation of RV in 6-day-old euthymic rats, lung was collected and analyzed by *in situ* hybridization with a  $^{35}\text{S}$ -labeled riboprobe to detect viral genomic and replicative DNA. Positive cells (black cells) are visualized among many alveolar septae.



**Plate 28.5** *In situ* hybridization, with a random-primed biotinylated probe, of a branch of a mesenteric artery collected at PID 28 after oronasal RV inoculation of 6-day-old athymic rats. Signal (brown nuclei and cells) is seen primarily in smooth muscle cells of the arterial wall.

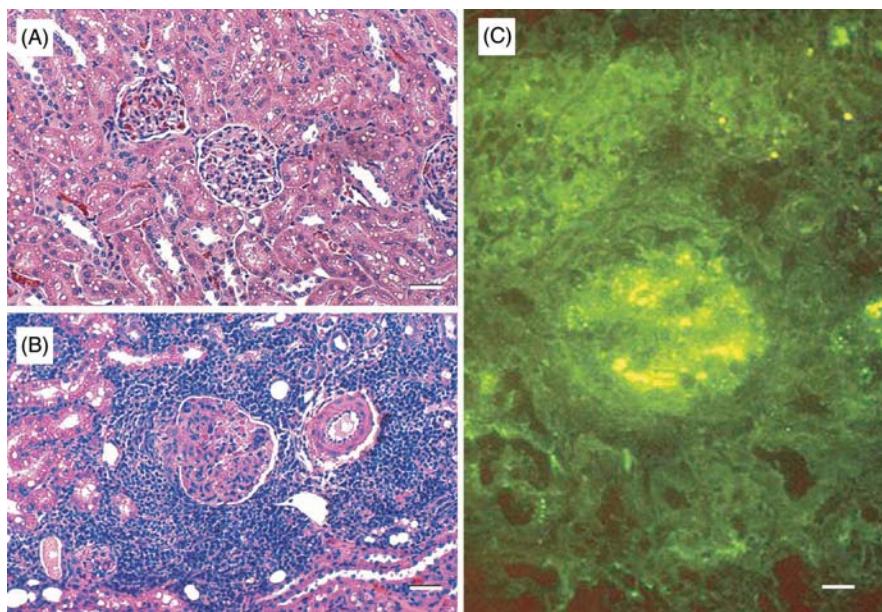


**Plate 32.1** Adult mink. (A) A sapphire mink of the Aleutian genotype. (B) A pastel mink of the non-Aleutian genotype. Both genotypes of mink are vulnerable to infection with AMDV, but the pathogenesis of AMDV in Aleutian mink is the most severe.

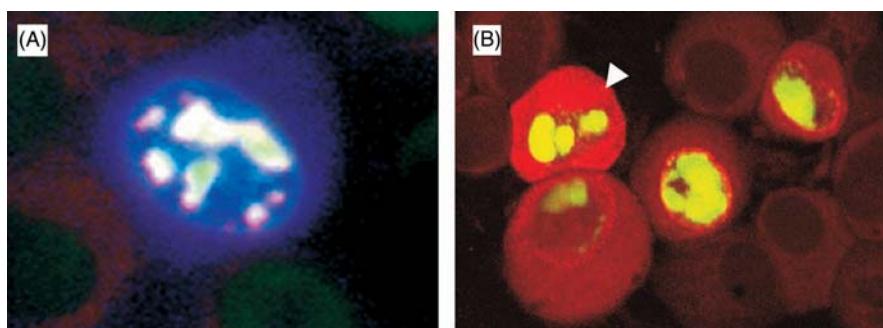


**Plate 32.4** Hypergammaglobulinemia induced by infection with ADV. The dramatic increase in gamma globulins is shown as a proportion of total immunoglobulin for two mink prior to infection with ADV at 0 days and 90 days post infection.

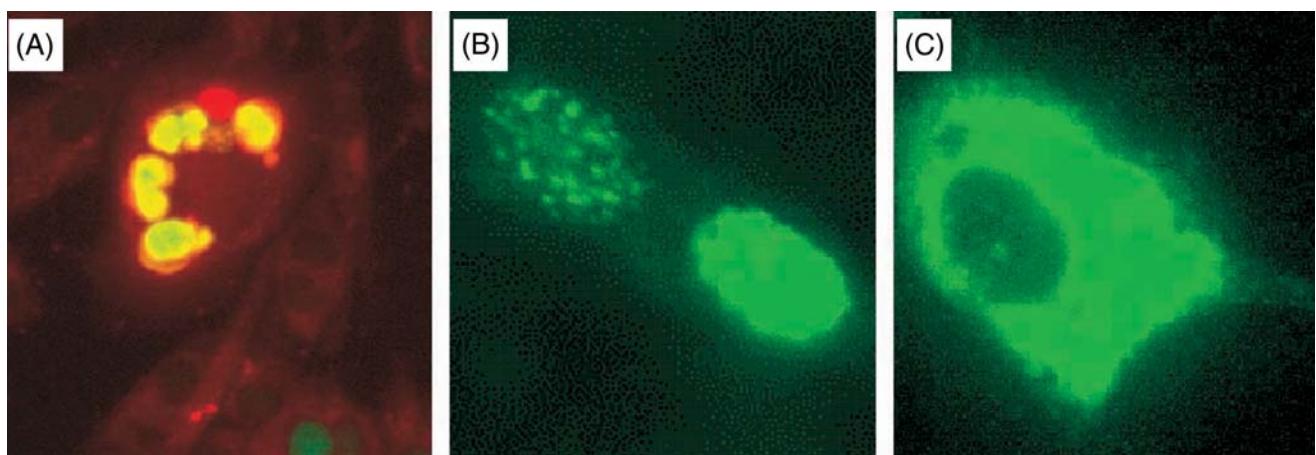
**Plate 32.3** Gross and histological pathology of mink kit lung following AMDV infection. (A) Normal lung; (B) lung from AMDV-infected Aleutian mink kit demonstrating extensive haemorrhaging; (C) histological section depicting the alveolus from an uninfected mink kit; (D) histological section of the alveolus from an AMDV-infected kit demonstrating an interstitial infiltrate, interstitial edema and hypertrophy of the alveolar type II pneumocytes (arrowhead). Bars represent 50  $\mu$ m.



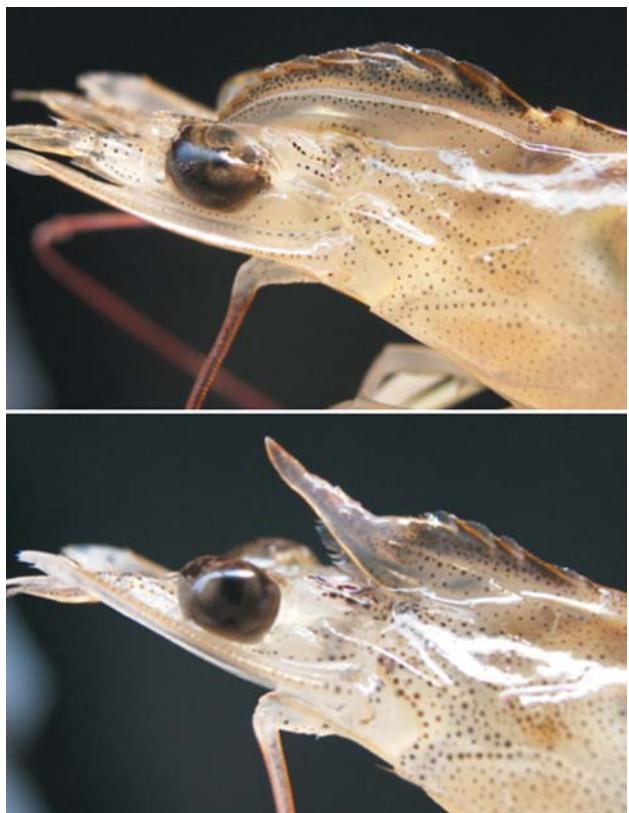
**Plate 32.5** *Histological changes in the kidney of AMDV-infected adult Aleutian mink. (A) Normal kidney. Bar represents 70 µm; (B) kidney from an ADV-infected mink demonstrating the extensive infiltration of plasma cells and lymphocytes resulting in a severe interstitial nephritis. Marked glomerular changes are also evident. Bar represents 17 µm; (C) immunofluorescence showing the extensive deposition of mink immunoglobulin in the glomerulus of an infected Aleutian mink. Bar represents 50 µm.*



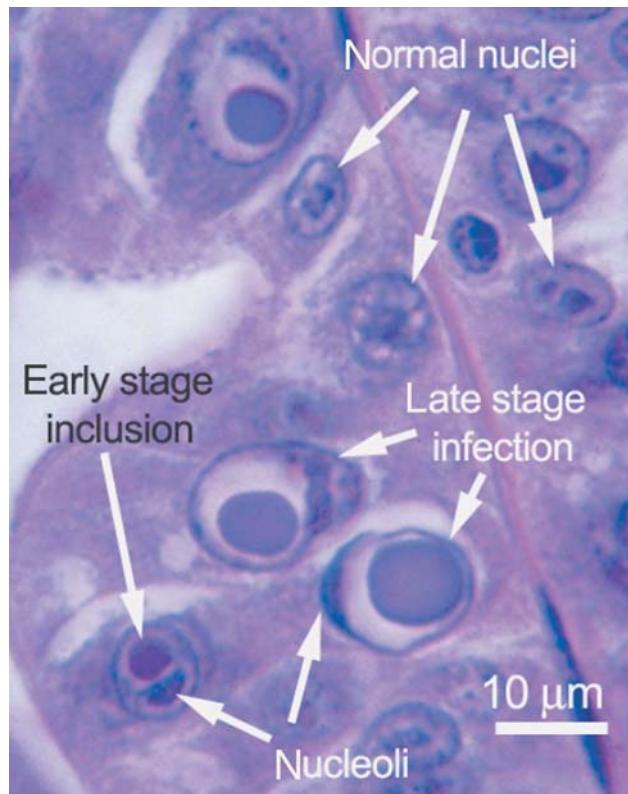
**Plate 32.6** *Localization of AMDV proteins in infected CrFK cells. (A) ADV-infected cell with nuclear staining of the replicative complex containing NS1 (green) and NS2 (red). Capsid proteins are localized around the nucleus periphery (blue). (B) ADV-infected cells showing localization of capsid protein (red) around the nucleus periphery and in the cell cytoplasm (arrowhead). The nuclear localization of NS1 is shown in green. Yellow represents co-localization of both capsid and NS1.*



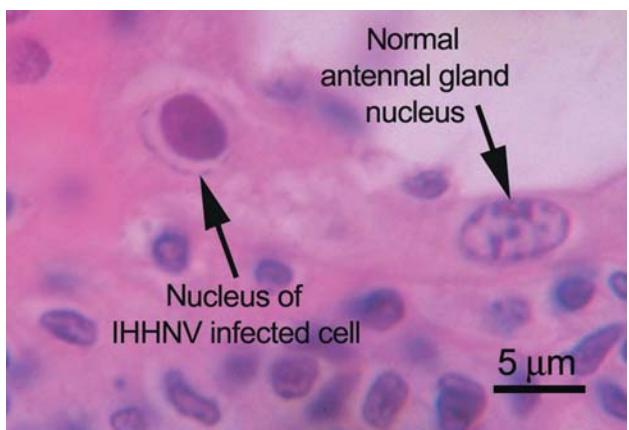
**Plate 32.7** *The role of caspase activity in nuclear localization of NS1. (A) Wild-type NS1 expression (red) following transfection causes apoptosis demonstrated by TUNEL (terminal deoxy-uridine nick end labeling) (green) that is dependent on caspase activity. (B) Wild-type NS1 (green) is expressed in the nucleus of transfected cells. (C) Following pretreatment of cells with broad-spectrum caspase inhibitors, wild-type NS1 (green) localizes in the cell cytoplasm.*



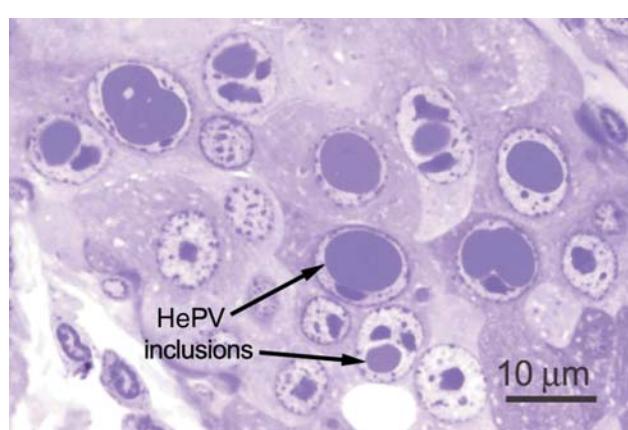
**Plate 35.1** Runt deformity syndrome in *Penaeus vannamei*. Two shrimp specimens showing deformed rostra – one curved down and the other up and both shorter than normal.



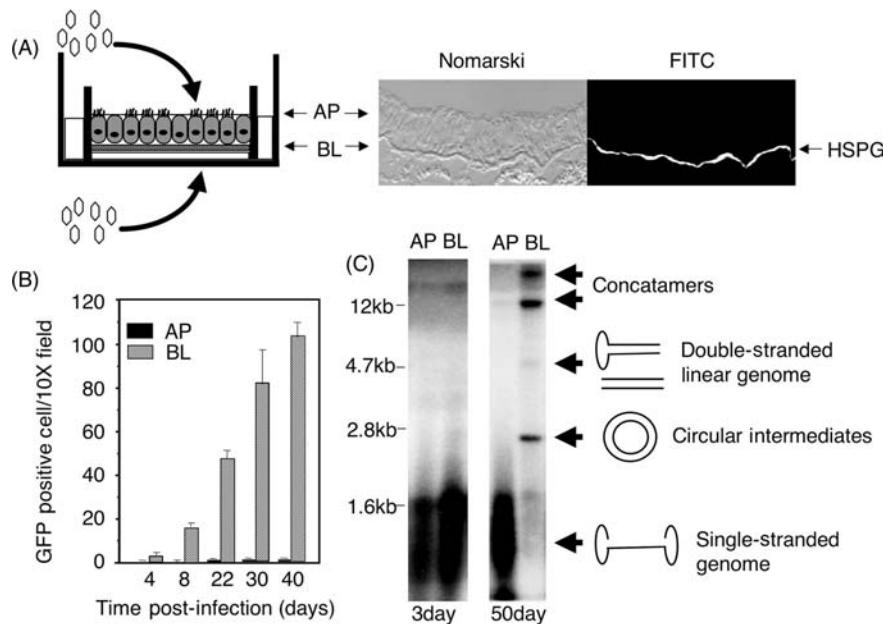
**Plate 35.3** Histopathology of hepatopancreatic parvovirus (HPV) in hepatopancreatic tubule epithelial cells of *Penaeus monodon* from Thailand. The basophilic intranuclear inclusions accompanied by a marinated nucleolus and chromatin are typical for all types of HePV reported from shrimp. Note that the early stage inclusion is eosinophilic rather than basophilic.



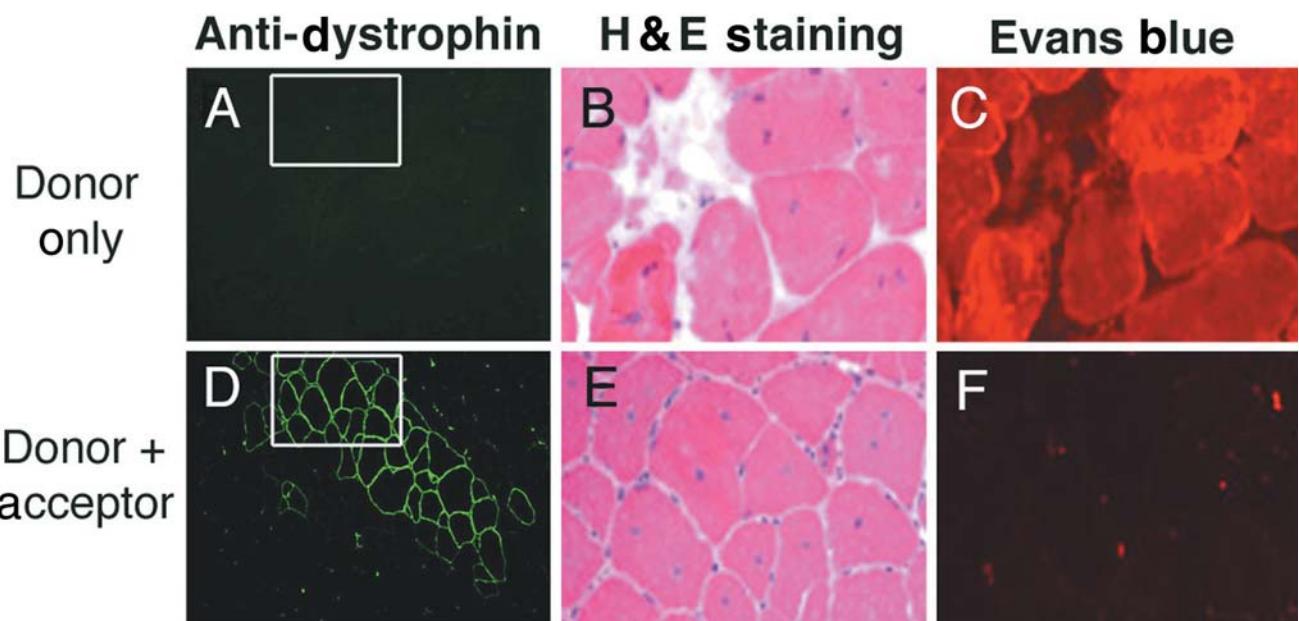
**Plate 35.2** Cowdry type A inclusion of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in a nucleus of an antennal gland cell of *Penaeus monodon* from Thailand. Note the eosinophilic central inclusion separated from the marginated chromatin by a lightly stained zone that occurs as an artifact in tissues fixed with Davidson's fixative (Lightner, 1996b).



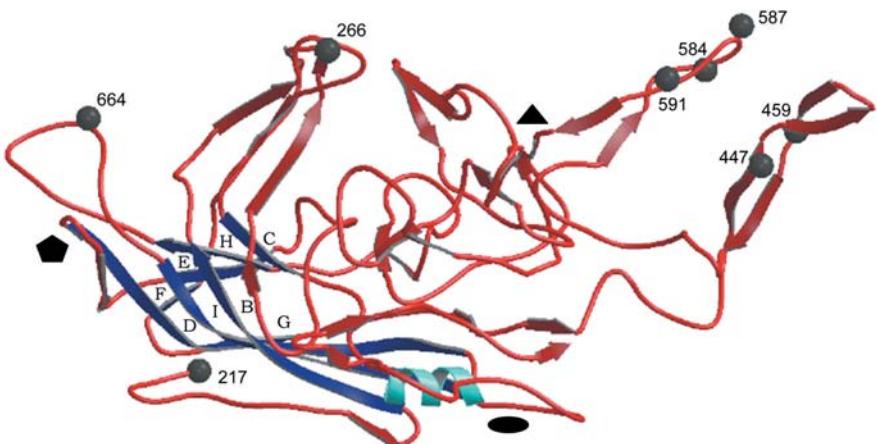
**Plate 35.4** Semithin section of hepatopancreatic tissue showing hepatopancreatic parvovirus (HePV) histopathology in *Penaeus monodon* from Thailand. Note that some nuclei have two nucleoli or what appear to be two or more HePV inclusions that have fused together.



**Plate 38.3** Impaired intracellular processing, but not receptor-mediated endocytosis, represents a significant obstacle for AAV2 transduction from the apical membrane of human polarized airway epithelia. **(A)** Polarized human airway epithelia are cultured at an air-liquid interface and used as a model to study the polarity of rAAV2 transduction from the apical (AP) or basolateral (BL) membranes. Immunofluorescent localization of HSPG in human bronchus demonstrates predominant staining of this primary attachment receptor for AAV2 at the basolateral membrane (right panel of A). An identical pattern of staining is seen in primary polarized airway epithelial cultures (data not shown). **(B)** rAAV2 GFP encoding virus transduces polarized human airway epithelia from the apical surface (AP) much less efficiently than from the basolateral surface (BL). **(C)** Southern blot analysis of rAAV2 genomes from polarized airway epithelia following 3 and 50 days after infection. In spite of the lack of HSPG expression on the apical surface of polarized human epithelia, entry of rAAV2 from the apical surface at 3 days post infection is only 3- to 4-fold less efficient than entry from the basolateral surface. By 50-day post infection, the majority of viral DNA following apical infection remains as single-stranded viral genomes. In contrast, most of the viral genomes following basolateral infection are converted into double-stranded forms that migrate at 2.8 kb (circular monomer), 4.7 kb (linear monomer) and >12 kb (concatamers). Data presented in this plate were adapted from previous publications (Duan et al. 1998b, 2000b).



**Plate 39.4** Trans-spliced minidystrophin expression prevents sarcolemma damage in *mdx* skeletal muscle. Serial sections from donor-infected (Panel A) or donor- and acceptor-co-infected (Panel D) muscle samples were evaluated by antidystrophin immunostaining. Panels B, C, and F are enlarged photomicrographs of the H&E staining and Evans blue dye uptake in the boxed region in Panels A and D, respectively.



**Plate 40.1** AAV2 VP3 monomer. The approximate 2-, 3-, and 5-fold icosahedral axes are shown by the filled oval, triangle, and pentagon, respectively. Some of the positions for peptide insertions (Girod et al., 1999; Shi et al., 2001; Wu et al., 2000) that successfully displayed the new epitope on the capsid surface, are shown by the black balls and labeled with the amino acid number (based on VP1 numbering). The 217 position indicates the most N-terminal amino acid whose atomic coordinates could be deduced by X-ray crystallography. The strands in the eight-stranded antiparallel  $\beta$ -barrel domain are shown as arrowed ribbons in blue and are labeled BIDG and CHEF for the two sheets. The small helix (residues 293–303) that is observed in all parvoviruses is shown in cyan. The rest of the VP3 polypeptide is shown in red, with strands shown as arrowed ribbons and the remaining residues as coils. (The plate was kindly prepared by Mavis Agbandje-McKenna.)

# Looking down the rabbit hole: understanding the binding, entry, and trafficking patterns of AAV particles in the cell

JOHN A. CHIORINI

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Understanding the processes by which a virus enters and traffics through a cell is critical for defining the life cycle of a virus. For many viruses, the first cells encountered are epithelial cells lining the lung, gut, or reproductive tract (for review see Bomsel and Alfsen, 2003). As such, they take advantage of the normal signaling and trafficking molecules already existing in these cells.

While the adeno-associated viruses (AAVs) have a similar size, structure, and genomic organization to other parvoviruses, because of their dependence upon a helper virus and their ability to enter a latent phase in their life cycle, they have developed a unique relationship with their host cell. Most AAVs were reported originally as contaminants of laboratory stocks of adenovirus, thus our understanding of their natural biology and cell tropism is limited. Furthermore, because of the cryptic lifestyle of dependoviruses much of our understanding of their interactions with their host comes from studies of recombinant particles. To date, 11 full length isolates have been reported and they have a broad range of sequence identity with some more than 90 percent identical while others are less than 60 percent (Srivastava *et al.*, 1983; Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997, 1999; Rutledge *et al.*, 1998; Xiao *et al.*, 1999; Gao *et al.*, 2002; Bossis and Chiorini, 2003; Farkas *et al.*, 2004). However most appear to have aspects unique to their biology presumably due to distinct interactions and transduction pathways with their host cells. We are just beginning to elucidate the molecular interactions between AAVs and their host cell as well as the factors that regulate and define the specificity observed *in vivo* for each isolate. The focus of this chapter will be the entry and trafficking pathways of AAV particles that lead to nuclear entry.

Although viral entry is not the only determinant of tropism, considerable effort has been made to identify the structures that mediate cell recognition. Rather than using a unique viral receptor for entry, many viruses are now thought to interact with different classes of molecules to gain entry into the cell. These receptors range from ubiquitous cell surface-associated carbohydrates to cell-specific transmembrane proteins. One class of molecules involved in viral entry is collectively referred to as attachment receptors or 'co-receptors'. The carbohydrate moieties found on the cell surface that are used by several viruses as attachment receptors include glycoproteins, glycosphingolipids, or the carbohydrate chains found on proteoglycans.

In general, the structural motifs of carbohydrates are more diverse than proteins thereby offering a broad array of structures for viral attachment. These molecules are not static and their presentation on the cells' surfaces varies with cell differentiation and maturation, all of which can affect viral attachment. Furthermore, their polarized surface expression or presence in the extracellular matrix or fluids, such as saliva or bronchoalveolar lavage fluid, can affect and block the virus's attachment to a cell.

An increasing number of viruses appear to act as lectins as they attach to the carbohydrate moieties on the surface of the cell, binding via peptides in their envelope or capsid proteins. Viral surface proteins are multimeric, meaning that they have several lectin sites. Therefore, they can interact with several receptor molecules simultaneously on the host-cell surface, which increases their binding affinity (Weis and Drickamer, 1996). A second group of protein receptors, referred to as the 'principal' receptors, mediate entry either alone, or in conjunction with the attachment receptors.

Some protein receptors for viruses include integrins, which can function as attachment and principal receptors, cell junction-associated proteins, and cell adhesion molecules.

AAV entry appears to involve many interactions. AAV type 2 (AAV2) is the best-characterized AAV. Studies by Summerford *et al.* identified the ubiquitously expressed glycosaminoglycan, heparan sulfate, as a carbohydrate attachment receptor for AAV2 (Summerford and Samulski, 1998). This molecule is composed of repeating disaccharide subunits of D-glucosamine and uronic acid and is highly polymorphic with the sulphated structural motifs primarily responsible for its protein binding and regulatory properties (Hallak *et al.*, 2000). As an attachment molecule, its cell surface expression is vital for the binding of AAV2. Binding of AAV2 to the surface of target cells is efficiently competed with by soluble heparin (Summerford and Samulski, 1998). *In vivo*, differences in the degree of expression of heparan sulfate proteoglycans (HSPG) have been correlated with a difference in transduction efficiency of AAV2 for slow and fast muscle fibres (Pruchnic *et al.*, 2000). AAV3 is highly related to AAV2 and also binds heparin. However, the overall affinities and specificity differ (Handa *et al.*, 2000; Rabinowitz *et al.*, 2002).

For AAV2 an important heparin-binding site has been mapped to amino acids 585–588 on the outside of the 3-fold axis (Opie *et al.*, 2003; Kern *et al.*, 2003). This positioning may allow the HSPG chain to wind around the particle and interact with multiple sites on the same particle. Indeed, such an interaction is supported by structural data for the capsid of AAV2 (Xie *et al.*, 2002). Interestingly, while a mutation in this region blocks virus binding and transduction *in vitro*, it appears to alter but not ablate transduction *in vivo*. Kern *et al.* reported that a mutation of two residues important in heparin binding results in a vector with improved specificity for heart tissue compared with wt virus, which can direct gene transfer to both the heart and liver (Kern *et al.*, 2003).

AAV2 also binds fibroblast growth factor receptor 1 (FGFR1) and its expression in conjunction with HSPGs has been shown to enhance AAV2 transduction (Qing *et al.*, 1999). Whether AAV2 binds to FGFR1 directly or as part of a complex as well as its role in AAV2 binding or entry is not clear. Furthermore, a novel 150 kDa protein has been found on AAV2 permissive cells (Mizukami *et al.*, 1996).

Identification of an important heparin-binding domain on the surface of the AAV2 particle has allowed investigators to develop new recombinant vectors based on replacement of this binding region with that of another epitope. Girod *et al.* demonstrated that an RGD (arginine-glycine-aspartic acid) integrin-binding epitope could be inserted at amino acid 587 and alter the tropism of AAV2 (Girod *et al.*, 1999). Instead of heparin-binding activity, the virus now had an integrin-binding activity. This same site has been shown to accept other functional peptides and likewise altered the tropism of the original AAV2 vector (Shi *et al.*, 2001). The surface of the parvovirus particle consists of a

series of loop structures interspersed on a conserved  $\beta$ -barrel framework. The loops and unstructured segments have been extensively studied as insertion sites for peptides and several sites that can tolerate an insertion but do not alter the heparin-binding activities of AAV2 have been identified (Gavin *et al.*, 1999; Muller *et al.*, 2003; Shi *et al.*, 2001; Wu *et al.*, 2000). Insertions in these regions essentially expand the tropism of the vector. For example, the insertion of an ApoE-derived ligand into the amino terminus of the VP2 peptide resulted in a vector whose transduction activity could be competed for by heparin or ApoE but demonstrating increased transduction activity both *in vitro* and *in vivo* (Loiler *et al.*, 2003). While the alterations studied thus far have focused on cell surface-binding interactions, future changes may affect other rate limiting steps in the viral trafficking pathway to improve cell specificity or overall transduction.

Not all AAVs interact with HSPGs. For example, AAV4 and AAV5 both use different forms of sialic acid for cell attachment. In a series of studies, both AAV4 and AAV5 required the  $\alpha$ 2–3 form of sialic acid for cell attachment and transduction of recombinant particles (Kaludov *et al.*, 2001; Walters *et al.*, 2001). However, they both exhibited different specificities. Treatment of cells with specific glycosylation inhibitors, resialation experiments with neuraminidase-treated erythrocytes, or cells genetically deficient in the addition of sialic acid demonstrated that AAV4 preferentially attached to an  $\alpha$ 2–3 sialic acid present on O-linked carbohydrate core while AAV5 attached to the N-linked type.

Not only is the interaction of AAV2 with HSPGs critical for attachment to the cell but it also defines the spread of the virus during transduction and the polarity of AAV2 entry. Heparan sulfate groups are commonly found in the extracellular matrix and cell basement membranes. Thus, the tropism of AAV2 is defined by the distribution of these molecules. In the brain, HSPGs exist in cell-associated forms such as syndecans (Hsueh and Sheng, 1999; Hsueh *et al.*, 1998) and glycan-1 (Liang *et al.*, 1999) as well as in the extracellular matrix. Following direct injection into the striatum, AAV2 transduction is tightly focused at the site of injection. However, if AAV2 is injected in the presence of soluble heparin to suppress interactions with the matrix, the spread of rAAV2 particles is dramatically increased (Nguyen *et al.*, 2001). In the lung, HSPG expression is primarily localized to the basal lateral surface and is not detected on the apical surface (Duan *et al.*, 1998). Thus, transduction is significantly more efficient when the virus is delivered through the basal lateral surface as compared with the apical surface *in vitro*.

In contrast, AAV1: AAV4: and AAV5: which do not bind HSPG, produce a wider pattern of transduction following injection into the striatum but their tropism is still defined by their attachment receptor interactions. AAV4 can only transduce polarized human airway lung epithelia from the basal lateral surface while AAV5 can transduce polarized airway

epithelia from either surface (Zabner *et al.*, 2000). The protective mucins secreted by airway epithelia are heavily glycosylated with an abundance of O-linked sialic acid. Binding and competition experiments demonstrate that AAV4 will bind and it is inhibited by purified muc-1 but not its deglycosylated form (Walters *et al.*, 2002). In contrast, AAV5 only weakly binds muc-1 and its transduction is not inhibited in competition experiments or by bronchoalveolar fluid (Rooney *et al.*, 2002; Walters *et al.*, 2002). Thus, while AAV5 can transduce lung cells via either the apical or basal-lateral surfaces, AAV4 can only transduce lung cells via the basal-lateral surface because of the apical protective layer of mucins.

Integrins are a class of surface molecules several viruses use to attach to and infect cells. Some integrins exhibit a basal lateral distribution while others apical patterns of expression. The classic integrin-binding motif RGD is present in many integrin ligands including fibronectin and collagen type 1 (Yamada, 1999). It is also the motif present in the adenovirus penton protein and binds to avb3 (Wickham *et al.*, 1993). Not all integrin-binding viruses contain this motif. Some will bind in an RGD independent manner. AAV2 is reported to bind avb5 integrin, an interaction important for virus internalization. However, the capsid of AAV2 lacks an RGD motif and binding is not affected by RGD peptide competition (Qiu *et al.*, 2000). But AAV2 internalization and transduction are inhibited by anti-avb5 antibody competition and dramatically increases in CS-1 cells engineered to express avb5. This supports a role for avb5 in AAV2 binding and entry (Sanlioglu *et al.*, 2000; Summerford *et al.*, 1999).

Using bioinformatics, the platelet derived growth factor (PDGF) receptors were identified as protein receptors for AAV5 (Di Pasquale *et al.*, 2003). This observation was confirmed by modulation of platelet-derived growth factor receptor (PDGFR) expression by transfection of expression plasmids, inhibitor treatment, or competition experiments that altered both viral binding and transduction. Furthermore, co-precipitation experiments indicated a direct interaction between AAV5 and PDGFR $\alpha$ . In addition to this direct interaction, the expression pattern of PDGFR correlated with the *in vivo* transduction pattern reported for AAV5. In the brain, AAV5 efficiently transduces neurons in the hippocampus, which is known to express high levels of PDGFR $\alpha$  (Oumesmar *et al.*, 1997). As noted earlier, AAV5 can also transduce polarized lung human airway epithelia via either the apical or the basal-lateral surfaces and it is an effective vector for lung gene transfer *in vivo* (Zabner *et al.*, 2000; Auricchio *et al.*, 2002).

Immunohistochemical staining has demonstrated that PDGFR $\alpha$  is expressed on the apical surface, while the beta form is found on the basal lateral surface, endowing AAV5 with multiple entrance routes into this cell type (Chiaroni and Zabner, unpublished). Immunohistochemical staining for PDGFR $\alpha$  in primate eyes has shown a correlation between expression and AAV5 transduction of rod photoreceptors (Lotery *et al.*, 2003).

Despite their differences, both avb5 and PDGFR are considered cell surface signaling molecules responsible for responding to extracellular stimuli. In the case of integrin, ligand binding recruits actin and forms signaling centres that stimulate mitogen-activated protein (MAP) kinase activation (Jockusch *et al.*, 1995; Yamada and Miyamoto, 1995). Multiple ligands also can bind to PDGFR leading to receptor dimerization and autophosphorylation of the tyrosine kinase domain, initiating signal transduction that affects calcium and lipid metabolism, gene expression, changes in cell morphology and cell replication (Claesson-Welsh, 1994). Just as the binding of a ligand to its receptor triggers a cellular response, that of the virus:receptor interaction may evoke a similar response. Current data demonstrate the importance of binding integrins or PDGFR for AAV2 or AAV5; respectively; yet, it is not clear if this type of ligand–receptor interaction also triggers intracellular signaling and if that signaling is important in the virus life cycle. Recent studies have suggested the binding of an AAV2 empty capsid inhibits mRNA levels of genes related to G2/M progression and induces genes involved in an antiproliferative response (Stilwell and Samulski, 2004). While most of these binding studies have focused on laboratory cell lines, the data does correlate with the minimal effects on the host observed with AAV vectors in both pre-clinical and clinical studies.

Following receptor attachment, AAVs are thought to enter the cell by endocytosis. Cell entry studies for AAV2 and AAV5 have observed particles in clathrin-coated vesicles (Bartlett *et al.*, 2000; Bantel-Schaal *et al.*, 2002). AAV2 trafficking has primarily been studied using cy3-labeled particles. Uptake of these labeled particles occurs rapidly in neuronal cells *in vivo* (Bartlett *et al.*, 1998). Rapid uptake is also observed in HeLa cells followed by processing through the late endosome and acidification of the compartment appears critical for escape and transduction. Treatment with drugs such as brefeldin A, which blocks early to late endosome transition, or ammonium chloride or Bafilomycin A1, which affect vesicle pH, inhibits AAV2 transduction (Bartlett *et al.*, 2000; Douar *et al.*, 2001). In contrast, adenovirus type 5 exits the early endosome and is not inhibited by brefeldin A (Leopold *et al.*, 2000).

Vesicle trafficking is a complex process that leads to particle deposition in many cell locations. In the lung, introduction of AAV2 particles has been described in end stage proteasomes and the nucleus. However, the addition of proteasome inhibitors shifts the trafficking pathway to increase AAV2 in the nucleus as assayed by increased transduction (Duan *et al.*, 2000). The addition of adenovirus proteins also is reported to increase nuclear entry of AAV2 and also may act by changing the trafficking of AAV2 through the cell (Xiao *et al.*, 2002). Which compartments the viruses pass through will depend on the initial cell surface interactions and cellular physiology.

While all of the trafficking studies to date have been done in immortalized cells, it will be important to confirm

their significance in primary cells as well. Careful analysis of these events will help to establish the permissive intracellular environment necessary for the life cycle of AAVs as well as the host defenses against viral infection.

Just as with receptor binding, not all dependent parvoviruses follow the same intracellular trafficking pattern. While more than one route is possible, at low multiplicity of infection (MOI) AAV5 enters the cell via microvilli or on the lateral surfaces near desmosomal structures in HeLa cells (Bantel-Schaal *et al.*, 2002). AAV5 particles also can associate with coated pits (Bantel-Schaal *et al.*, 2002). Time course experiments indicated that AAV5 interacts with structures resembling early endosomes that are eventually deposited in the cistern of the trans-Golgi network. Additionally, AAV5 is observed in the lysosomal compartment at high MOI (Bantel-Schaal *et al.*, 2002).

Trafficking studies have been difficult to interpret because of the high MOIs required for signal detection. In the case of AAV5, multiple interactions are observed but it is unknown which one(s) result in transduction. However, some of these trafficking patterns also are observed with advanced single particle/single label imaging techniques (Seisenberger *et al.*, 2001). In this study, AAV2-containing endosomes are reported to release the particles in a pH dependent manner, which then move through the cell in a directed manner indicating microtubule-dependent transport of viruses by motor proteins such as kinesin or dynein. This directed transport also took place in the nucleus.

The ultimate destination of the AAVs is in the nucleus. Whether the viral particles enter the nucleus intact or as naked DNA remains unclear, but the process of viral uncoating is a potentially rate-limiting step in AAV transduction. Currently, our understanding of the cellular components necessary for this step is deficient. Experiments with AAV2 suggest that the conversion of AAV2 from a single-stranded form to a double-stranded transcriptionally active form is the underlying reason for the long delay in transduction following AAV injection (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). Alternatively, recent studies suggest the slow transduction kinetics stem from a slow breakdown and release of the AAV genomes from the AAV particles (Thomas *et al.*, 2004). Interestingly, experiments with AAV5 have not detected particles in the nucleus (Bantel-Schaal *et al.*, 2002).

Our understanding of the natural biology of dependent parvoviruses is limited. In addition, it has not been possible to gain insight into virus tropism by following viral pathology. The use of new molecular and fluorescence approaches, however, in combination with classic biochemistry and genetics has benefitted the mapping of the viral pathway inside the cell. We are just beginning to understand the molecular interactions between viral and cellular components that control the finely regulated specificity observed for each virus and defined target cell. Furthermore, the cells' response to viral entry and the signaling induced by virus binding or entry and the role this may play in subsequent

steps in the trafficking pathway needs further refinement. While some binding receptors have been identified and their importance in primary cells confirmed, our understanding of intracellular trafficking events is mostly restricted to tissue culture cells, and should be re-examined in primary cells. Defining the cellular parameters that may affect binding, entry, and trafficking will add valuable information to optimize this process and improve the transduction efficiency of recombinant vectors.

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# A rolling-hairpin strategy: basic mechanisms of DNA replication in the parvoviruses

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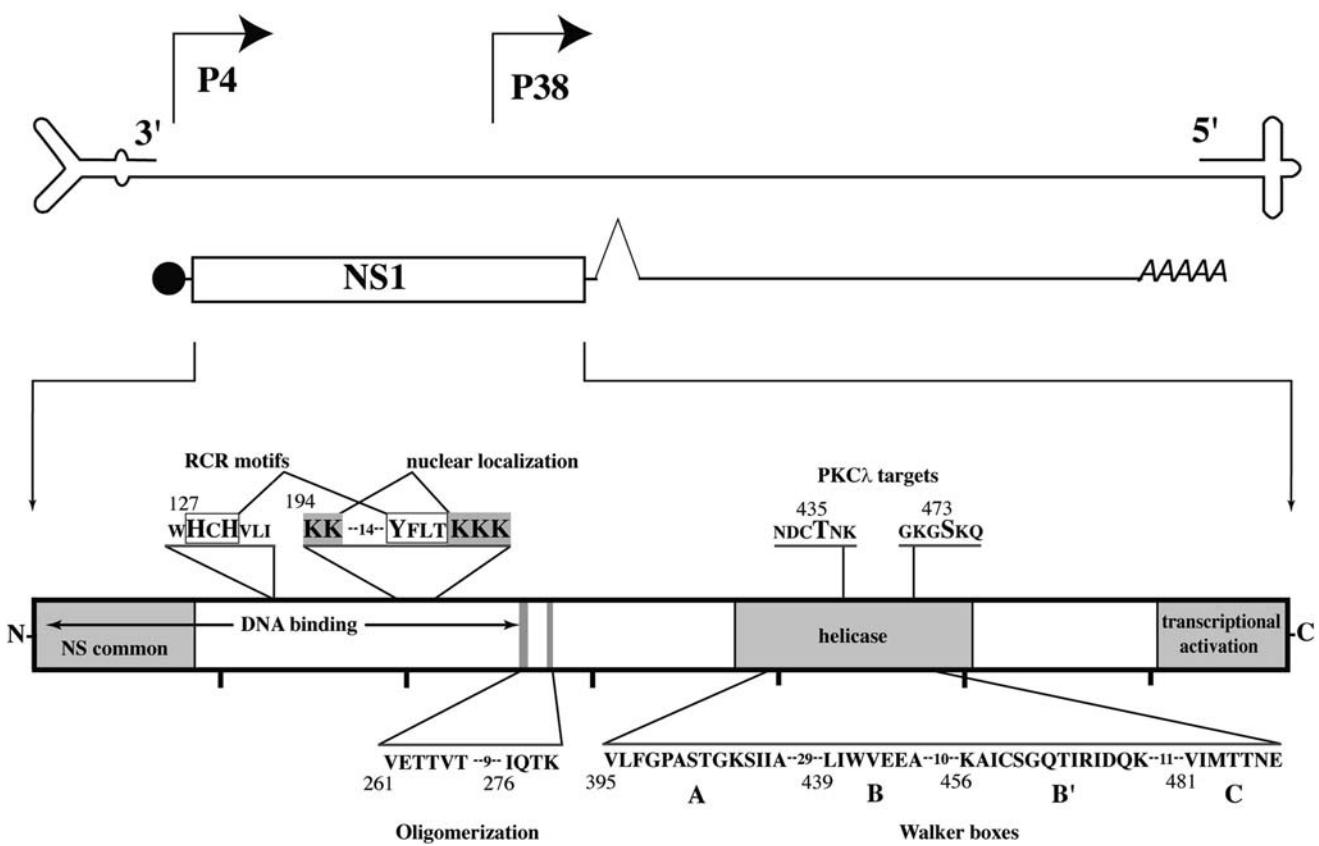
Parvoviruses are unique in having a DNA genome that is both single stranded and linear. This unusual chromosome lacks the structural constraints associated with duplex or circular DNAs, and so allows the maximum possible genetic information to be translocated into the particle and therein compressed within the smallest possible space. The overriding advantage of being able to compress their ~5 kilobase genome into the 280 Å diameter parvoviral virion is hard to assess experimentally, but it likely coincides with the fact that this is the maximum particle size that can be imported intact through the nuclear pore, and suggests a specific role for the intact capsid within the host cell nucleus. However, this single-stranded lifestyle demands a novel replication mechanism, called ‘rolling-hairpin replication’ (RHR), and largely restricts gene expression from newly-arrived genomes to dividing cell populations. These viruses subvert the synthetic machinery of their eukaryotic host for their own preferential replication, using an ancient unidirectional, strand-displacement mechanism, known as rolling-circle replication (RCR), that is employed by many other small prokaryotic and eukaryotic replicons, but is modified in the parvoviruses for the replication of a linear chromosome. Within their host cells, all RCR replicons amplify through a series of duplex replicative-form (RF) DNA intermediates, and in general employ distinctive origins of replication that contain small palindromic DNA sequences, which are able

to alternate between inter- and intrastrand basepairing at different phases of the replication process. In the parvoviruses, this ancestral use of palindrome rearrangement to mediate initiation has developed in a novel way, leading to the creation of self-priming telomeres at each end of the linear molecule. Some parvoviruses, such as adeno-associated virus 2 (AAV2), have inverted terminal repeat (ITR) sequences, so that equivalent palindromes are present at both ends of the genome, and the replication and resolution processes associated with each terminus are very similar, if not completely identical. In contrast, other viruses, such as minute virus of mice (MVM), have two physically and functionally disparate terminal palindromes, and their replication mechanism appears to be substantially more complex. However, the molecular principles that underlie both modes of replication are very similar, and these simple mechanisms generate a complex mixture of replication intermediates *in vivo*. In this chapter we explore the basic principles of RHR, predominantly from the perspective of the replication intermediates and resolution pathways used by the autonomously-replicating, or helper-independent, virus MVM. In the following chapters, AAV replication is analyzed in more detail, with particular reference to its alternative lifestyles, as either a latent, integrative molecule or a productive replicon, activated by helper-virus gene products.

## WAITING FOR S-PHASE: THE CONSEQUENCES OF PACKAGING A SINGLE-STRANDED DNA GENOME

Parvovirus particles contain a single copy of the linear, non-permuted DNA genome in which a relatively long single-stranded coding region (4–6 kb) is bracketed by short (121–418) palindromic terminal sequences capable of folding into hairpin duplexes, as illustrated for MVM in Figure 14.1. The viral genome is packaged with its 5'-associated NS1 molecule on the outside of the virion, anchored by a ~24 nucleotide long 'tether' sequence that is cleaved off during entry (Cotmore and Tattersall, 1989), but restored, as we shall see, by an early step in viral DNA replication. Viral genomes can be positive- or negative-sense with regard to transcription, some species packaging plus and minus strands with equal efficiency, while others, such as MVM, favor minus-sense DNA. Because of this disparity, a simple convention has been adopted by which the 5' end of the

strand encoding the non-structural proteins is described as the 'left end' of the genome, and its 3' end the 'right end'. The viral telomeres contain most of the *cis*-acting information required for both DNA replication and encapsidation, and, while viruses belonging to the *Parvovirus*, *Amdovirus*, *Bocavirus*, and *Breviadensovirus* genera have disparate palindromes at each end of their genomes, members of the *Dependovirus*, *Erythrovirus*, *Densovirus*, and *Iteravirus* genera have inverted terminal repeats. These telomere structures differ profoundly between virus species, as detailed in Chapter 7 of this volume, but they are invariably imperfect palindromes in which a fully, or predominantly, basepaired stem region terminates in some sort of axial asymmetry that can fold into a variety of secondary structures, such as the 'Y'-shaped and 'cruciform' configurations depicted at the left and right ends of MVM, respectively, in Figure 14.1. During replication these termini can be considered to serve as 'hinges', with the imperfectly base-paired or potentially cruciform regions surrounding the axis providing an



**Figure 14.1** Genetic strategy of MVM. At the top of the figure is a line diagram of the MVM genome with the structures of the terminal hairpins expanded approximately 20-fold relative to the coding sequences. Positions of the non-structural gene promoter (P4) and the capsid gene promoter (P38) are indicated. Below this, the R1 transcript that encodes NS1 is depicted, with the cap symbolized by a filled ball, a splice in non-coding sequence by a vertical caret, and its polyA tail by a string of As. At the bottom of the figure a block diagram indicates the positions of domains involved in DNA binding, oligomerization, ATPase, transcriptional activation, and helicase activity, with its component Walker boxes (Koonin, 1993). The rolling-circle initiator protein homologies are detailed, with the putative Mg<sup>2+</sup> coordination site centered around histidines 127 and 129, and the DNA-linking tyrosine at residue 210, in the sequence YxxxK. Two lysine clusters, straddling residue 210, that make up the bipartite nuclear localization signal are boxed in gray. Phosphorylation of residues T435 and S473 by PKC $\lambda$ , is known to be essential for helicase function.

energetically favorable environment for unfolding and refolding the hairpin. Copying the extreme 5' end of linear DNA molecules is problematic because DNA polymerases synthesize only in a 5'-to-3' direction and absolutely require a basepaired primer. Parvoviruses solve this conundrum by using their terminal hairpins as primers for complementary strand synthesis. The sequence information in each terminus is then duplicated by nicking the palindrome at the inboard end of the original hairpin and copying it in the opposite direction – a process initially described as hairpin transfer (Tattersall and Ward, 1976), which generates an inverted copy of the original palindrome. Although central to the process of replication, the complexity and diversity of the telomeres suggest that in individual viruses they may have been coopted to perform additional functions.

Parvoviruses accumulate in the host nucleus within hours of entering the cell, but unlike the DNA tumor viruses, they lack cell cycle-modulating proteins that would enable them to induce resting cells to enter S-phase. This is particularly restrictive because parvoviral promoters are packaged as single-stranded DNA, so that acquisition of a complementary strand necessarily precedes viral gene expression. Thus, the viruses generally remain silent within the nucleus until the cell progresses into S-phase of its own volition. For MVM, complementary strand synthesis occurs at the G1/S-phase transition and is dependent upon the onset of cyclin A expression and cyclin A-associated kinase activity (Bashir *et al.*, 2000, 2001). Expression from the MVM P4 promoter involves the host transcription factor E2F (Deleu *et al.*, 1999), which becomes available at the G1/S transition, so that gene expression in MVM appears uniquely tailored to be optimal during S-phase. This therefore implies that strategies must exist to allow viral genomes to persist within cells, without triggering host defense mechanisms, so that infected cells can progress into S-phase unchecked.

Parvoviral genomes appear to enter cell nuclei while still enclosed within intact particles, although these may be converted to an open, or ‘transitioned’, configuration during entry (Cotmore *et al.*, 1999; Vihtinen-Ranta *et al.*, 2002). Exactly how or when the incoming DNA is released from the virion remains uncertain. Since naked single strands are likely to be quite unstable and to be perceived as foreign or improperly replicated by the host repair surveillance apparatus, viral genomes must either be converted rapidly into a less obtrusive and more stable duplex form, or retained within the particle until actively uncoated during S-phase, a process itself perhaps catalyzed by the replicative machinery of the host cell.

At present, we have little experimental data to document how viruses, like MVM, which package negative-sense DNA predominantly, remain sequestered within non-dividing cells. However, recombinant AAV (rAAV) vectors, which encapsidate DNA strands of both polarities, have been shown to persist as particles in the nuclei of non-dividing cells for protracted periods (Thomas *et al.*, 2004), and to slowly give rise to transcriptionally-active, monomeric and concatemeric

duplex episomes in such cells (Yan *et al.*, 2000), suggesting that these genomes can be uncoated by S-phase independent mechanisms. Gene expression from rAAVs in non-dividing cells is currently the subject of close experimental scrutiny because of its potential importance to gene therapy applications, and is dealt with extensively in Part 4 of this volume (Chapter 38). In some instances, at least, duplex formation in such cells appears to be driven by the annealing of complementary incoming DNA strands (Nakai *et al.*, 2003). One interesting observation to emerge recently is that nuclei from non-replicating liver tissue may contain factor(s) that can uncoat rAAV2 virions *in vitro*, without generalized capsid protein degradation. Despite this, uncoating is normally impaired for rAAV2 vectors in liver cells, allowing particles to persist for at least 6 weeks. In contrast, rAAV6 and rAAV8 particles appear to be uncoated and expressed efficiently in liver, perhaps reflecting differences in subnuclear trafficking patterns (Thomas *et al.*, 2004). This resurrects the intriguing possibility that viral tissue-specificity may, in some instances, be determined by capsid-specific uncoating processes (Previsani *et al.*, 1997). *De novo* synthesis of complementary DNA by cellular repair pathways is also considered a likely mechanism for stabilizing input genomes in non-dividing cells, but this may require protracted incubation since repair-mediated synthesis of positive-sense complementary strands of MVM cannot be detected in synchronized cell populations prior to S-phase, at least in short-term culture (Bashir *et al.*, 2000).

## THE VIRAL NON-STRUCTURAL PROTEINS

Parvoviral genomes therefore appear to await activation in the cell nucleus, without disturbing normal cell-cycle control. Once the cell successfully enters S-phase, MVM genomes are rapidly converted into duplex transcription templates by the cellular replication machinery and mRNAs encoding the non-structural (NS) genes are expressed from the viral P4 promoter (Tullis *et al.*, 1994). MVM encodes two types of non-structural proteins; a long-lived, pleiotropic 83 kDa nuclear phosphoprotein, NS1, which is a site-specific DNA-binding protein that dominates both DNA replication and many other aspects of the viral life cycle and three distinct forms of a 25 kDa NS2 polypeptide, NS2P, NS2Y, and NS2L (Cotmore and Tattersall, 1990). NS1 is the replication initiator protein and is equivalent to the Rep68/78 polypeptides encoded by AAV. Recently our understanding of the structure and biochemistry of these proteins has been greatly informed by atomic structures obtained by X-ray diffraction for the nuclease and helicase domains of AAV5 and AAV2 Rep proteins, respectively (Hickman *et al.*, 2002, 2004; James *et al.*, 2003). As shown in Figure 14.1 MVM NS1 is a multidomain protein of 672 amino acids. It binds sequence-independently to single-stranded DNA and RNA and site-specifically to duplex DNA at reiterations of the tetranucleotide sequence 5'-ACCA-3',

which are present in the viral replication origins and repeated at multiple sites throughout the viral genome in more or less degenerate forms (Christensen *et al.*, 1995; Cotmore *et al.*, 1995). Amino acids in the amino-terminal 275 residues of NS1 mediate both these sequence-specific and non-specific interactions with the DNA (Lorson *et al.*, 1996) and its single-strand nickase activity (Nuesch *et al.*, 1995). Nicking occurs via a nucleolytic transesterification reaction that liberates a basepaired 3' nucleotide to prime the polymerase and hence initiate DNA replication, while leaving NS1 covalently attached to the new 5' end. However, site-specific binding by NS1 can only occur if it assembles into homodimers or higher order multimers. This happens naturally upon addition of ATP, presumably mediated by the helicase domain of NS1, and can be achieved experimentally *in vitro* by the addition of various tags or the use of antibodies that cross-link sequences in either terminus (Christensen *et al.*, 1995; Cotmore *et al.*, 1995; Lorson *et al.*, 1996). NS1 carries an additional bipartite dimerization motif, between residues 261 and 279, physically identified both by deletion mutagenesis in a yeast two-hybrid system and by fine mapping in a peptide enzyme-linked immunosorbent assay (Pujol *et al.*, 1997). This motif, in its intact form, appears to be essential for MVM viability, although its exact role in NS1 multimerization remains unexplored. Although NS1 may exist in a variety of oligomeric states *in vivo*, structure predictions based on isolated AAV Rep helicase and nuclelease domains suggest that it probably assembles into hexamers to fulfill both of these functions (James *et al.*, 2003; Hickman *et al.*, 2004). NS1 also exists in a variety of differentially phosphorylated forms *in vivo* (Corbau *et al.*, 1999; Lachmann *et al.*, 2003; Nuesch *et al.*, 2003), which appear to reflect its various roles in the viral life cycle, reviewed in detail in Chapter 19. Thus specific phosphorylations at T435 and S473, introduced by the atypical lambda isoform of protein kinase C (PKC $\lambda$ ), are essential to activate its helicase function while somewhat impairing site-specific DNA binding (Dettwiler *et al.*, 1999; Nuesch *et al.*, 2001). A second PKC isoform, PKC $\eta$ , is also known to phosphorylate specific NS1 peptides *in vivo* and to potentiate its ability to initiate RCR *in vitro* (Lachmann *et al.*, 2003). Inactivation of either of these kinases *in vivo* by the introduction of dominant negative mutants drastically impairs viral replication (Lachmann *et al.*, 2003; Nuesch *et al.*, 2003).

During the nicking reaction, NS1 becomes covalently linked to the 5' end of the DNA at the nick site through the hydroxyl of tyrosine residue 210 (Nuesch *et al.*, 1995) and from this location at the nick, is thought to organize the replication fork and function as the replicative 3' to 5' helicase (Wilson *et al.*, 1991; Jindal *et al.*, 1994; Nuesch *et al.*, 1995; Christensen and Tattersall, 2002). Its helicase activity maps to a domain situated between amino acids 394 and 500 in NS1 (Anton and Lane, 1986; Astell *et al.*, 1987), which belongs to helicase superfamily III and contains the expected Walker A, B, B' and C boxes (Gorbatenya *et al.*, 1990; Koonin, 1993; James *et al.*, 2003; Iyer *et al.*, 2004). Finally, NS1 carries

an acidic transcriptional activation domain near its carboxy-terminus. This acts to upregulate transcription from the viral P38 promoter when NS1 is bound to a particularly potent series of 5'-ACCA-3' motifs, called the *tar* sequence, positioned upstream of the P38 transcription unit (Gu and Rhode, 1992; Christensen *et al.*, 1995; Lorson *et al.*, 1996) and via direct interactions between NS1 and a variety of transcription factors including Sp1, TF11A, and TBP (Kraday and Ward, 1995; Lorson *et al.*, 1998).

While NS1 is the only non-structural protein essential for productive replication in all cell types, one or more of the NS2 isoforms is also absolutely required in rodent cells, both in culture and in the whole animal (Naeger *et al.*, 1990; Brownstein *et al.*, 1992; Cater and Pintel, 1992; Cotmore *et al.*, 1997). In murine cells, NS2 appears to influence multiple steps in the viral life cycle, apparently reprogramming the host cell for efficient DNA amplification, single-strand progeny synthesis, capsid assembly and virion export (Naeger *et al.*, 1990, 1993; Cotmore *et al.*, 1997; Eichwald *et al.*, 2002; Miller and Pintel, 2002), although there is no evidence for the direct involvement of NS2 in any of these processes. NS2 initially accumulates approximately three times faster than does NS1, so that it is the predominant species in early S-phase, but it is turned-over rapidly (with a half life of ~1 hour) via a proteasome-mediated pathway, causing its relative abundance to diminish rapidly as the infectious cycle progresses and P38-driven transcription comes to predominate (Cotmore and Tattersall, 1990; Miller and Pintel, 2002). Exactly how these NS2 species influence the cell remains uncertain, but they have been shown to interact *in vivo* and *in vitro* with various proteins, including two 14-3-3 family members (Brockhaus *et al.*, 1996), the survival motor neuron protein (SMN) (Young *et al.*, 2002b) and the nuclear export factor Crm1 (Bodendorf *et al.*, 1999; Ohshima *et al.*, 1999), the latter interaction being particularly important for early virion export from the nucleus (Eichwald *et al.*, 2002; Miller and Pintel, 2002).

As the viral non-structural gene products accumulate, they usurp the cellular replication apparatus, causing host cell DNA synthesis to be terminated and viral DNA amplification to initiate (Cotmore and Tattersall, 1987). Interference with host DNA replication may possibly be mediated by direct effects on replication proteins, such as DNA polymerase  $\alpha$  (Ho *et al.*, 1989), which are not essential for MVM replication (Christensen *et al.*, 1997b), but may also result from the extensive nicking of host DNA (Op De Beeck and Caillet-Fauquet, 1997) or the restructuring of the nucleus that accompanies viral infection (Oleksiewicz *et al.*, 1996; Cziepluch *et al.*, 2000; Bashir *et al.*, 2001). Early in infection, parvoviruses establish characteristic nuclear replication foci (Oleksiewicz *et al.*, 1996, 1998), subsequently termed autonomous parvovirus-associated replication (APAR) bodies, and shown to be unrelated to other known nuclear domains (Cziepluch *et al.*, 2000). NS1 co-localizes with replicating viral DNA in these structures, together with a number of cellular replication proteins that are known to be

essential for viral DNA synthesis, such as proliferating nuclear cell antigen (PCNA), RPA and DNA polymerase δ (Christensen *et al.*, 1997b; Bashir *et al.*, 2001). Somewhat surprisingly, because it is not required for viral DNA synthesis, the polymerase α-primase complex, also co-localizes in autonomous parvovirus replication (APAR) bodies, supporting the notion that MVM simply coopts cellular replication complexes that are at least partially preformed. Cyclin A is massively redistributed to these foci, while cdk2 is not, suggesting that cyclin A may be playing some sort of structural role. It has been suggested (Bashir *et al.*, 2001) that this sequestration of cyclin A could be responsible for parvovirus-induced cell cycle arrest (Op De Beeck *et al.*, 1995, 1997), since it could lead to failure of cdk1 activation in G2-phase, thus preventing cell cycle progression.

Within a few hours of the start of S-phase, translation of P38-driven transcripts leads to the accumulation and nuclear translocation of the viral capsid proteins. These are assembled into intact empty particles from oligomeric precursors within the nucleus, reviewed in Chapter 20 and 21. When expressed in the absence of the Rep proteins, assembled AAV particles are first observed within the nucleoli, and certainly nucleolar components are heavily recruited during many parvoviral infections since these structures rapidly become disorganized (Singer and Rhode, 1977; Walton *et al.*, 1989), allowing their constituent proteins, such as fibrillarin, to become dispersed and degraded (Cotmore and Tattersall, unpublished). As infection progresses the viral APAR microdomains begin to coalesce with the contents of other, formerly distinct, nuclear bodies, such as Cajal bodies, promyelocytic leukemia (PML) gene product oncogenic domains (PODs), and speckles, forming progressively larger nuclear inclusions that are active sites of viral replication and virion assembly. These have been dubbed SMN-associated APAR bodies (SAABs), to reflect the eventual translocation of SMN from Cajal bodies into these structures, which ultimately spread throughout the nucleoplasm (Young *et al.*, 2002a). Full virions are exported from the nuclei to the cell exterior prior to nuclear disintegration (Cotmore *et al.*, 1997; Eichwald *et al.*, 2002; Miller and Pintel, 2002; Maroto *et al.*, 2004), as discussed in Chapter 20. Progression out of S-phase is prevented, and the cells remain actively synthesizing viral DNA (Cotmore and Tattersall, 1987) until disruption of the host environment leads ultimately to cell lysis, via necrotic or apoptotic mechanism (Rayet *et al.*, 1998; Ran *et al.*, 1999), with the concomitant release of residual virus.

## FEATURES COMMON TO BOTH ROLLING-CIRCLE AND ROLLING-HAIRPIN REPLICATION MECHANISMS

Many small circular replicons, including bacteriophages of the family Microviridae, eubacterial plasmids, single-stranded DNA viruses of spiroplasma and chlamydia, plant

geminiviruses and mammalian circoviruses, replicate via a unidirectional, strand-displacement mechanism, called rolling-circle replication (RCR), in which successive rounds of replication are both initiated and terminated by the introduction of a site-specific single-strand nick. These nicks are introduced by a replicon-encoded nuclease, variously called the nickase, relaxase, mobilization protein (mob), transsterase, or Rep. Ilyina and Koonin recognized that a cluster of minimal sequence motifs were common to these proteins (Ilyina and Koonin, 1992; Koonin and Ilyina, 1993), and from database analyses predicted the existence of a considerable family of RCR initiator proteins, spanning eubacteria, archaea, and eukarya, with the NS1 and Rep proteins of the parvoviruses positioned on an outer branch of the tree. Specifically, they predicted the importance of three motifs. Even though parvovirus replicons are linear, rather than circular, and parvoviral initiators show only vestigial traces of being able to carry out the joining reactions common to their RCR counterparts (Smith and Kotin, 2000), two of these three sequence motifs are present in their initiator proteins. The two motifs are an HUHUUU cluster, (where U is a hydrophobic residue), presumed to bind a Mg<sup>2+</sup> ion required for the nicking activity, and a YxxxxK motif, containing the active-site tyrosine that attacks the phosphodiester bond. During RCR the initiator protein nicks a single strand of DNA at a specific sequence in the replication origin, carrying out a trans-esterification reaction that forms a 5'-phosphate linkage between the DNA and the conserved active site tyrosine in the initiator, and liberates the 3'-OH adjacent to the nick site. This base-paired 3' nucleotide then primes unidirectional strand displacement synthesis, while the initiator protein that made the nick remains covalently attached to the 5' end of the parental strand. When the newly synthesized strand completes one round of replication it returns to the nick site, and at this point the original initiator complex, still attached to the parental strand, attacks the regenerated duplex nick site, or in some cases a nearby second site, carrying out a topoisomerase-like nicking-joining reaction. During this second reaction, it cleaves the new site, and is itself transferred across the analogous phosphodiester bond, becoming covalently attached to the new 5' end, while effectively ligating the 5' end of the parental strand to which it was originally attached to the new 3' nucleotide. The exact mechanism of this second reaction varies somewhat between replicons, sometimes involving a second active site tyrosine in the initiator, as seen in the gene A protein of φX174 (Baas and Jansz, 1988), but more commonly using the analogous active-site tyrosine in a second initiator protein molecule, present as part of a multimeric nickase complex (Rasooly *et al.*, 1994).

Strategies used by RCR initiators to engage the nick site are also seen in parvovirus replicons. Thus, most RCR origins are presented as duplex DNA, which must be melted before they can be cleaved by the initiator protein. To achieve this RCR initiators typically bind site-specifically to canonical

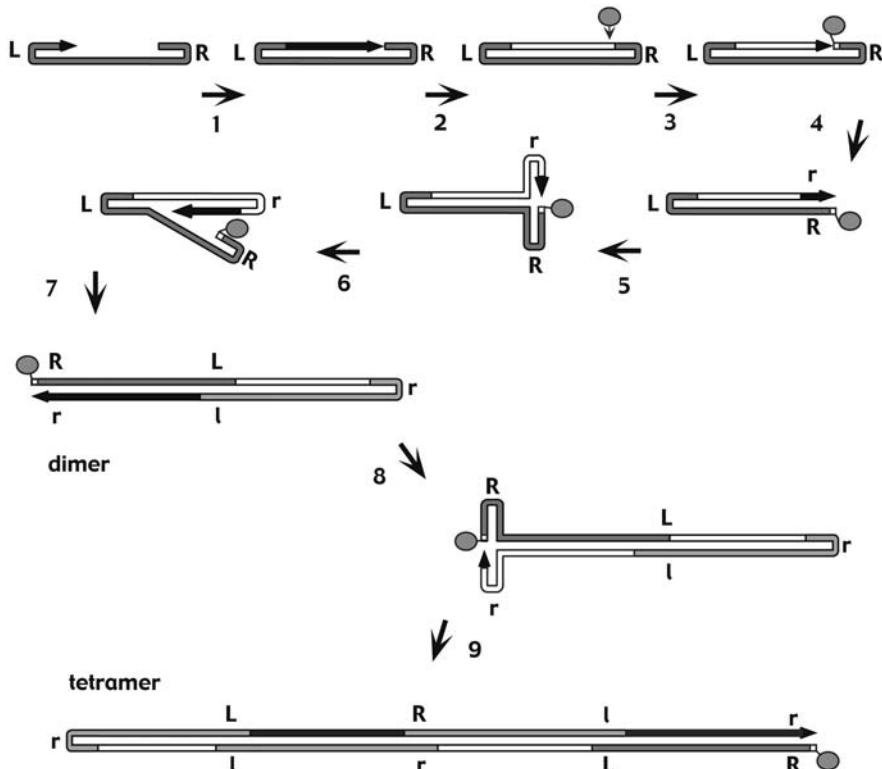
duplex DNA-binding sequences positioned in the origin next to the initiation site, and melt the latter via a process that consumes energy in the form of adenosine triphosphate (ATP), and which is often assisted by the ability of the separated strands to reconfigure into stem-loop structures with the nick site presented on an exposed loop. Like many of their RCR counterparts, parvovirus initiators have an associated helicase activity, which allows them to melt the origin prior to nicking, and subsequently to serve as the 3' to 5' helicase in the replication fork.

Strand displacement synthesis, as seen in all RCR replicons, appears inherently susceptible to template strand switching between homologous sequences, although the molecular basis for this remains poorly defined and may be complex. For example, unidirectional forks may be inherently less stable than their bidirectional counterparts, or template switching may be promoted by the fact that replication displaces long stretches of single-stranded DNA, which can invade and compete for the fork. Alternatively, the fork itself may be prone to strand switching, perhaps because it is organized by multimeric initiator complexes that can bind multiple DNA strands, as has been demonstrated for AAV Rep68/78/DNA complexes (Muzychuk and Berns, 2000). Inverted repeat sequences are always particularly susceptible to switching, as shown by the recent elegant demonstration that mutations introduced into

palindromic sequences flanking the nick site of porcine circovirus are readily corrected by copying the other arm of the palindrome (Cheung, 2004). Template strand switching during MVM replication *in vivo* results in, for example, the structures of type II defective genomes (Faust and Ward, 1979), which appear to be formed when replication forks, mostly those proceeding leftward from the right-end origin, stall and then reform using the nascent 3' end as primer but the displaced strand as a template. This reverses the direction of synthesis within the genome, creating an extensive hairpin molecule in which a single-stranded loop is flanked by complementary copies of the right-end hairpin. This type of strand jumping by the replication fork is inherent in the 'rabbit ear' step of parvovirus telomere regeneration, discussed below, and forms the basis of a heterocruciform resolution model that attempts to explain how sequences at the left-end of MVM are conserved in a single orientation.

## ROLLING-HAIRPIN SYNTHESIS

Parvoviruses replicate their linear genome through a series of duplex, concatemeric intermediates by the unidirectional, quasi-circular, rolling-hairpin mechanism depicted



**Figure 14.2** Parvoviral 'rolling-hairpin' DNA replication scheme. The parvoviral genome is represented by a continuous line, shaded dark gray for the original genome, light gray for progeny genomes and black for newly synthesized DNA, the 3' end of which is indicated by the arrowhead. The gray sphere represents an NS1 molecule. The letters L and R represent left-end and right-end palindromic sequences, respectively. Upper and lower case represent 'flip' and 'flop' versions of these sequences, respectively, which are inverted complements of one another.

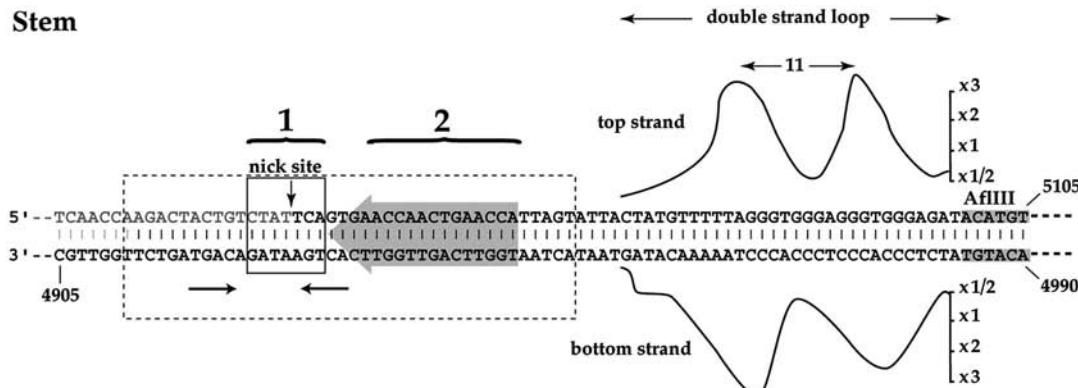
in Figure 14.2, as originally suggested nearly 30 years ago (Tattersall and Ward, 1976). Like all RCR replicons, parvoviruses rely heavily on the synthetic machinery of their host cell for their own preferential amplification. Since MVM replication forks are aphidicolin-sensitive, require PCNA, are unidirectional, and result in the synthesis of a single, continuous DNA strand, synthesis is probably mediated by DNA polymerase  $\delta$  and its accessory proteins (Bashir *et al.*, 2000; Christensen and Tattersall, 2002). In general, the parvoviruses are unique among RCR replicons in having a linear genome that is capped at each end by short imperfect palindromic sequences. These terminal sequences are repeatedly unfolded and refolded during replication, first creating duplex hairpin telomeres, in which the 3' nucleotide of the strand is paired to an internal base to create a DNA primer and then unfolding to allow the hairpin to be copied. During RHR they serve effectively as 'toggle-switches', reversing the direction of synthesis at each end of the genome, thus modifying the ancient rolling-circle strategy for the amplification of the linear parvoviral chromosome.

The rolling-hairpin model depicted in Figure 14.2 rationalizes, in a simple progression of forms, a wealth of experimental data obtained over the last 30 years from both *in vivo* and *in vitro* studies (reviewed in Cotmore and Tattersall, 1995, 1996). Many steps in the model represent intermediates that are readily observed *in vivo* as, for example, monomer or dimer RF molecules with telomeres in the form of covalently-closed 'turn-around' hairpins or 'extended-form' duplex palindromes. However, the fact that such forms accumulate means that the rolling-hairpin mechanism must pause after their completion, at least some of the time. In reality, a continuum of replicative intermediate (RI) DNA forms are generated *in vivo*, which are represented in the model rather simply by partially single-stranded forms, for example, the product of step 6 in Figure 14.2. However, during peak amplification in the infected cell, the RI forms generated commonly achieve very high molecular weights, and have complex branched structures, which appear to represent both the synthesis of multimeric concatemers and their concomitant processing (Cotmore and Tattersall, 2005a).

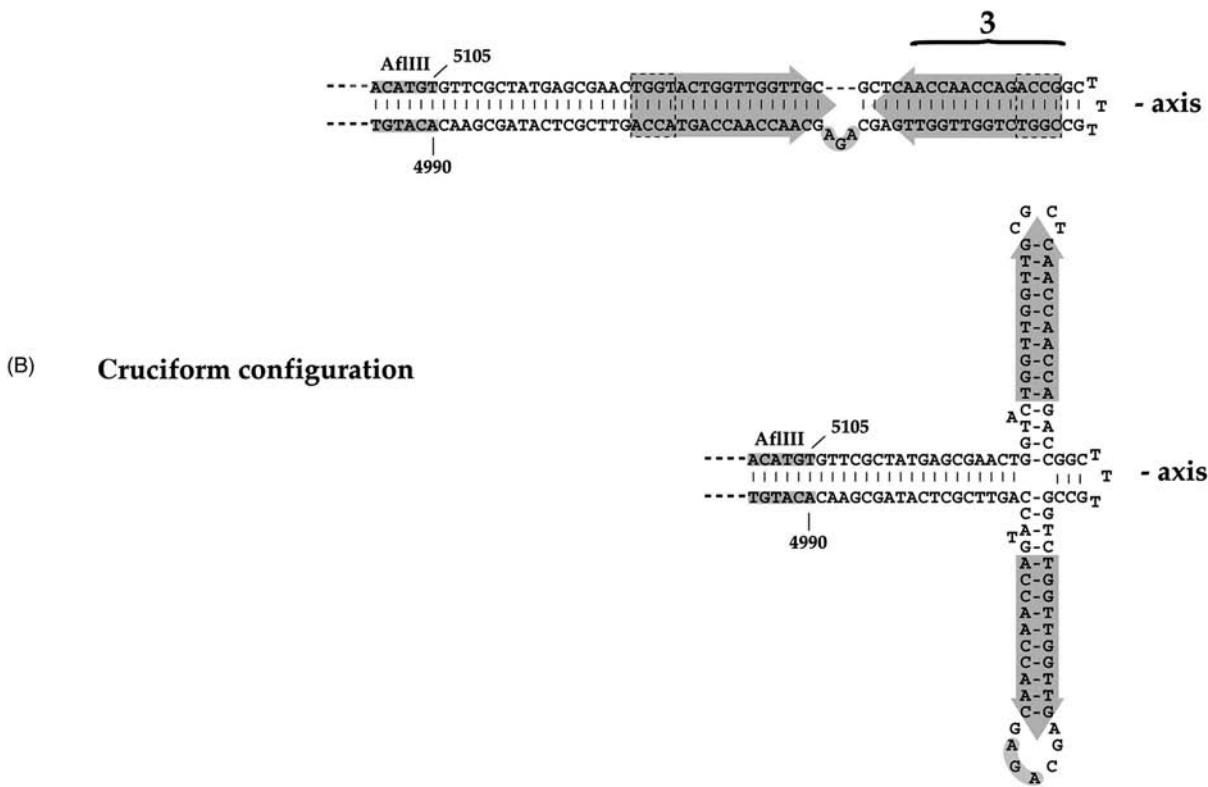
As shown in step 1 of Figure 14.2: the left-end (3') hairpin of the incoming negative-sense MVM genome primes complementary strand synthesis, converting virion DNA into the first duplex intermediate. This is a monomer-length, duplex molecule in which the two strands are covalently cross-linked at the left end by a single copy of the viral telomere and it probably functions as the initial viral transcription template. However, synthesis of this intermediate necessarily precedes NS1 expression, so that when the cellular fork reaches the right (5') end of the genome it is unable to displace and copy the right-end hairpin. This allows the 3' end of the new DNA strand to be ligated to the 5' end of the hairpin by a host ligase, creating the covalently continuous duplex molecule (Lochelt *et al.*, 1989;

Cotmore *et al.*, 1989; Baldauf *et al.*, 1997), shown in step 2 of Figure 14.2. This step resynthesizes the ~24 nucleotides originally present as the 'tether' sequence, mentioned earlier, which was located on the outside of the incoming virion attached to an NS1 molecule, but which is lost during cell entry. Replication beyond this point requires expression of NS1, which nicks the covalently-closed right-end hairpin, as illustrated in step 3 of Figure 14.2 in a reaction that is assisted by a host DNA-bending protein from the high mobility group 1/2 (HMG1/2) family (Cotmore and Tattersall, 1998). This nick is introduced into a complex replication origin, OriR, created by sequences in and immediately adjacent to, the right hairpin, as discussed in greater detail below. The left-end telomere of MVM also contains sequences that can give rise to a replication origin in a subsequent higher-order duplex intermediate, but these are inactive in the hairpin configuration of the terminus present in the first monomer duplexes (Baldauf *et al.*, 1997), so that NS1-mediated replication invariably initiates at the right end of the genome. Nicking liberates a base-paired 3' nucleotide that supports the assembly of a new replication fork, while leaving NS1 covalently attached to the 5' end of the DNA at the nick, where it is believed to function as the 3'-to-5' replicative helicase (Christensen and Tattersall, 2002). NS1 also recruits the cellular RPA (replication protein A) complex, which is essential both for the establishment of the new fork and for binding and stabilizing displaced single strands, so that the availability of this factor appears rate-limiting for the processivity of the ensuing strand-displacement mechanism (Christensen *et al.*, 1997a; Christensen and Tattersall, 2002).

A complex sequence of melting and re-annealing reactions then occurs, as depicted in steps 4 and 5 of Figure 14.2 in which the replication fork, now assisted by NS1 first unfolds and copies the hairpin (step 4). This process, called 'hairpin transfer', replaces the original sequence of the terminus with its inverted complement. Unfolding of the hairpin requires NS1 (Willwand *et al.*, 1997), but whether this unfolding is mediated simply via its processive helicase activity in front of the fork, or by specific destabilization of the duplex following DNA binding at one of its 5'-(ACCA)n-3' recognition sites, is uncertain. Since the terminal sequences are imperfect palindromes and this inversion occurs with every round of replication, progeny genomes comprise equal numbers of each terminal orientation, dubbed 'flip' and 'flop' (represented by 'R' and 'r' in Figure 14.2). In MVM the entire sequence of reactions from the introduction of the NS1-mediated nick to the generation of a duplex extended-form telomere was originally referred to as 'hairpin resolution', but is now more commonly termed 'terminal resolution' in accord with the use of this term to describe an essentially similar process in AAV (Snyder *et al.*, 1990; Cotmore and Tattersall, 1996). A general rule seems to be that viruses with identical termini, like the AAVs and B19, replicate both ends by terminal resolution and thus generate flip and flop forms of each telomere, while viruses with

**Stem**

## (A) Linear configuration



## (B) Cruciform configuration

**Figure 14.3** The structure of MVM OriR, cellular co-factors and the mechanism of initiation. The DNA sequence of the MVM right-end palindrome is shown in the flip sequence orientation in both its linear (A) and cruciform (B) configurations. Size constraints require that this structure is divided into two parts, around the single AfIII site in the stem, which is represented in both halves. In the linear form, the perfect duplex stem is interrupted by a single three-base insertion, AGA, shown shaded in gray. Above the sequence, recognition elements that are essential for nicking are indicated by horizontal brackets, and numbered 1–3. Gray arrows denote ACCA sequences that bind NS1, the direction of the arrow indicating the position of the ~20 base 5' sequence that is protected from digestion with DNase I by NS1 complexes bound at this site. The sequence of the entire NS1 DNase I footprint at the nick site is also shown boxed with dashed lines, as are those ACCA sequences in the axis-proximal NS1 binding sites that are disrupted in the formation of the internal cruciform. Inverted tetranucleotide repeats, surrounding the nick site, that may help to stabilize its melted structure are underlined in bold. Compared to naked DNA, NS1 binding results in enhanced DNase I cleavage at most points between the two specific NS1 footprints, but addition of HMG1 alters this pattern, so that cleavage shows a 10–11 base periodicity on both strands, which is characteristic of looped double-helical DNA (Cotmore et al., 2000). The position of this induced loop is graphed on the hairpin stem for each strand as a pattern of alternate over- and undercutting, with the Yaxis representing the intensity of the resulting cleavage product relative to naked DNA (set at 1X) and to NS1-only bound DNA (which is constant at ~2X). DNase is positioned over the minor groove and cuts phosphate groups symmetrically to either side, but in looped DNA all grooves on the inside of the circle narrow due to compression, while those on the outside become correspondingly wider, so that this pattern of over- and undercutting indicates which nucleotides are on the inside and outside of the loop. In panel B, the triplet insert AGA, now positioned at the dyad axis of the cruciform is again shaded in gray.

disparate termini, like MVM, replicate their right-end by terminal resolution and their left end by an asymmetric mechanism, called junction resolution, which conserves a single-sequence orientation and requires quite different structural arrangements and co-factors to activate the NS1 nickase. However, the original isolation of AAV DNA intermediates containing covalently linked plus and minus strands, which, under denaturing conditions, yielded single strands at least four times the length of the genome (Straus *et al.*, 1976), indicates that duplex concatemers requiring some form of junction resolution are also produced during AAV replication.

The extended-form duplex termini generated in step 4 of Figure 14.2 are then melted out and individual strands caused to fold back on themselves, creating hairpinned 'rabbit ear' structures (step 5 of Figure 14.2). This process is greatly enhanced by NS1 (Cossions *et al.*, 1996; Willwand *et al.*, 1998), which binds directly to symmetrical copies of its duplex recognition sequence that surround the axis of the extended palindrome, as illustrated in Figure 14.3 (Willwand *et al.*, 2002). This refolding is dependent upon ATP hydrolysis and so requires the NS1 helicase function as well as its site-specific binding activity (Willwand *et al.*, 1997). Rabbit-ear formation allows the 3' nucleotide of the newly synthesized DNA strand to pair with an internal base, thus repositioning the fork, in what is essentially a strand-switching maneuver and consequently priming synthesis of additional linear sequences (step 6 of Figure 2). At the left end of the genome, NS1 is probably required to unfold the hairpin, but this has not been formally proven. However, there is evidence to suggest that NS1 is directly involved in melting-out and reconfiguring the resulting extended-form left-end duplexes into rabbit ear structures, although this reaction appears less efficient than the equivalent reaction at the right-end terminus (Willwand *et al.*, 1998). The result of rolling-hairpin synthesis is that the coding sequences of the virus are copied twice as often as the termini and duplex dimeric (step 7) and tetrameric (step 9) concatemers are generated, in which alternating unit length genomes are fused, through a single palindromic junction, in left-end:left-end and right-end:right-end orientations.

## THE MVM RIGHT-END ORIGIN, OriR; A SUBSTRATE FOR TERMINAL RESOLUTION

Duplex turn-around forms of the right end of all known parvovirus genomes are resolved to an extended-form configuration containing two copies of the palindrome by terminal resolution. First suggested by Cavalier-Smith as a theoretic solution to the problem of maintaining the sequence of linear chromosome ends during DNA replication (Cavalier-Smith, 1974), this type of resolution has been recapitulated *in vitro* using MVM monomer RF and virion DNA substrates (Baldauf *et al.*, 1997).

The right-end hairpin of MVM contains 248 nucleotides. Size constraints require that, in Figure 14.3, this is shown in two sections, divided at the single AflIII site. This structure is most favorably arranged as an almost perfect duplex (Figure 14.3: panel A), with just three unpaired bases at the axis and a single mismatched region positioned 20 nucleotides from the axis. Here a three nucleotide insertion (AGA or TCT) on one strand separates opposing pairs of NS1 binding sites, creating a 36 basepair palindrome that can potentially assume an alternate cruciform configuration with little change in free energy (as illustrated in Figure 14.3: panel B). Whether or not this cruciform rearrangement is required for viability has yet to be demonstrated experimentally, but it would be expected to significantly destabilize the duplex, facilitating its ability to function as a hinge. Hairpins lacking the three nucleotide insertion but still able to rearrange into a cruciform structure support NS1-mediated terminal resolution *in vitro* with efficiencies that are comparable to wild-type termini (Willwand *et al.*, 1997). Such hairpins are also functional *in vivo* when transfected as part of an infectious plasmid clone, although the resulting virus is out-competed by wild-type virus (Costello *et al.*, 1995). However, substitution of an unrelated four nucleotide asymmetry restores its *in vivo* replication efficiency to wild-type levels, indicating that it is the presence of the mismatch, rather than its sequence, that is important (Costello *et al.*, 1995). This, therefore, favors the suggestion that the asymmetry simply helps to promote instability of the duplex.

Fully duplex linear forms of the right-end hairpin sequence also function as NS1-dependent origins (Cotmore and Tattersall, 1998; Cotmore *et al.*, 2000). However, in many other parvoviral telomeres the only elements absolutely required for origin-function are an initiator binding site positioned next to a nick site, so that the minimal sequences required for nicking occupy less than 40 basepairs. In contrast, the minimal MVM right-end origin is around 125 basepairs in length, and includes almost the entire sequence of the right-end hairpin (Cotmore *et al.*, 2000). This is because at least three specific recognition elements are involved, as shown in Figure 14.3A. These include the consensus nick site, 5'-CTWWTCA-3' (element 1), positioned seven nucleotides upstream from a duplex NS1-binding site (element 2), that is oriented in such a way that the bound NS1 complex extends over the nick site, and a second NS1-binding site (element 3), located immediately next to the axis of the hairpin. While this second binding site is over 100 basepairs from the nick site, it is absolutely required for NS1-mediated cleavage (Cotmore *et al.*, 2000). The consensus nick site for MVM, 5'-CTWWTCA-3', was derived simply by comparing the somewhat disparate sequences present in the origins at the two ends of the genome, and while it is known that this entire consensus element cannot be deleted (Cotmore *et al.*, 2000) or inverted (Christensen and Tattersall, 2002), the importance of individual nucleotides has not been determined experimentally. The

exact position of the nick within this consensus appears slightly variable *in vivo*, plus or minus one nucleotide, (Cotmore *et al.*, 2000), but analysis of cleavage products generated *in vitro* from 5'-labeled substrates suggests that the position indicated in Figure 14.3 is preferred (Christensen and Tattersall, 2002). During nicking this site is predicted to be exposed as a single strand, perhaps stabilized as a minimal stem-loop structure by the tetranucleotide inverted repeats that flank the site. In a closely related virus LuIII, there is a two-base insertion, 5'-AT-3', immediately upstream of the indicated position of the nick. This insertion impairs the efficiency of NS1-mediated replication initiation *in vitro*, and creates a virus that generates a slightly different spectrum of duplex replicative-form DNAs *in vivo*, and subsequently packages approximately equimolar plus- and minus-sense DNA strands (Cotmore and Tattersall, 2005a), as discussed later.

Degenerate oligonucleotide selection studies suggest that optimal forms of the NS1-binding site should contain at least three tandem copies of the 5'-ACCA-3' tetranucleotide repeat sequence, but that 50 percent degeneracy in one of these motifs or insertion of a single base between them has little effect on affinity (Cotmore *et al.*, 2000). This suggests that NS1 binding is modular, with each tetranucleotide motif being recognized independently by different molecules in the NS1 complex, a concept that is supported by the recently determined X-ray structure of Rep nuclease complexed with oligonucleotides representing its binding motif (Hickman *et al.*, 2004). Viewed from this perspective, the NS1-binding site that orients NS1 over the nick site in the right-end origin, 5'-(ACCA)(ACTG)A(ACCA)-5', is clearly recognized as a high affinity site despite its single base insertion and the degeneracy of the second tetranucleotide motif. In the presence of ATP, which promotes the assembly of NS1 into higher order multimers, NS1 binds asymmetrically over this sequence, protecting a 41 basepair region from digestion with DNaseI (shown boxed in Figure 14.3A). This footprint extends five nucleotides 3' of the ACCA box repeat, but projects 22 nucleotides beyond its 5' end, so that the footprint terminates 15 basepairs beyond the nick site, apparently positioning NS1 to nick the origin. However, nicking does not occur unless the distant, axis-proximal NS1-binding site (element 3 in Figure 14.3) is also present in the origin and the entire complex is activated by addition of HMG1, as discussed below. The axis-proximal NS1-binding site is particularly interesting because it is closely juxtaposed to a third NS1-binding site, which makes up the inner arm of the cruciform in such a way that the ~20 base 5' projections of NS1 from both sites should overlap. This conflict might either force NS1 molecules to compete for binding at the two sites, or force the palindrome to reconfigure into a cruciform, thus creating room for both 5' extensions. In the event, footprints suggest that neither scenario is realized, rather inverted juxtaposition of the two sites increases the binding affinity of NS1 to each,

without shifting the footprints from their expected positions, suggesting that a more stable rearranged or higher order NS1 complex is formed.

In the absence of NS1, HMG1 binds the hairpin sequence-independently, causing it to bend, as can be measured by circular DNA ligation assays, but without protecting any specific region from digestion with DNaseI. However, HMG1 can be shown to bind directly to NS1 *in vitro* (Christensen, personal communication) and it effectively mediates interactions between the NS1 molecules bound to their various recognition elements in the origin, so that HMG1 is absolutely required for formation of the cleavage complex (Cotmore and Tattersall, 1998; Cotmore *et al.*, 2000). The ability of the axis region to reconfigure into a cruciform does not appear to be critical for this process, since the aforementioned third NS1 recognition sequence, positioned immediately inboard of the AGA asymmetry, can be mutated without impairing nicking efficiency *in vitro*, even though this mutation completely disrupts the potential cruciform structure (Cotmore *et al.*, 2000). Cleavage is also absolutely dependent upon the correct spacing of the various elements, since insertions or deletions of more than one base at the AflIII site in the stem (Figure 14.3) are lethal, but sequence substitutions at this position are tolerated. DNaseI footprinting studies show that addition of HMG1 induces only very slight shifts in the sequences protected by NS1, but the conformation of the intervening DNA changes, folding into a double helical loop that extends for ~30 basepairs through a characteristic G-rich element in the hairpin stem. Between this G-rich element and the nick site there is a stretch of five thymidine residues that are included in the loop and the site it flanked by a region rich in alternating A and T residues, which would be expected to provide enhanced flexibility. Mutant origins that do not reconfigure into a double-helical loop upon addition of HMG1 also fail to nick (Cotmore and Tattersall, unpublished), presumably because creation of the loop allows the terminus to assume a specific 3D structure and thus indicating that the configuration of this entire telomere complex is required to activate the nickase. Specific bending patterns of this type have also been reported in some resolvase and transposase nicking complexes that similarly require coordination by cellular DNA bending proteins (Salvo and Grindley 1988; Sarkis *et al.*, 2001).

Following nicking, a replication fork is established at the newly-exposed 3'-nucleotide that proceeds to unfold and copy both arms of the hairpin. Thus, the ultimate product of terminal resolution is a duplex extended-form terminus, containing two complete copies of the palindrome. Such molecules can be shown to support repeated rounds of terminal resolution *in vitro*, leading to the displacement of single copies of the palindrome linked at their 5' termini to an NS1 molecule (Baldauf *et al.*, 1997; Willwand *et al.*, 1998), but this would be highly counterproductive if it were to occur *in vivo*. Thus a mechanism probably exists to

promote the selection of hairpin over linear substrates. While this may simply reflect the enhanced efficiency with which hairpin substrates are nicked *in vitro* (Cotmore and Tattersall, 1998), there may also be mechanisms in place *in vivo* to promote rapid progression to the next step in rolling-hairpin synthesis, namely rabbit-ear formation. As mentioned above, this refolding reaction is mediated by NS1, requires ATP hydrolysis, and is dependent upon the presence of NS1-binding sites that flank the symmetry axis in the extended-form terminus. If such termini are generated *in vitro* in an NS1-supplemented cellular replication extract, rabbit-ear formation does sometimes occur, but it is very inefficient (Baldauf *et al.*, 1997), perhaps because of the limited supply of a critical cellular factor(s) or because recombinant NS1 does not reflect the full spectrum of post-translationally modified forms of NS1 observed during infection (Corbau *et al.*, 1999). As discussed in detail in Chapter 19 of this volume, it does seem likely that changes in the post-translational modification of NS1 could modulate its oligomeric state and that switching from processive synthesis to rabbit-ear formation at the end of terminal resolution may require different types of NS1 complexes. However, rabbit-ear formation creates a new DNA primer and so should immediately be followed by the assembly of a new replication fork. Thus it is tempting to speculate that the replication complex responsible for hairpin transfer, with its associated NS1 helicase complex, remains essentially intact and associated with the terminus during rabbit-ear formation and is ready to recommence displacement synthesis following completion of the refolding process.

Individual genomes are excised from duplex concatemers generated during rolling-hairpin synthesis and their telomeric sequences reduplicated, by a combination of terminal resolution and junction resolution. Thus concatemeric intermediates, such as those shown in Figure 14.2, p. 176, may support multiple forks simultaneously *in vivo*. Since both linear and hairpin forms of the right telomere support initiation, resolution of duplex right-end:right-end junctions may occur symmetrically on the basepaired duplex junction sequence, or after this duplex sequence has melted locally and transiently reconfigured into two hairpin structures.

Which reaction predominates is unclear, since both reactions generate equal numbers of sequences in flip and flop orientations and the products of the two reactions appear identical. *In vivo*, right-end junction resolution proceeds more efficiently than left-end:left-end resolution, so that dimer duplexes containing right-end junctions are underrepresented *in vivo* (Cotmore and Tattersall, 2005a). Resolution of left-end:left-end junctions appears more complicated, since it proceeds asymmetrically and results in the generation of left-end termini in a single-sequence orientation. However, it simply recapitulates the nicking and rabbit-ear formation steps seen in terminal resolution, but using an asymmetric template created by inactivation of

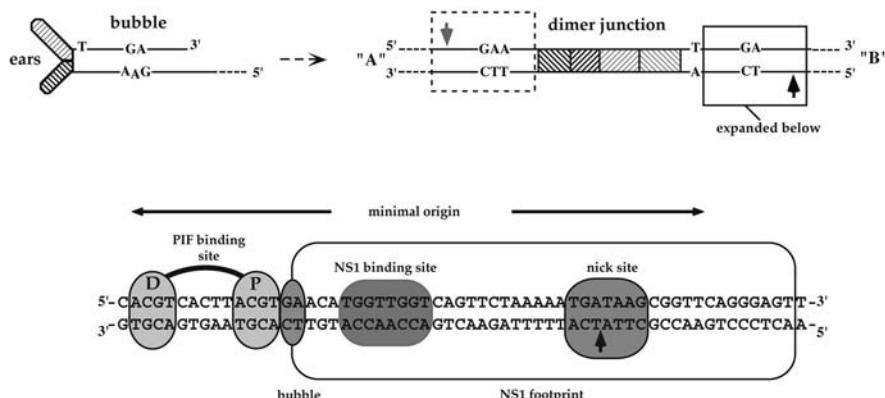
the nick site associated with one arm of the hairpin. In order to understand this process, we first explore the structure of the left hairpin of MVM, and of the left-end:left-end junctions in concatemeric RF molecules.

## THE MVM LEFT-END ORIGIN; SETTING UP ASYMMETRIC JUNCTION RESOLUTION

In negative-sense MVM genomes the 121 nucleotide left-end hairpin of MVM exists in a single flip sequence orientation, as shown in Figure 14.4. This Y-shaped telomere contains small internal palindromes, which fold into the 'ears' of the Y, and a 43-basepair duplex stem region that is interrupted by an asymmetric thymidine residue and a mismatched 'bubble' sequence, where the triplet 5'-GAA-3' on the inboard arm is opposed to the doublet 5'-GA-3' in the outboard strand. Sequences from this hairpin are involved in both replication and transcriptional control, with the elements involved in these two processes effectively segregating in the two arms of the hairpin.

This telomere, and presumably the analogous extremity of most other paroviruses that have disparate left and right termini, cannot function as a replication origin in its hairpin configuration. Instead, it generates a single competent origin when the hairpin is extended and copied during replication to form the fully basepaired palindromic junction sequence that spans adjacent genomes in dimer RF, illustrated in Figure 14.4 (Cotmore and Tattersall, 1994). Within this structure the sequence from the outboard arm, surrounding the GA dinucleotide, serves as an origin, called  $OriL_{TC}$ , while the equivalent sequence from the inboard arm containing the bubble trinucleotide, called  $OriL_{GAA}$ , does not (Cotmore and Tattersall 1994). Instead, the inboard arm and the hairpin configuration of the terminus appear to function as upstream control elements for the viral P4 transcriptional promoter (Faisst *et al.*, 1994; Perros *et al.*, 1995). This ability to segregate one arm of the palindrome which cannot be nicked by NS1 appears essential to the virus. Thus, mutated genomes carrying active origins on both arms of the telomere are non-viable, but allow the selection of viable variants in which the origin associated with the inboard arm has been inactivated by a variety of secondary mutations (Burnett, Cotmore and Tattersall, unpublished).

As seen in Figure 14.4: the minimal linear origin is ~50 basepairs long, extending from two 5'-ACGT-3' motifs spaced five nucleotides apart at one end, to a position some seven basepairs beyond the nick site. While the actual sequence of the 'GA' doublet is relatively unimportant, insertion of any third nucleotide here inactivates the origin, indicating that the bubble is a critical spacer, rather than a recognition element in its own right. Within the origin there are three essential recognition sequences: an NS1-binding site that orients the NS1 complex over its



**Figure 14.4** Junction resolution reaction for the left-end of MVM. (Top left) A schematic representation of the 3' end of single-stranded MVM DNA, showing its three major palindromic elements folded into a stem plus ears structure in which the stem contains the mismatched bubble and asymmetric T residue described in the text. This is unfolded and copied during replication to form the junction fragment that spans adjacent genomes in dimer RF (top right). Hatched boxes represent palindromic sequences that originally formed the internal ears of the hairpin. The boxed sequence, expanded below, represents the minimal active replication origin from the outboard arm (with the bubble dinucleotide), designated  $OriL_{TC}$ , while the sequence in the dashed box represents the inactive corresponding sequence in the inboard arm (with the bubble trinucleotide) which is dubbed  $OriL_{GAA}$ . The potential nicking sites on each side of the junction are denoted by vertical arrowheads. The lower panel depicts the sequence of  $OriL_{TC}$ , the active form of the minimal left-end origin, showing the different elements involved in replication. The PIF binding site (see text) overlaps a consensus binding site for the CREB/ATF family of host transcription factors. Other boxes indicate sequences involved in the bubble dinucleotide spacer element, the (ACCA)2 NS1 binding site, and the nick site, a specific sequence required for nicking and covalent attachment of NS1. Sequences protected by NS1 from DNase I digestion are boxed (Christensen *et al.*, 1995).

consensus nick site, 5'-CTWWTCA-3', which is positioned 17 nucleotides downstream, and the two ACGT motifs.

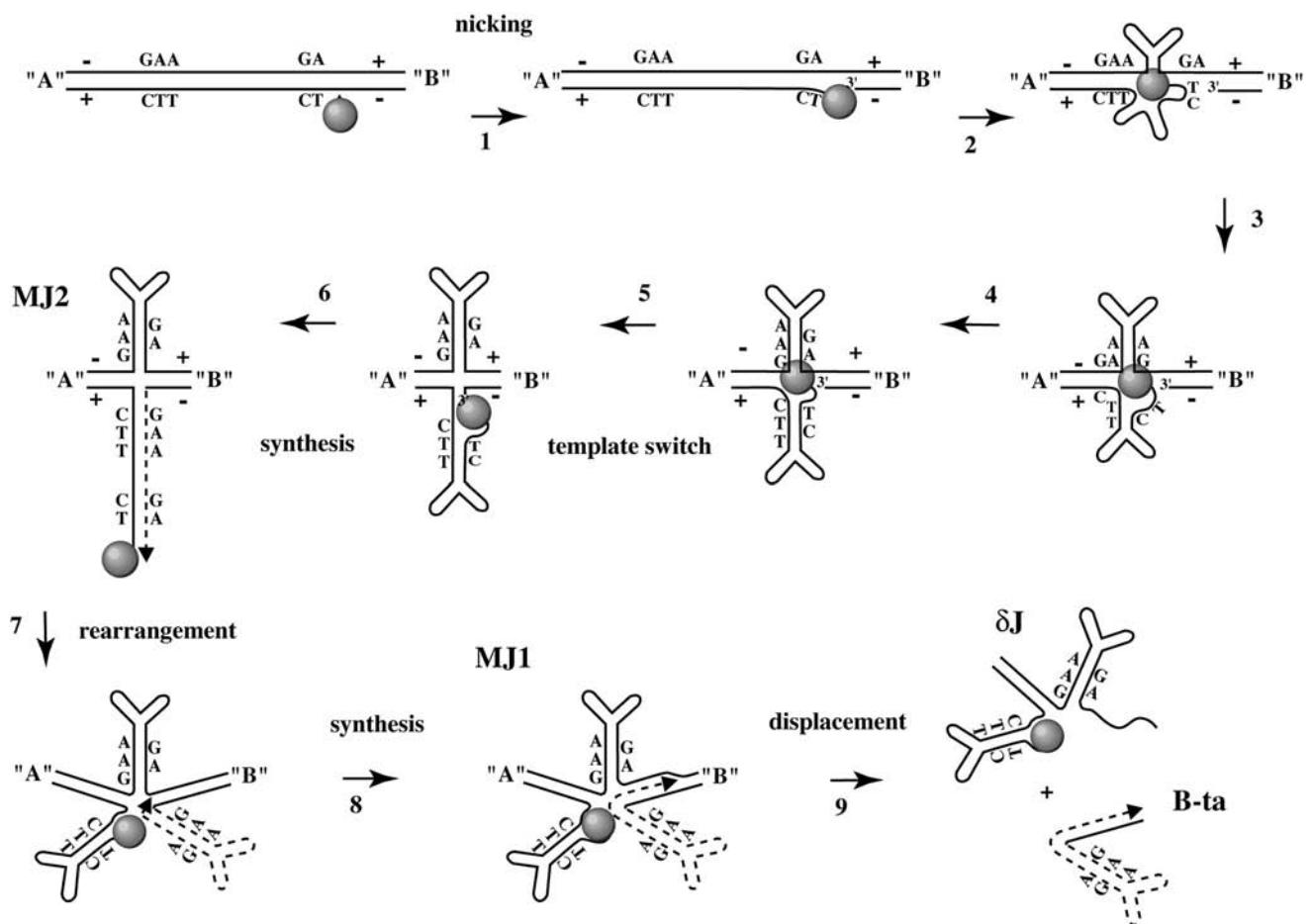
These bind a heterodimeric cellular factor variously called PIF, for parvovirus initiation factor (Christensen *et al.*, 1997a, 1999) or GMEB, for glucocorticoid modulating element-binding protein (Zeng *et al.*, 1998), which is abundantly expressed in many cell types (Zeng *et al.*, 2000).

PIF is a site-specific DNA-binding heterodimeric complex comprising p96 and p79 subunits, which functions as a transcriptional modulator in the host cell. It binds DNA via a novel 'KDWK' fold (Bottomley *et al.*, 2001; Surdo *et al.*, 2003) first recognized in DEAF-1, the drosophila homeobox protein deformed enhancing activating factor (Gross and McGinnis, 1996) and its binding specificity is unusual because it recognizes two ACGT half-sites, but the spacing between them can vary substantially, from one to nine nucleotides, with an optimal spacing of six nucleotides (Christensen *et al.*, 1997b; Burnett *et al.*, 2001). This factor stabilizes the binding of NS1 on the active form of the left-end origin,  $OriL_{TC}$ , but not on the inactive form,  $OriL_{GAA}$ , because the two complexes are able to establish contact over the bubble dinucleotide, but this interaction does not occur when a single extra intervening residue is inserted (Christensen *et al.*, 2001). The left-end hairpins of all other members of the *Parvovirus* genus have bubble asymmetries and PIF-binding sites, although details of the spacing vary somewhat between species, suggesting that a similar origin segregation mechanism is used throughout this branch of the parvovirus family.

## ASYMMETRIC JUNCTION RESOLUTION THROUGH A CRUCIFORM INTERMEDIATE: SYNTHESIS OF NEW TERMINI IN A SINGLE SEQUENCE ORIENTATION

Given the precise location of the active origin,  $OriL_{TC}$ , in the dimer junction, synthesis of new copies of the left-end hairpin in the correct, flip, sequence orientation is clearly not a straightforward process because a replication fork progressing from this site through the linear bridge structure would be expected to synthesize new DNA in the flop orientation. However, the MVM dimer junction is resolved asymmetrically *in vivo* and *in vitro* (Cotmore and Tattersall, 1992; Cotmore *et al.*, 1993), and it now appears that this process occurs via a novel cruciform intermediate, a maneuver that fulfills two functions, it both allows synthesis of new DNA in the single, correct, sequence orientation and it creates a structure that can ultimately be resolved by NS1 (Cotmore and Tattersall, 2003). Based on the observed *in vivo* products and the kinetics with which a series of resolution intermediates were generated *in vitro*, the 'heterocruciform model' (Cotmore and Tattersall, 2003) suggests that resolution is driven by the helicase activity of NS1, and depends upon the inherent instability of the duplex palindrome, a property that allows it to switch between a linear and a cruciform structure.

According to this model, outlined in Figure 14.5, NS1 initially introduces a site-specific single-strand nick in  $OriL_{TC}$ ,



**Figure 14.5** The heterocruciform resolution pathway. After nicking the initiation site in the B arm of the dimer bridge (step 1), NS1 associates with RPA to function as a 3'-to-5' helicase (step 2), unwinding the lower strand of the palindrome and allowing the exposed single strands to fold-back on themselves, creating a cruciform intermediate (step 3). Branch migration proceeds (step 4), eventually passing the inactive initiation site in the A arm. At this point the exposed 3' nucleotide can switch templates and anneal to its complement in the lower cruciform arm (step 5). A replication fork assembling at this time will copy and unwind the cruciform arm, synthesizing a palindrome in the flip orientation on the end of the negative-sense B strand (step 6). This heterocruciform structure corresponds to the MJ2 intermediate. In a second duplex-to-hairpin transition, the palindromic heterocruciform arm of MJ2 is then melted out and both strands fold back on themselves (step 7), allowing the exposed 3' end to basepair with inboard sequences in the B arm. A replication fork established at this 3' end would copy the lower strand of the B arm (step 8), creating the MJ1 intermediate and progressively displacing the upper strand, leading to the eventual release of a newly-synthesized B turn-around form (step 9). The residual  $\delta J$  intermediate is partially single-stranded, having an intact upper strand paired to an NS1-associated lower strand from the A arm. Since this complex carries the active helicase, it is presumed to be a dynamic structure in which the bridge palindrome is periodically reconfigured into a cruciform structure, as shown.

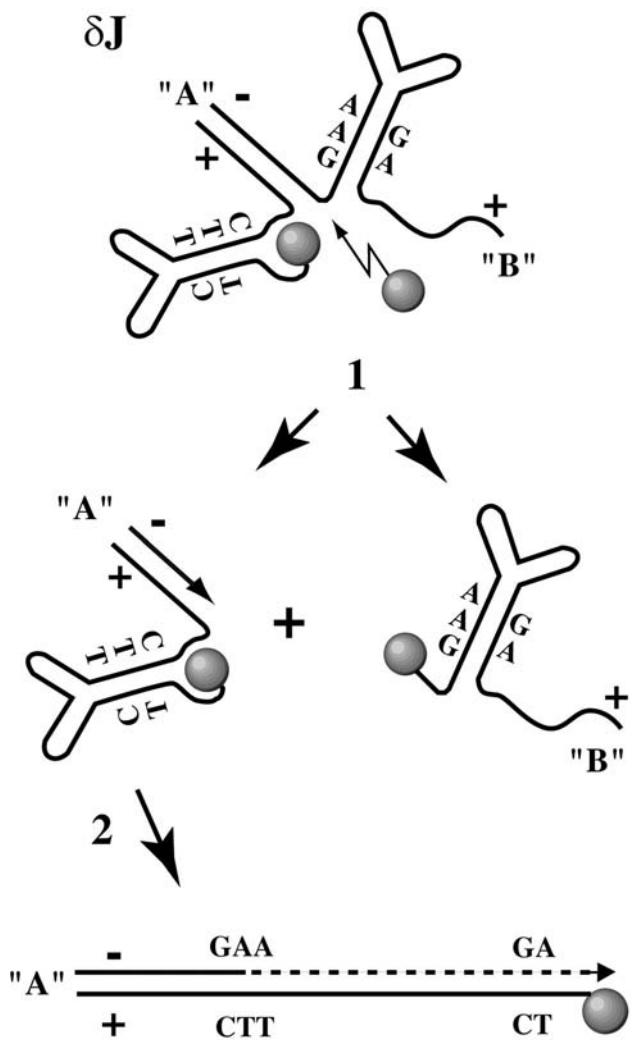
in the B arm of the junction, becoming covalently attached to the DNA at the 5' side of the nick and exposing a base-paired 3' nucleotide (step 1 of Figure 14.5). Two outcomes are then possible, depending upon the speed with which a replication fork is assembled. If the fork assembles rapidly, while the junction remains in its linear configuration, 'read-through' synthesis simply copies the upper strand, regenerating the duplex junction and displacing a positive sense single strand that feeds back into the replicating pool. This promotes amplification of duplex MVM DNA, but does not lead to synthesis of new terminal sequences in the correct orientation or to junction resolution. In order to

generate a structure that can be resolved, the initial nicking event must be followed by melting and rearrangement of the junction palindrome into a cruciform, driven by the 3' to 5' helicase activity of the 5'-linked NS1 complex (steps 2–4 of Figure 14.5). Once this cruciform extends to include sequences beyond the nick site, the primer exposed at the nick site in OriL<sub>TC</sub> undergoes a template switch by annealing with its complement in the lower cruciform arm (step 5 of Figure 14.5). If a fork assembles after this point, the resulting synthesis unfolds and copies the lower cruciform arm (Figure 5: step 6), creating a heterocruciform intermediate that contains the newly-synthesized telomere in the

flip sequence orientation, attached to the lower strand of the B arm. In kinetic analyses *in vitro*, this modified junction, dubbed MJ2, was seen to accumulate, along with the product of its subsequent rearrangement, MJ1 (steps 7 and 8), both ultimately giving way to a third intermediate, called  $\delta J$  (step 9). The lower arm of the MJ2 heterocruciform is an extended-form palindrome, essentially identical to those generated by terminal resolution reactions. Once synthesized, this becomes susceptible to rabbit-ear rearrangement, which repositions its 3' nucleotide so that it primes strand displacement synthesis back along the B arm of the junction (creating MJ1 intermediates, as in step 8) and leading to the release of a duplex form of the B arm with its left-end hairpin in the turn-around configuration (step 9). The residual sequences, now termed the  $\delta J$  intermediate, consist of the intact upper strand of the junction, paired to the lower strand of the A arm, which has an intact copy of the left-end hairpin, terminating in a 5' NS1 complex.

To this point, the sequence of the resolution reaction is relatively easy to follow *in vitro*, but the mechanism of the next step, even though it proceeds with reasonable efficiency, remains largely conjectural. Again, it is based on the ability of the 5'-linked NS1 helicase to destabilize the remaining duplex, allowing both palindromes to fold back on themselves, as depicted in Figure 14.6: and on the known ability of NS1 to nick single-stranded forms of its left origin without the help of its usual stabilizing co-factor, PIF (Nuesch *et al.*, 2001). According to this aspect of the model, the NS1 helicase would create a dynamic structure in which the nick site in the normally inactive A arm is transiently but repeatedly exposed in a single-stranded form, allowing NS1 to engage this site. This nick would leave NS1 covalently attached to a positive-sense B arm, which would then be released from the complex by strand displacement synthesis from the new 3' nucleotide exposed at the nick site (step 1 of Figure 14.6), generating a new extended form of the A arm (step 2).

However, when MVM genomes replicate *in vivo*, this single strand nick may not inevitably occur, because both ends of dimer RF bear efficient right-end hairpin origins. Thus, *in vivo*, forks may proceed back toward the dimer junction from the right end of the genome, copying the top strand of the B arm, before the final resolving nick occurs. This would effectively bypass dimer bridge resolution, recycling the top strand into a replicating duplex dimer pool. Interestingly, the single-strand nick would release a positive-sense DNA strand with its left hairpin in the flop orientation (step 3), which has not been described in MVM RF, but has been identified in the closely related virus, LuIII. Unlike MVM, LuIII packages DNA strands of both senses with equal efficiency (Bates *et al.*, 1984) and while the left-end hairpins of its negative sense strands are all in the flip orientation, in positive-sense DNA flip and flop orientations are expressed with equal frequency (Chen *et al.*, 1989). We have recently shown that, when compared with the right origin of MVM, the equivalent LuIII sequence has a two-base insertion immediately 3' to the nick site, which



**Figure 14.6** Introduction of a single-strand nick and resolution of the  $\delta J$  intermediate. The initiation site in the A arm of the palindrome is periodically exposed as a single strand during duplex-to-hairpin rearrangements of  $\delta J$ . This allows NS1 to attack the initiation site in  $OriL_{GAA}$  without the help of a cofactor (step 1). Nicking leads to the release of a positive-sense B strand, and leaves a base-paired 3' nucleotide on the A arm to prime assembly of a fork which will copy the hairpin, creating an extended-form of the A arm (step 2).

substantially impairs its efficiency. Thus, in LuIII, the reduced efficiency with which forks are generated at the right end of the genome may favor the single-strand nicking event, by simply allowing more time for it to occur.

## SYNTHESIS OF PROGENY SINGLE-STRANDS

Inhibitor studies suggest that displacement of single-stranded progeny genomes occurs predominantly (Myers and Carter, 1980) or exclusively (Zhou and Muzyczka, 1998) during active DNA replication, while genetic disruption of

the capsid gene indicates that single-stranded DNA only accumulates in cells that are also assembling viral particles (Rhode, 1976). These observations led to the hypothesis that the displacement of single strands might be concomitant with packaging and that the preassembled particle may simply sequester the genome in a 5'-to-3' direction as it is displaced from the moving fork (Cotmore and Tattersall, 1989). More recent studies indicate that low levels of non-packaged single-stranded AAV DNA can be generated, at least *in vitro* (Ward and Linden, 2000), but these are such active replication intermediates that they fail to accumulate. This has led to a re-evaluation of the packaging process and, as will be discussed in detail in Chapter 21, current data suggest that packaging likely proceeds in a 3'-to-5' direction (King *et al.*, 2001; Cotmore and Tattersall, 2005b), presumably using newly-displaced single-strands. At present it is not clear whether such strands are released into the nucleoplasm, so that packaging complexes are physically separate from replication complexes, or if the complex, branched, replication intermediates observed *in vivo* (Cotmore and Tattersall, 2005a) serve as combined replication and packaging substrates. In the latter scenario, newly displaced progeny genomes would simply be retained in the replication complex via interactions between their 5'-linked NS1 molecules and NS1 or capsid proteins physically associated with the replicating DNA.

Like MVM, most members of the genus *Parvovirus* encapsidate predominantly negative-sense DNA, but one virus, LuIII, packages approximately equal numbers of plus and minus strands. As discussed above, this characteristic maps to a two-base insertion immediately 3' to the cleavage position of the LuIII right-end nick site, which effectively impairs origin efficiency. These findings suggest that strand selection for encapsidation does not involve specific packaging signals, defined as *cis*-acting sequences that mediate the initiation of packaging, but they rather lend support to an alternative, mathematical, model postulated some years ago by Chen and colleagues (Chen *et al.*, 1989), called the Kinetic Hairpin Transfer model. This explains the distribution of the strands and terminal conformations of the packaged genomes of all known parvoviruses in terms of the efficiency with which each individual type of terminus can undergo reactions that allow it to be copied and reformed. In its simplest form, the model postulates that the relative efficiency with which the two genomic termini are resolved and replicated determines the distribution of amplified replication intermediates generated during the infectious cycle, and, ultimately, the efficiency with which single-stranded DNAs of characteristic polarity and terminal orientation are excised, which will then be packaged with equal efficiency. While most aspects of this model accord with the new experimental data, the mechanism of selection appears slightly more complex, since preferential excision of particular unit-length strands is only apparent during the packaging phase of genome amplification (Cotmore and Tattersall, 2005a). Thus, in viruses that

package strands of one particular sense, replication appears biphasic, with both positive and negative sense unit-length strands being excised at early times, followed by a switch in the mode of replication that allows the exclusive synthesis of single-sense strands of the packaged sense. A modified form of the kinetic hairpin transfer model, which we call the 'preferential strand displacement' model, suggests that this switch is simply brought about by the onset of packaging, and provides strong evidence that the substrate for packaging is a newly-displaced single-stranded DNA (Cotmore and Tattersall, 2005a).

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# Replication of adeno-associated virus DNA

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Among the DNA viruses that infect the cells of higher organisms, the parvoviruses are markedly handicapped with respect to replicating their genomes. They neither possess their own replication machinery, nor are they able to direct their host cell towards the S-phase of the cell cycle. Depending on their solution to this limitation, parvoviruses have traditionally been divided into two groups: the adeno-associated viruses (AAV), which for the most part only replicate in cells co-infected with a helper virus, and the autonomous parvoviruses, which are capable of replicating without a helper virus but only in actively replicating cells. The helper viruses which enable AAV replication must do so by altering the environment of the infected cell and/or by directly providing viral factors to support AAV replication. Adenovirus, the herpes viruses, papilloma virus, and vaccinia have all been shown to support AAV DNA replication (Atchison *et al.*, 1965; Buller *et al.*, 1981; Hoggan *et al.*, 1966; McPherson *et al.*, 1985; Schlehofer *et al.*, 1986; Walz *et al.*, 1997).

Our knowledge of AAV replication is derived primarily from cells in culture or from cell-free assays. The cellular target of AAV in the infected host organism is not known. Consequently, it remains possible that there may be cell types in the human host that can support productive AAV replication in the absence of a helper virus. For example, recently helper virus-free replication of AAV in raft cultures of epithelial cells has been demonstrated (Meyers *et al.*, 2000).

It is also interesting to consider goose parvovirus, characterized as an autonomous virus because it can replicate very efficiently without helper virus in specific cell types in young poultry. However, its sequence and potential secondary structure are quite similar to that of AAV2 (Brown *et al.*, 1995). Furthermore, like AAV, it can be induced to replicate in non-dividing cells if those cells are co-infected with duck plague herpes virus (Kisary, 1979).

Despite an inability to replicate on its own, AAV does quite well with respect to the number of copies of its genome produced per infected cell during a productive infection. AAV can produce 1–2 million copies of its genome per cell, resulting in perhaps  $10^6$  mature DNA-containing virus particles per cell or approximately  $10^4$  infectious units (IU)/cell (Rose and Koczot, 1972; Laughlin *et al.*, 1982; Carter *et al.*, 1983).

When AAV DNA replication is considered, it is necessary to note that this may be accomplished by several different mechanisms. As mentioned above, the AAV DNA replication observed in productive infection requires that the cell be co-infected by one of several helper viruses, principally adenovirus or herpes virus. A lesser amount of replication is observed when certain AAV-infected cell lines are treated with 'genotoxic' agents. Replication may involve somewhat different mechanisms in each of these two cases. If there is no helper agent, then AAV is able to establish a latent infection in cultured cells by integrating its genome site-specifically into chromosome 19 through a mechanism that is thought to involve a replicative process (Kotin *et al.*, 1990). It is certainly the case that productive replication differs from the more limited replication that is believed to play a role in site-specific integration into chromosome 19.

In addition, in the absence of a productive infection, transduction of recombinant adeno-associated virus (rAAV) vectors is observed. Since the AAV genome is single-stranded, transduction necessarily requires the synthesis of a second strand. It is unclear whether this second-strand synthesis is the same as the second-strand synthesis that initiates productive replication.

The major focus of this review will be productive replication. Studies of replication associated with integration are discussed in the following chapter (Chapter 16). A final point is that almost all studies of AAV DNA replication have

focused upon AAV2. It seems likely that the mechanisms determined for AAV2 will also be applicable to the other AAVs, but that has not been investigated.

This review is divided into three parts: the first is a brief historical survey of the principal early results that culminated in the basic model for the mechanism of AAV DNA replication. The second and third focus on subsequent studies that were informed by the model, and which were aided greatly by the development of infectious clones of AAV. The second section surveys studies which focus principally on the AAV genome and its interaction with AAV factors. The third surveys studies that focus principally on the interaction of AAV with non AAV factors.

## ORIGINAL STUDIES THAT LEAD TO THE BASIC MODEL OF AAV DNA REPLICATION

This section of the review is brief. A much more complete coverage of the early work can be found in several excellent previous reviews (Hoggan, 1970; Berns and Hauswirth, 1979; Carter, 1990b; Carter *et al.*, 1990).

In 1965 both Hoggan and Atchison and his colleagues noted that a small particle found contaminating stocks of adenovirus was not a defective adenovirus but rather a separate virus that was dependent on adenovirus for its replication (Atchison *et al.*, 1965; Hoggan *et al.*, 1966). There had been several earlier reports of what appear, in retrospect, to have been AAV contaminants of viral stocks, but they had not been unequivocally identified as a separate virus or shown to be dependent upon adenovirus for their replication. This inability to replicate by themselves gave rise to the notion that AAV was a defective or dependovirus.

The first report of a helper effect for AAV other than from adenovirus was one in which infectious canine hepatitis virus served as a helper in a co-infected cell line derived from dog kidney cells (Smith and Gehle, 1967). It was soon noted that AAV could grow in the presence of herpes virus (Blacklow *et al.*, 1970; Buller *et al.*, 1981). Later studies were to implicate vaccinia virus as a helper (Schlehofer *et al.*, 1986) and most interestingly, it was noted that treatment of cultured cells with certain 'genotoxic' agents rendered cells permissive for the complete AAV replication cycle (Schlehofer *et al.*, 1986; Yakinoglu *et al.*, 1988; Yakobson *et al.*, 1989). This last helper effect gave significantly lower yields of virus than did adenovirus or herpes. At present, adenovirus and herpes virus are considered to be the main helpers for AAV replication.

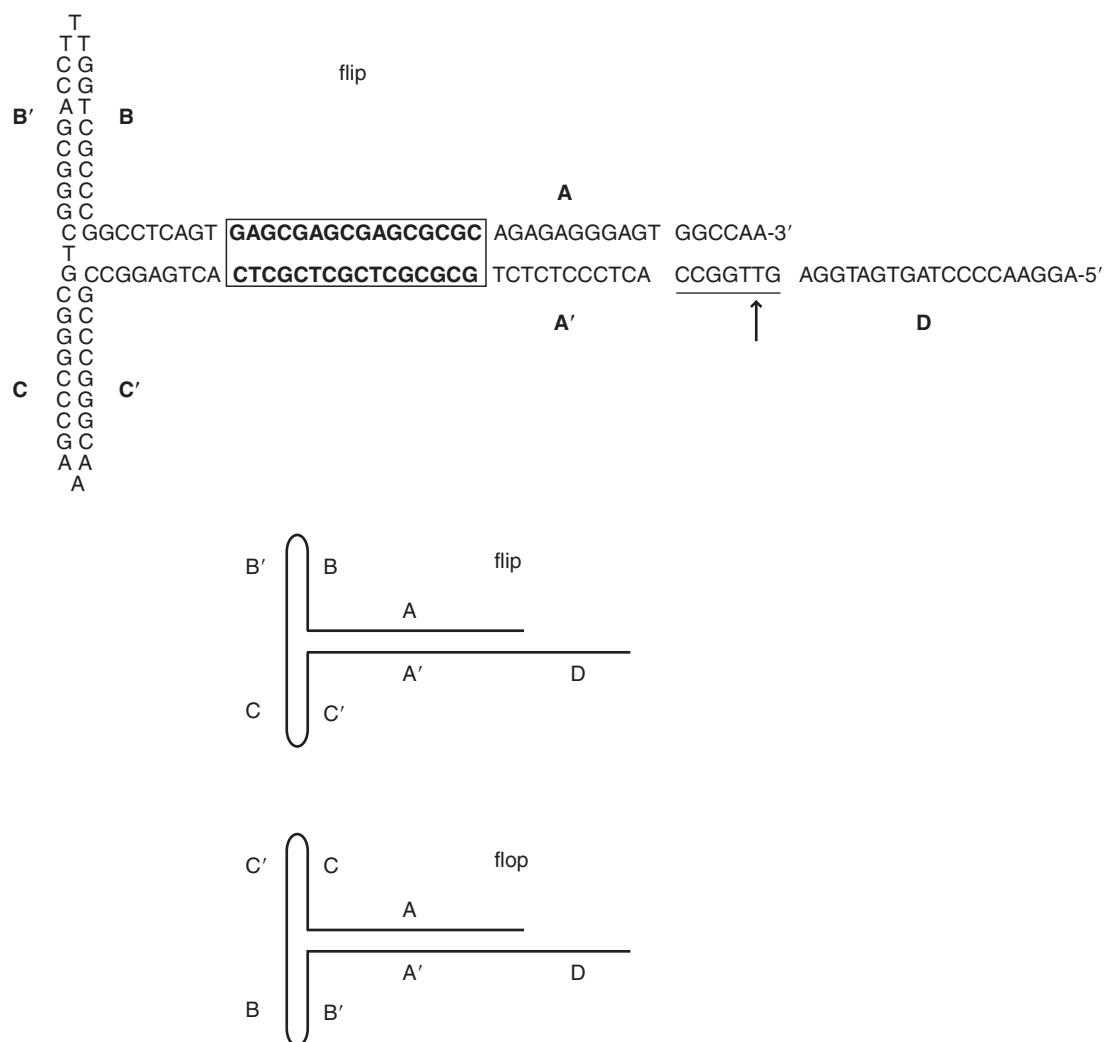
A model for AAV DNA replication developed, based principally on a knowledge of the sequence and potential structure of the viral genome. In addition AAV was noted to have properties in common with a group of rodent viruses, Kilham rat virus, H1, and minute virus of mice (MVM). These properties included its size, the morphology of the intact particle, its resistance to heat and chemical

agents, and its density. These viruses were members of the autonomous parvoviruses and led investigators to think about AAV DNA replication in the context of what was being discovered about the autonomous viruses.

When AAV DNA was first isolated from AAV particles it was thought to be double-stranded. However several groups determined that AAV particles contained single-stranded genomes and that a particle was equally likely to contain a strand of either polarity (Mayor *et al.*, 1969; Rose *et al.*, 1969; Berns and Rose, 1970; Berns and Adler, 1972). In particular Crawford *et al.* noted that, in comparison with the DNA content of MVM particles, which are of the same size, this putative double-stranded genome contained twice as much DNA as the particle's expected capacity (Crawford *et al.*, 1969). These investigators proposed that the DNA in the capsids was single-stranded but was annealing upon isolation. This was shown to be correct by mixing viral particles that had been grown in either the presence or absence of bromodeoxyuridine. Upon extraction many of the duplex molecules were of an intermediate density, indicating that they had annealed during extraction (Rose *et al.*, 1969). Since growth of virus in bromodeoxyuridine-containing media allowed separation of the viral strands in CsCl, since one strand has more thymidine, it was possible to demonstrate that approximately equivalent numbers of particles possessed strands of each polarity (Berns and Rose, 1970). This was confirmed by showing complete duplex association of the DNA isolated from a batch of particles, i.e. no DNA of either sense was left over (Carter *et al.*, 1972).

An obvious question is whether both plus and minus strands are infectious. The alternative possibility would be that a cell must be infected by both a plus and a minus strand, which upon annealing would form a double-stranded infectious genome. Several sorts of assays suggested that both plus and minus strands were infectious in themselves. The dose response curve for infection indicates single hit kinetics for AAV. This implies that a single particle can generate a productive infection, supporting the notion that either a plus or minus strand is infectious (Blacklow *et al.*, 1971). Experiments using either plus or minus strand containing particles, which, because they had been grown in bromodeoxyuridine (BudR), could be separated by CsCl centrifugation, also suggested either strand was infectious. However, with separation on CsCl gradients, it is impossible to completely eliminate cross-contamination. The question was definitively answered at a later time using two clones of the AAV genome, inserted in opposite orientations into a pEMBL plasmid. Infection of each clone separately with bacteriophage f1 generated virus stocks containing either plus or minus copies of the AAV genome exclusively. Transfection of the DNAs extracted from either of these viruses into adenovirus-infected 293 cells led to accumulation of AAV DNA (Samulski *et al.*, 1987).

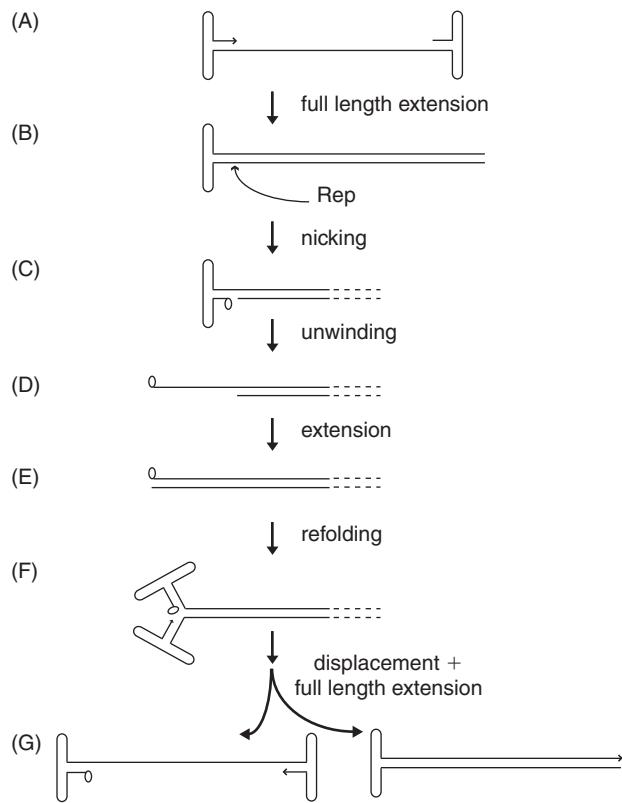
The idea that the AAV genome was simply single-stranded was first challenged by Carter *et al.*, based on experiments in which digestion with S1 nuclease and chromatography



**Figure 15.1** Structure of the AAV2 inverted terminal repeat (ITR). This structure is also referred to as the terminal repeat (TR) and is present at both ends of the AAV genome. Shown is ITR in its most likely secondary structure. The box identifies the sequence known as the Rep binding site (RBS) also referred to as the Rep binding element (RBE). The underlined sequence designates the recognition sequence for the terminal resolution site (TRS). The arrow indicates the Rep-dependent cleavage site. The schematic shows the alternation in sequence order between the flip and flop structures. The sequence shown is in the flip orientation. The sequence designated as the D-region is also repeated at each end of the genome but is not part of the palindrome.

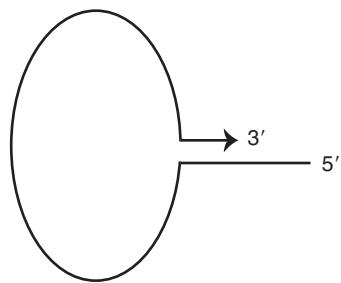
on hydroxylapatite suggested that a small part of the genome was duplex (Carter *et al.*, 1972). Later Gerry *et al.* partially digested a double-stranded form of the AAV genome with exonuclease III, and found that the products were able to self-anneal to form double-stranded circles, suggesting that the termini of the AAV genome possessed direct repeats (Gerry *et al.*, 1973). This finding, coupled with the observation that the single-stranded forms could also self-anneal to form a single-stranded circle, led to the hypothesis that the ends of the viral genome are inverted repeats, which each contained an inverted repeat (Koczot *et al.*, 1973). It was also suggested that self-complementarity implied identical ends, and that identical ends might allow bidirectional replication, which could produce individual plus and minus strands by a system of back and forth replication (Koczot *et al.*, 1973).

Sequencing the termini of AAV confirmed that the ends were inverted terminal repeats with internal inverted repeats (Lusby *et al.*, 1980). The termini were found to be 145 bases in length, of which the outer 125 bases were capable of self-basepairing. This 125 base region could self-anneal to form a T-shaped structure, consisting of a stem in which bases 0–41 pair with bases 85–125. The central 44 bases contain two small internal palindromes, which can self-anneal to form the arms of the T. Only seven bases of the outer 125 bases are left unpaired in the model, three bases at the end of each arm of the T and the central nucleotide between the two arms, which is also the central base of the whole palindrome. The full 145 base sequence is referred to as the inverted terminal repeat (ITR) or the terminal repeat (TR) (Figure 15.1).



**Figure 15.2a** Schematic model for AAV DNA replication. (Rep cleavage is indicated although, when the model was first proposed, it was not known that cleavage was by the AAV Rep protein.) (A) Single stranded AAV genome after uncoating. (B) After full length extension from the 3' primer a duplex with one hairpinned end is created. (C) The hairpin is nicked at the TRS by Rep68 or Rep78, opposite the original 3' terminus leaving the Rep protein attached. (D) The hairpinned end is unwound. (E) The 3' end formed by Rep cleavage is extended to the end of the template strand. (F) The ends of each strand are refolded into their alternative self-base pairing hairpin structures. (G) full length DNA synthesis from the 3' primer on the left end of the genome produces one complete single-stranded genome and one duplex genome. Each of these can serve as a substrate for additional replication by the same mechanisms. Rep designates either Rep68 or Rep78. Rep is not shown to scale.

A problem in the replication of any linear DNA molecule is the difficulty of copying the 3' end of the template strand. It had been noted by Cavalier-Smith that for a linear DNA molecule with hairpin ends, a hairpin transfer model would overcome this problem, allowing replication of the full length molecule (Cavalier-Smith, 1974). As adapted for parvoviruses by Tattersall and Ward, this hairpin transfer model provides a description of how the AAV genome might replicate (Tattersall and Ward, 1976). A consequence of the hairpin transfer model is that, if the hairpin is internally asymmetric, any replication process that incorporates this mechanism will necessarily continually reverse the asymmetry in the hairpin. When sequencing the termini it was noted that the two internal palindromes occurred in two



**Figure 15.2b** Repair of missing ITR sequences. Shown is a single-stranded copy of an AAV genome in which most of one ITR is missing. Remaining ITR sequences allow limited basepairing and synthesis of missing ITR regions.

forms, designated flip and flop, in which the internal palindromic sequences from one arm were directly switched for those of the other arm (Spear *et al.*, 1977; Lusby *et al.*, 1980, 1981; Figure 15.1). The fact that the flip and flop sequences are precisely the expected inversions of asymmetry lent strong support to the notion of a replication mechanism involving hairpin transfer (Straus *et al.*, 1978). It was noted that not only were both orientations seen (Lusby *et al.*, 1980), but that the distribution of flip and flop at the two termini were independent of each other (Lusby *et al.*, 1981). This was followed by the observation that a cloned AAV genome in which the termini were in one orientation generated both flip and flop progeny viruses when transfected into cells, providing further evidence for the hairpin-transfer mechanism (Samulski *et al.*, 1982).

The notion of bidirectional replication from each end of the AAV genome coupled with hairpin transfer suggested that the termini might serve as origins of replication. This suggestion was supported by the studies of Hauswirth and Berns, who showed by pulse chase experiments with tritiated thymidine that the first regions of the genome to be synthesized were at the termini. Plus and minus strands each contained a continuous gradient of label from their 5' to 3' ends (Hauswirth and Berns, 1977). Subsequent assays, demonstrating that mutants with extensive deletions in their terminal sequences were not able to replicate or be complemented *in trans*, reinforced the notion that these sequences contained the viral origins of replication (Samulski *et al.*, 1983; Senapathy *et al.*, 1984).

Rose and colleagues noted that in infected cells one can find duplexes with one hairpinned end, as well as concatemers of alternating plus and minus strands. Taken all together the data permit a fairly complete description of a mechanism by which the AAV genome can be duplicated (Straus *et al.*, 1976). The model for the mechanism of AAV DNA replication derived from these and many other early studies is, in its basic form, still accepted (Figure 15.2a).

Upon uncoating, the incoming AAV genome is single-stranded and has the potential to form identical hairpins at each end. The 3' hairpin serves as a primer for full-length extension. Depending on whether the original genome was

a plus or minus strand, the 3' hairpin can be at either end of the genome, and therefore priming and DNA synthesis can occur from either end. The product of this 'primer extension' is a full-length duplex genome with one end in a closed hairpin conformation. The hairpinned end has one copy of the viral ITR, while the open end contains two copies, one on each strand. The hairpin end must then be nicked at a site near the junction between the palindromic and non-palindromic domains of the ITR (i.e. at a point opposite the end of the original viral hairpin). Nicking is followed by synthesis from the newly created 3' terminus, so that the ITR is copied. This process converts the closed hairpin end to a duplex open end with one copy of the now inverted ITR on each strand. The process of nicking and extension is referred to as 'terminal resolution'. Both ends of the duplex genome can now fold back on themselves. At each end there will be one hairpin with a 3' end that can prime synthesis as described above, allowing the whole process to be repeated. Duplex dimers are created if extension from a 3' terminus, on a genome with a closed hairpin at its other end, reaches that closed hairpin before it has been terminally resolved. Extension will continue through the hairpin, resulting in a dimer of two inverted copies of the AAV genome with one duplex copy of the ITR at the junction. This model, based only on the structure of the AAV genome and of replication intermediates as found in productively infected cells, predicted key steps that were as yet without experimental support. In addition outstanding questions included the nature of the factor that effected hairpin transfer, and the details of the synthetic machinery that replicated the genome.

## STUDIES FOCUSING ON THE AAV GENOME

Shortly following the presentation of this model, infectious clones of AAV were created, making possible many investigations. The existence of a model for the mechanism of replication has of course influenced subsequent experimentation and analysis. The results of these investigations have seemed generally compatible with the basic model. This section will focus on studies based primarily on the sequence and structure of the AAV genome and, briefly, with its interaction with the AAV Rep proteins as far as this relates to replication.

Complete duplex copies of the AAV genome were cloned into pBR-derived plasmids, despite the considerable difficulties involved in manipulating the inverted terminal repeats (Samulski *et al.*, 1982; Laughlin *et al.*, 1983). When these plasmids were transfected into 293 cells and the cells subsequently infected with adenovirus, infectious AAV was produced that was indistinguishable from that produced by co-infection of adenovirus (Ad) and AAV virions. DNA was extracted from cells that had either been transfected by plasmid or infected by AAV virions. A comparison was performed by agarose gel electrophoresis and Southern blotting

using AAV specific probes. The pattern of bands in the plasmid transfected/Ad infected cells was indistinguishable from that produced in AAV infected/Ad infected cells. In the absence of adenovirus co-infection there were no detectable AAV specific bands in either the plasmid transfected or the AAV infected cells. Apparently whether the AAV genome is supplied in a single-stranded form or in a plasmid construct, the pathways of productive replication are indistinguishable. The surprise in these assays was that a copy of the AAV genome must have been released from the plasmid backbone.

Shortly after the discovery of AAV it had been known that the virus was able to establish a latent state in cells when conditions were not permissive for AAV replication (Hoggan *et al.*, 1972). It had also been shown in cultured cells that the AAV genome could become integrated into the genome of its host cell (Cheung *et al.*, 1980). This integration was subsequently shown to occur preferentially at a site on chromosome 19, which was designated *AAVS1* (Kotin *et al.*, 1990). When adenovirus infects cells that are latently infected with AAV, the AAV genome becomes detectable, presumably because it is released from its chromosomal locus and begins to replicate productively. This release from the chromosome, which is believed to occur upon helper virus infection, has been given the term 'rescue'. The rescue step in latently infected cells would seem to be the first step of the replication pathway for AAV. The release from the plasmid vector described above, which also occurs upon helper virus co-infection, is also termed 'rescue'. It is thought to occur by a mechanism that is similar to release from the chromosome. Consequently, the availability of infectious clones of AAV made developing assays to assess the contribution of various parts of the AAV genome to rescue more feasible. However, in this, as in subsequent work, it is difficult to distinguish the effects of modifications to the AAV genome on rescue from their effects on replication. Assays for both are based on analysis of the structure and quantity of a product, the newly synthesized AAV genome, which is the combined result of both rescue and replication.

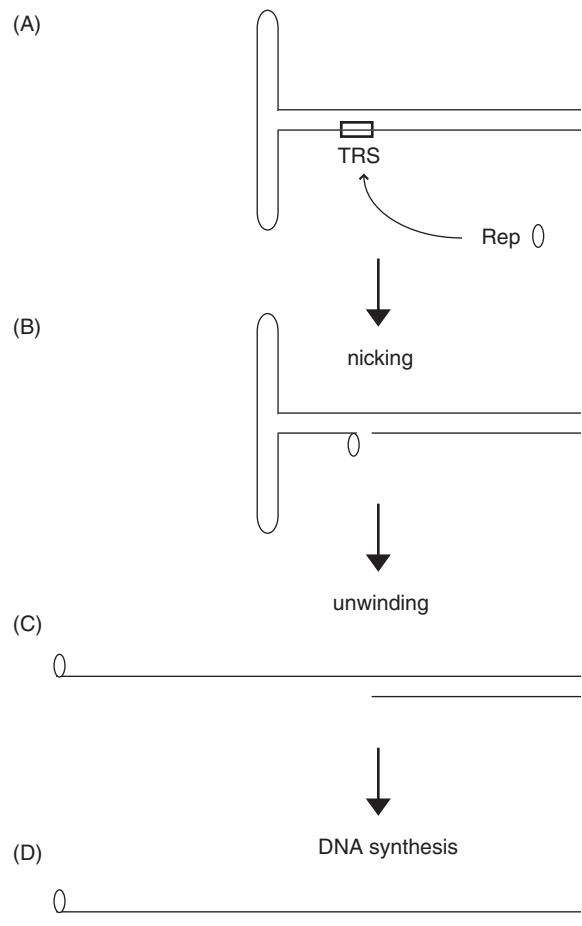
Samulski and his colleagues made several early observations that defined which AAV sequences were necessary for the rescue and replication of AAV genomes from plasmid vectors. Using constructs with large deletions in one or other ITR, they showed that it was possible to achieve rescue and replication as long as the remaining ITR was largely intact. In the case of a construct containing a large deletion in one ITR, only a relatively small deletion was tolerated in the other ITR. In all cases the product was a virus with wild-type DNA. They concluded that a mechanism must exist to correct deletions in the ITR, and postulated that the single-stranded form of the genome might assume a panhandle configuration in which the intact ITR would serve as a template for the repair of the other (Figure 15.2b) (Samulski *et al.*, 1983). They suggested that rescue was most likely due to cleavage near the ITRs, possibly by an AAV-encoded Rep protein. Senapathy and Carter made similar observations, and postulated a mechanism for the rescue and repair process

that involved a double-strand excision and ligation, followed by hairpin transfer (Senapathy *et al.*, 1984).

The availability of infectious clones made it possible to demonstrate a critical step in the AAV DNA replication model, namely the hairpin-transfer mechanism originally described by Cavalier-Smith (Cavalier-Smith, 1974). Conclusive evidence that hairpin transfer does occur as hypothesized was provided by assays that employed a closed hairpin form of the viral ITR, which was constructed from the cloned AAV genome (Snyder *et al.*, 1990). This ITR structure is the presumed hairpin ITR initially described by Straus *et al.* as a component of a replication intermediate found in co-infected cells (Straus *et al.*, 1976). The hairpinned substrate was incubated with extracts from cells infected by both AAV and adenovirus. The hairpin was nicked near the junction between palindromic and non-palindromic sequences, and the newly created 3' terminus was extended by DNA synthesis. By this mechanism, which is referred to as 'terminal resolution', the palindrome is necessarily inverted (Figure 15.3). The terminal resolution assay also suggested that a protein became covalently attached to the 5' terminus at the nick site (Snyder *et al.*, 1990).

Using Ad/AAV co-infected cell extracts, Ashktorab and Srivastava showed that proteins bound to the AAV hairpin. This binding was specific for the hairpinned form of the ITR, (i.e. they did not detect binding to the double-stranded duplex), and only occurred if the extract had been prepared from cells infected with both adenovirus and AAV (Ashktorab and Srivastava, 1989). Several groups had shown that the left half of the AAV genome contained a function that was necessary for replication. (Hermonat *et al.*, 1984; Senapathy *et al.*, 1984; Tratschin *et al.*, 1984). This activity seemed likely to be directly involved in replication since mutations in this region reduced RNA production only somewhat, but had a severe effect on DNA replication. The protein coded by the left open reading frame (ORF) of AAV is designated the Rep protein. This is now known to be the factor that bound to the hairpinned ITR in the assays of Ashktorab and Srivastava, that nicks the hairpinned ITR to effect hairpin transfer, and that is bound to the 5' terminus at the nick site.

The Rep protein has been reviewed in detail elsewhere (Muzyczka and Berns, 2001). A great deal of data has accumulated regarding the activities of the AAV Rep protein. The left ORF of AAV, which is the only reading frame that codes for non-structural proteins, encodes four proteins, designated Rep78, Rep68, Rep52, and Rep40, in accordance with their apparent molecular weights upon gel electrophoresis. The four proteins differ from each other in their start sites and splicing patterns. Either Rep78 or Rep68, but not Rep52 or Rep40, is required for AAV DNA replication (Chejanovsky and Carter, 1989) Rep78 and Rep68, but not Rep52 or Rep40, bind to a site in the ITR designated the Rep-binding site (RBS) (Snyder *et al.*, 1993; Chiorini *et al.*, 1994b, 1995b; McCarty *et al.*, 1994b; Davis *et al.*, 2000). This binding is apparently stabilized by additional contacts with a site at the tip of one arm of the T structure (Ryan



**Figure 15.3** Schematic of terminal resolution. (A) Shown is the AAV ITR in the hairpinned form believed to be an intermediate in AAV DNA replication. (B) Site-specific and strand-specific cleavage at the TRS. (C) Unwinding of the hairpin by a DNA helicase activity. (D) Extension of the 3' primer to the end of the substrate. This synthesis inverts the orientation of the nucleotides between the TRS and the end. It also makes possible a refolding of the ITR into a hairpin structure with a 3' primer (see Figure 15.2). Bold line indicates newly synthesized DNA. Small oval indicates Rep linkage to 5' nucleotide at cleavage site. Rep designates either Rep68 or Rep78.

*et al.*, 1996). Complexes containing multiple copies of Rep bound to the AAV ITR have been observed (McCarty *et al.*, 1994b; Kyostio *et al.*, 1995; Smith *et al.*, 1997; Li *et al.*, 2003). Rep nicks in a strand-specific manner at a site designated the terminal resolution site (TRS), which is defined as a sequence of seven nucleotides, shown in Figure 15.1 (Im and Muzyczka, 1990; Brister and Muzyczka, 1999, 2000; Davis *et al.*, 2000; Smith and Kotin, 2000). Rep is subsequently found linked to the nucleotide on the 5' side of the cleavage site (Figure 15.2; Wistuba *et al.*, 1995; Kube *et al.*, 1997; Prasad *et al.*, 1997; Dubielzig *et al.*, 1999). For convenience, in the remainder of this chapter 'Rep' will designate either Rep78 or Rep68, but not Rep52 or Rep40 except where specifically noted. Rep also binds to the p5

promoter (McCarty *et al.*, 1994a). In addition Rep was found to be able to unwind the ends of the duplex AAV genome, creating the hairpin primer that is the proposed origin for replication of the full-length genome (Snyder *et al.*, 1993; Brister and Muzyczka, 1999; Davis *et al.*, 2000). Phosphorylation of Rep has been observed, but its possible role in the AAV life cycle is unknown (Collaco *et al.*, 1997). It is noteworthy that both the AAV Rep protein and the NS1 proteins of the autonomous parvoviruses contain motifs that are characteristic of rolling-circle replication proteins (Koonin, 1993; Koonin and Ilyina, 1993).

The hairpin ends of the AAV genome seem able to form primers for replication of the full-length AAV genome. An immediate question is whether the sequence, or merely the structure, of the hairpin is important. This question was examined in relation to the arms of the T. Substituting nucleotides in the arms of the T hairpin with a different sequence that was still able to fold into the same structure allowed the production of virus (Lefebvre *et al.*, 1984). Some models of AAV DNA replication postulate that the ends of the genome basepair create a panhandle structure for initiation. To test whether the two ends of the genome must be complementary, mutants were created in which the two ends were different. Replication of these constructs was quantitatively similar to that seen when both ends were the same. When chimeric plasmid constructs were used in which the left end was mutant and the right end was wild-type, chimeric molecules were still found to be present after many rounds of DNA replication (Bohenzky and Berns, 1989). Therefore the ends do not need to basepair perfectly in order for replication to occur. Bohnzky and colleagues compared the wild-type genome to ITR mutants with alternate sequences but still possessing the wild-type structure with the wild-type genome, and saw the same kinetics of DNA accumulation (Bohenzky *et al.*, 1988). This was true whether the transfected DNA was annealed double-stranded viral DNA or plasmid DNA. Rescue of mutant and wild-type genomes was also found to be equally efficient. Additional experiments, however, demonstrated that the wild-type sequence does have an advantage over the mutant sequences.

When cells were co-transfected with a mixture of reannealed viral DNA in which the genomes contained either two wild-type or two mutant ends, the wild type was dominant. This was not seen when plasmid DNAs were co-transfected. It is not clear why there is this difference between transfected reannealed viral DNA and transfected plasmid DNA. It is also not clear why this method gives wild-type dominance, but it is clear that the wild type has some advantage (Bohenzky and Berns, 1989). In experiments in which the left end was wild type and the right end mutant, progeny virus had wild-type termini at both ends. With the reverse organization, namely mutant at the left end and wild type at the right end, there was no change in the progeny. Therefore in these experiments it was not simply that wild type was always dominant over the mutant, or that the left ITR was always dominant over the right ITR

(Bohenzky *et al.*, 1988). At present there is no satisfactory explanation for these observations.

In addition to the palindromic part of the ITR, the adjacent D-region was also found to be necessary in *cis* for high efficiency rescue and replication of the AAV genome (Wang *et al.*, 1995). Rescue and replication do happen in the absence of these regions, but to a much lesser extent. Restoration of the TRS, which had been partly altered by removal of the D-region, did not restore replication and rescue. In addition replacement of the D-regions with two 20-nucleotide inverted repeats, which would allow basepairing between the two 'D-regions' at each end of the genome, did not restore rescue and replication. This indicates that it cannot be solely an ability to basepair with each other that determines the role of the D-regions, but that their specific sequence is important. An additional function for the D-region was observed when performing a modified Hirt extraction on adenovirus infected 293 cells transfected with AAV derived constructs (Wang *et al.*, 1996). No detectable single-stranded DNA was produced from transfection with plasmids containing an AAV genome with the D-region deleted. In a nicking assay it was found that Rep-mediated cleavage at the TRS was independent of D-region sequences. This last result suggests that replication and Rep dependent nicking at the TRS can be uncoupled, and that in the D-region-deleted construct the lack of efficient AAV replication is not a consequence of impaired nicking at the viral TRS (Wang *et al.*, 1997). The same group also found that in the 20-nucleotide D-region, the 10 nucleotides proximal to the hairpin were sufficient for efficient rescue, replication, and encapsidation *in vivo* (Wang *et al.*, 1997). No assay has yet detected a functional role for the other 10 nucleotides. The required proximal 10 nucleotides were also necessary and sufficient for interaction with a cellular protein, which was termed the D-region binding protein (Wang *et al.*, 1997). Interactions with this protein will be described more fully in the following chapter (Chapter 16).

While the hairpinned ITRs of the AAV genome can serve as an initiation structures (i.e. a 3' primer) for replication, several assays have demonstrated that Rep-dependent initiation of replication can occur without a hairpin. The terminal resolution assay of Muzyczka and his colleagues described above involves initiation from a nick at the TRS. Hong *et al.* used as substrate a plasmid construct containing an AAV genome in which the outermost 55 nucleotides from each ITR had been deleted (Hong *et al.*, 1992). When incubated with an extract made from Ad/AAV co-infected cells, this construct replicated as an intact plasmid, i.e. there was no rescue and therefore no possibility of a terminal hairpin. It has been shown that the AAVS1 site on chromosome 19 into which AAV integrates site-specifically, contains a Rep-binding site and a nearby sequence that is homologous to the AAV TRS. Using HeLa cell extracts supplemented with an AAV Rep protein, Urcelay *et al.* demonstrated that the AAVS1 site from chromosome 19 would, when cloned into a plasmid construct, support replication

of that construct in either linear or circular form (Urcelay *et al.*, 1995). Similarly other workers have shown that a minimal origin consisting of only the AAV Rep-binding site, a TRS, and the intervening nucleotides, was sufficient to support replication (Smith *et al.*, 1999; Yoon *et al.*, 2001). In these last assays the Rep-binding site and TRS were at least 1000 nucleotides from the nearest 3' end, forestalling the possibility of an adjacent hairpin 3' terminus. Musatov and colleagues have shown *in vivo*, that a plasmid containing only the AAV AD domain of the ITR replicates in a circular form (Musatov *et al.*, 2002).

Using HeLa extracts supplemented with a Rep68-maltose-binding protein fusion, it was also shown that replication could initiate from a terminal viral ITR that was missing a TRS, demonstrating that replication did not require nicking at the TRS as a first step (Ward and Berns, 1995). The TRS minus construct also demonstrated that most of the terminal hairpin structure could be deleted. Provided a small terminal sequence capable of folding into a hairpin remained, replication could initiate. It seems that Rep-dependent initiation of replication at a Rep-binding site can occur efficiently from either an adjacent terminal 3' hairpin or from a nearby TRS. It should be noted that the spacing between the Rep-binding site and the nearby TRS is important for efficient nicking (Brister and Muzycka, 2000). The one constant in all these assays is a Rep-binding site.

It has become evident from more recent investigations that the viral ITRs are not the only sequences that have potential rescue and replication functions. When plasmids containing segments of the AAV genome are transiently transfected into adenovirus infected cells, a region of the AAV genome between nucleotides 190 and 540 acts in *cis* as a Rep-dependent element to promote replication (Nony *et al.*, 2001). Nony *et al.* termed this region the *cis*-acting replication element (CARE). Tullis and Shenk also noted that a *cis* acting element from the left side of the AAV genome was required for efficient replication of AAV DNA (Tullis and Shenk, 2000). They observed that a deletion between nucleotides 194 and 1882 reduced the accumulation of monomer length duplex DNA forms by 14-fold. In a related observation Wang *et al.* showed that a plasmid construct which contained the entire AAV genome except for the left ITR could nevertheless support excision and replication of the AAV sequence, suggesting that there is an additional potential excision site on the left side of the genome (Wang and Srivastava, 1997). This site was mapped to the p5 promoter region, and nicking was shown to occur between nucleotides 287–288. Deletion of the RBS at p5 abolished this ITR-independent rescue and replication of AAV sequences. What role these internal sequences might play in the AAV life cycle is at present unclear, as is the mechanism by which they might contribute to replication and rescue.

Tullis and Shenk also made the unexpected observation that genomic sizes of less than 3.5 nucleotides produced 3–8-fold fewer single-stranded progeny genomes than the wild-type length molecule (Tullis and Shenk, 2000). They

suggested that efficient production of single-stranded genomes may be in part dependent on the time it takes the replication complex to proceed from one hairpin terminus to the other. It had been suggested previously by Muzyczka and colleagues that terminal resolution did not occur until elongation was complete (Ni *et al.*, 1998). This notion was based on the absence of terminally nicked elongation intermediates. Tullis and Shenk suggest that this observation is an example of the sorts of coordination that might exist in the AAV replication process, and which might be disrupted by altering the length of the genome.

Within the adenovirus/AAV co-infected cell, AAV genomes can apparently interact with each other. Recombination between replicating genomes was first observed by Senapathy and Carter (Senapathy and Carter, 1984), who noted that this recombination is dependent on the extent of overlap between different genomes. In cells infected with AAV alone, recombination would not normally be detectable since all the virus infecting a cell would generally have the same sequence. However when Gao and his colleagues examined non-human primates for the presence of previously uncharacterized AAVs, they found AAV genomes in multiple tissues and possible evidence of recombination (Gao *et al.*, 2003). These genomes showed a great diversity of sequence, primarily in the hypervariable regions of the capsids. Gao and his colleagues suggested that this diversity may be caused by homologous recombination between distinct, co-infecting parental viruses. Recombination by AAV is not restricted to recombination with other AAV genomes; recombination between AAV and SV40 in co-infected cells has also been observed (Grossman *et al.*, 1984, 1985).

In the making of recombinant AAV vectors, many groups have noted that unfortunately the presence of even very small regions of overlap between the recombinant vector and the *rep/cap* construct in the producing cell can lead to the production of wild-type virus. Circular concatemers of rAAV genomes are also observed (Duan *et al.*, 1999; Yang *et al.*, 1999). The majority contained one ITR and were apparently created by recombination between the ITRs. A possible useful consequence of recombination (or end-to-end joining) has been the use of paired vectors to overcome the size constraints for packaging in one virion (Duan *et al.*, 2000; Sun *et al.*, 2000; Nakai *et al.*, 2000). It is at present unclear whether the recombination pathways available in the non-productively infected cell, i.e. with rAAV vectors, is the same as that available in the presence of Rep and of a productive infection.

Some fraction of the replicated and packaged AAV genomes are defective (de la Maza and Carter, 1980; Hauswirth and Berns, 1979). These are referred to as defective interfering or DI particles. In general they consist either of a hairpinned form of one end of the genome or of a structure with both the left and right ITRs and varying amounts of internal deletions. In the autonomous parvovirus, MVM, mapping of deletion mutants showed that they occurred preferentially between short direct repeats

(Hogan and Faust, 1984). This result suggests that they are formed either by recombination or by slippage during replication. It is possible that a similar mechanism may be responsible for the internal deletions seen in AAV DI genomes. It seems likely that the hairpin genomes are the result of replication turning back upon itself.

Production of high titer AAV and rAAV stocks requires that the *Rep* and *Cap* genes also be amplified in order to produce adequate amounts of these proteins (Chiorini *et al.*, 1995a; Fan and Dong, 1997). In the case of AAV, the *Rep* and *Cap* genes are amplified as the genomes replicate. In the case of rAAV genomes in which *Rep* and *Cap* may be supplied by plasmid co-transfection, this may not be the case. The importance of amplification was demonstrated by Chiorini *et al.* who showed that higher virus titers could be achieved in Cos cells by expressing *Rep* and *Cap* from a plasmid with an SV40 origin (Chiorini *et al.*, 1995a). Chadeuf *et al.* demonstrated that the success of a HeLa derived *rep/cap* cell line, HeRC32: as a producer for rAAV vectors was dependent upon a 100-fold amplification of the endogenous *rep/cap* sequences that was induced by adenovirus (Chadeuf *et al.*, 2000). However, simply increasing the level of *Rep* does not always lead to increased DNA synthesis. In the presence of higher levels of *Rep*, Li *et al.* observed decreasing yields of an rAAV vector. This was due not only to decreased levels of *Cap* production but also to substantially lower levels of the replicated rAAV genome (Li *et al.*, 1997).

When low molecular weight DNA is extracted from AAV/adenovirus co-infected cells, much of the AAV DNA is in a single-stranded form. It has been noted that the capsid gene must be present in order to observe this single-stranded AAV (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984). Presumably this represents genomes that have been sequestered by intact capsids or capsid proteins. Chejanovsky and Carter noted that AAV mutants that did not express Rep52 or Rep40: while showing normal amounts of double-stranded 'replicative form' AAV genomes did not accumulate single-stranded genomes (Chejanovsky and Carter, 1989). They also observed greatly reduced AAV production. It is unclear whether the absence of single-stranded genomes reflects a failure to produce these forms, or the fact that, if they are not sequestered by encapsidation, they may anneal to each other during DNA extraction. While Rep40 and Rep52 are not required for DNA replication they are apparently needed for efficient assembly of the complete virion.

These last results were further explored using transfected plasmids. In these assays, Rep78 alone was sufficient for the production of AAV virions in HeLa cells, but the addition of a Rep52 construct increased virus yields by about 350 fold (Holscher *et al.*, 1995). In this report, as in a previous report (Holscher *et al.*, 1994), the expression of Rep52 along with Rep78 did not strongly increase the synthesis of AAV DNA, but there may have been some enhancement. Interestingly in the autonomous parvovirus MVM, the small non-structural protein NS2 contributes to the

amount of DNA replication (Naeger *et al.*, 1990). Holscher *et al.* also demonstrated by transfection that either Rep68 or Rep78 alone could support a complete replication cycle. The functional difference, if one exists, between Rep68 and Rep78 with respect to DNA replication remains unknown.

An additional point about DNA replication is that it seems linked to subsequent events in the AAV life cycle. The packaging of the AAV genome, which does require the small Rep proteins to achieve efficient levels, appears intimately coupled to DNA synthesis. In a cell-free packaging assay, either depletion of Rep78 and Rep68 by immunoprecipitation or the addition of the polymerase inhibitor aphidicolin significantly reduced packaging activity (Zhou and Muzychka, 1998).

## STUDIES FOCUSING ON THE ROLE OF NON-AAV FACTORS IN AAV DNA REPLICATION

AAV has only one ORF that encodes non-structural proteins, and is therefore dependent upon host factors for all aspects of its life cycle. The replication of AAV in cultured cells is also normally dependent upon co-infection with a 'helper' virus. AAV was originally isolated as a contaminant of an adenovirus preparation and adenovirus has proved to be a potent helper for AAV replication. It was later noted that the herpes viruses could also function as very efficient 'helpers' for AAV replication (Buller *et al.*, 1981; Blacklow *et al.*, 1971). The ability of either virus to function as a helper renders immediately confusing the question of what the 'helper' is providing since adenovirus and the herpes viruses are rather dissimilar. This conundrum led to the view that the helper virus, rather than providing specific replication functions, was in large part altering the cellular 'milieu' to make the cell permissive for AAV replication. The replication factors would most likely be cellular. This view was given support by the subsequent findings that AAV replication in cultured cells could be supported by vaccinia (Schlehofer *et al.*, 1986) and papilloma viruses (Walz *et al.*, 1997) and particularly by the observation that cells could be rendered permissive for AAV replication by treatment with certain genotoxic agents in the complete absence of helper virus co-infection (Schlehofer *et al.*, 1986; Yakobson *et al.*, 1987; Yakinoglu *et al.*, 1988). The following section covers investigations of AAV DNA replication that relate to AAV's interaction with helper viral and cellular functions, although in both of these areas our knowledge remains limited. In addition, AAV itself has effects on the cell which, while apparently not sufficient to render a cell permissive for AAV replication, are likely to play an important role in productive replication.

### Adenovirus as helper

The interaction of AAV and adenovirus was investigated extensively during the early years. For the most part that work will not be discussed in this review, and instead the

reader is referred to excellent previous reviews by Carter (Carter, 1990a; Carter *et al.*, 1990).

The recent independent development, by several groups, of helper plasmids that provide an apparently complete helper effect for the production of rAAV vectors also provides a definitive assay for determining which adenovirus genes are needed for the 'helper' effect (Xiao *et al.*, 1998; Grimm *et al.*, 1998; Matsushita *et al.*, 1998; Salvetti *et al.*, 1998). When transfected into 293 cells, plasmids containing the adenovirus E2a, E4, and VA RNA genes can provide a complete helper effect, given that 293 cells already possess copies of the adenovirus E1a and E1b genes. In some cases help delivered in this way has given higher titers of rAAV than was provided by adenovirus. Xiao *et al.* suggested that this increased efficiency occurs because there is no adenovirus infection to compete with AAV for cellular factors.

VA RNA improves protein synthesis, apparently by blocking the antiviral effects of interferon (Kitajewski *et al.*, 1986; O'Malley *et al.*, 1986). E1a is thought to induce cells to enter S-phase, which is necessary for making the cellular replication machinery available (Muzyczka, 1992). E1a is also necessary for transcription of other adenovirus genes and for transcription from the AAV genome (Chang *et al.*, 1989). The E2a-region encodes the adenovirus single-stranded binding protein. Its role is less clear. It has been shown to play a role in transcription from the AAV genome (Chang and Shenk, 1990; Carter *et al.*, 1992). In cell-free assays the adenovirus single-stranded binding protein has been shown to play a role in rescue and in processive replication (Ward *et al.*, 1998). The role of E1b in providing a helper function is also unclear. The E1b-55k protein has been found complexed with the E4orf6 protein. An adenovirus mutant in the E1b-55k protein was unable to support replication of an AAV genome from the integrated state in the cell, but was able to support replication of the AAV genome when the cell was infected with an AAV virus (Ostrove and Berns, 1980). This result suggested that the E1b-55k protein provides a helper function for rescue. The role of E4orf6 is also unclear but is apparently of prime importance. The E4-region was originally shown to be necessary to help AAV DNA replication using an adenovirus deletion mutant (Carter *et al.*, 1983). With this mutant as the helper, accumulation of both AAV replicative form and single-stranded DNA were both greatly diminished.

It had been noted that there may be low level replication of the AAV genome in 293 cells (Wang and Srivastava, 1998) which, as mentioned, contain copies of the E1a and E1b genes. The same workers noted that this 'autonomous' replication could be enhanced by mutations in the p5 promoter that allowed increased expression of the Rep gene. Allen *et al.* used 293 cells in a series of assays designed to provide a further refinement of the adenovirus helper effect (Allen *et al.*, 2000). With no adenovirus infection and AAV promoters driving expression from Rep and Cap constructs they could detect no virus production from a recombinant AAV construct. These workers then substituted

the constitutively active MT and CMV promoters for the p5 and p40 promoter sequences in their Rep and Cap helper constructs. With Rep and Cap expressed from the substituted promoters these authors now saw the level of rAAV production enhanced to 6 percent of the level seen with adenovirus infection. Therefore part of the adenovirus helper effect is certainly due to its ability to induce rep/cap expression from AAV promoters. However this is only part of the effect. When they also co-transfected a construct in which the adenovirus E4orf6 gene was coupled to a CMV promoter, they achieved a level of rAAV production that was more-or-less equivalent to that obtained with adenovirus infection. They achieved an even greater efficiency when the Rep and Cap genes were expressed from separate plasmids. However, the E4orf6 construct had almost no helper effect when Rep and Cap were expressed using the native p5 and p40 promoters. Their conclusion was that, with high levels of Rep and Cap, the E4orf6 construct could provide as much help as adenovirus when measured by rAAV production. Since their assays were performed in 293 cells, the adenovirus E1a and E1b gene products were presumably also present. However, when they examined total nucleic acid extracted from the cell pellets of transfected cells they observed that the levels of AAV DNA synthesis in the E4orf6 transfected cells, as determined by Southern blot analysis, was substantially less than in the adenovirus infected cells, despite the similar levels of rAAV production.

Interestingly, some of the earliest experiments aimed at defining the adenovirus helper effect, performed by injecting adenovirus DNAs and messenger RNAs (mRNAs) into cultured cells, also identified the E4-region as providing a direct helper effect for AAV DNA replication (Richardson and Westphal, 1981). Injection of DNA fragments containing early region genes directly into VERO cells demonstrated that the E2a and E4 genes were sufficient to provide a complete helper effect if given in high enough copy number. At lower copy number either E1a or E1b were required, leading to the notion that these genes may function in part by increasing the expression of the E2a and E4 genes (Richardson and Westphal, 1984).

Transduction studies also point towards a central role for the E4orf6 gene. Adenovirus co-infection substantially increases transduction by rAAV. Using rAAV that expressed lacZ and a panel of adenovirus mutants and cell lines, it was determined that the adenovirus gene required for this enhancement was E4orf6. This conclusion was supported by subsequent assays in which only this adenovirus gene was employed (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). These groups demonstrated a correlation between the presence of the E4orf6 gene and second-strand synthesis of the incoming single-stranded rAAV genome, and suggested that this correlation explained the E4orf6 gene's enhancement of transduction. One group (Ferrari *et al.*, 1996) provided evidence to support this notion by demonstrating that UV light, which also enhances transduction, also promoted second-strand synthesis. They speculated that E4orf6 might perform

the same function in the infectious pathway as it does in the transduction assays. Another group proposed that it functions by removing a cellular block to the synthesis of duplex genomes from single-stranded substrates, and that the gene may perform an analogous function in *Rep*-dependent replication of the AAV genome (Qing *et al.*, 1997; see below).

It has also been shown that induction of *E4orf6* expression in 293 cells leads to an increase in the percentage of cells in S-phase, apparently by blocking the S-phase to G2 transition (Grifman *et al.*, 1999). However, while *E4orf6* expression increases transduction, blocking the S/G2 transition by other means did not enhance rAAV transduction significantly, indicating that blocking the S/G2 transition cannot entirely explain the *E4orf6* enhancement. *E4orf6* expression correlates with a decrease in cyclin A, which has been implicated in the control of cellular DNA replication. The increase in rAAV transduction seen with *E4orf6* expression was reduced by increasing cyclin A levels using an expression plasmid.

Aside from its relationship with AAV, studies of *E4orf6* function have shown that it prevents concatemerization of adenovirus (Weiden and Ginsberg, 1994), and that it is involved in the disruption of the Mre11-Rad50-NBS1 DNA repair complex (Stracker *et al.*, 2002). This is probably how the *E4orf6* gene prevents concatemerization of adenovirus and should be considered as a possible explanation for part of its role as a helper factor for AAV DNA replication.

Since the adenovirus polymerase was not required for adenovirus helper activity (Richardson and Westphal, 1981), it has been assumed that the machinery used to replicate the AAV genome in adenovirus co-infected cells was of cellular origin. Furthermore, the notion that AAV DNA replication occurs by a single-stranded mechanism implies that it is components of the cellular leading strand fork that perform replication.

Using AAV Rep protein expressed from a recombinant baculovirus and a 'no end DNA' substrate to represent a replication intermediate, Ni *et al.* demonstrated that extracts from adenovirus-infected cells would support AAV DNA replication if Rep protein was added (Ni *et al.*, 1994). Apparently the Rep protein is required to recruit cellular replication factors to the viral genome and/or to unwind the viral ITR. Using an extract from uninfected but stressed HeLa cells, they later demonstrated by immunodepletion experiments that the Rep-dependent DNA replication activity relied upon components of the cellular leading strand replication machinery, i.e. replication protein A (RPA), replication factor C (RFC), proliferating cell nuclear antigen (PCNA) (Ni *et al.*, 1998). As expected the activity was not dependent upon the polymerase alpha-primase complex, supporting the notion that lagging strand activity was not involved. Dependence upon RFC indicates that, also as expected, either polymerase delta or polymerase epsilon are involved.

Using an open-ended duplex AAV genome, also representative of a replication intermediate, and an *Escherichia coli*-produced Rep-maltose binding protein fusion, it was shown that an extract from uninfected cells was also able to replicate

the AAV genome in a Rep-dependent manner (Chiorini *et al.*, 1994a; Ward *et al.*, 1994). However, this replication was much less extensive than that seen in an extract from adenovirus infected cells. This drop in replication efficiency was not due to a deficiency in initiation in the cell-free system, but rather resulted from a deficiency in the processivity of the replication forks (Ward and Berns, 1996). This problem could be overcome by addition of the adenovirus single-strand binding protein (Ad-DBP) to the uninfected cell extract, raising the possibility that in adenovirus-infected cells, the Ad-DBP may play a direct role in DNA replication (Ward *et al.*, 1998).

It is interesting to note a parallel between the AAV DNA replication seen in cell-free assays using adenovirus helper factors, and the proposed mechanism for adenovirus DNA replication in adenovirus-infected cells. In both AAV DNA replication (Ward and Linden, 2000) and adenovirus DNA replication (Lechner and Kelly, 1977), a replication cycle is constructed in which extensive replication occurs from single-stranded templates. The AAV assays suggested that duplex AAV genomes are preferentially dissociated into single strands prior to replication, and that DNA replication mostly occurs from single-stranded templates. One advantage of such a mechanism might be that it would forestall the possibility of a lagging strand complex associating with the displaced strand and commencing lagging strand replication. Emphasizing the importance of the single-stranded genome in AAV DNA replication, it has been noted in the herpes virus helper system, that single-stranded, but not double-stranded, AAV genomes were capable of supporting the establishment of AAV genome-Rep78-ICP8 complexes that are necessary for localization of the AAV genome to the replication compartments of AAV/HSV co-infected cells (Heilbronn *et al.*, 2003).

## Herpes virus as helper

The nature of the helper effect provided by herpes simplex virus (HSV) co-infection has also been difficult to define, although recent reports in which HSV was used as a helper in the production of rAAV vectors have convincingly demonstrated that HSV provides a very efficient helper effect (Conway *et al.*, 1997, 1999). When transfected into cultured cells seven HSV genes are entirely sufficient to support the replication of plasmids containing an HSV DNA replication origin (Challberg, 1986). By transfecting plasmids coding for each of these seven genes into cells infected with AAV, Heilbronn and her colleagues identified the *UL5*, *8*, *29*, and *52* genes as being sufficient for complete AAV helper activity (Weindler and Heilbronn, 1991). The *UL29* gene product has single-strand DNA-binding activity, but inclusion of the other herpes genes was a somewhat puzzling result since the *UL5*, *8*, and *52* gene products are components of the lagging strand replication machinery. It is expected that AAV would only be replicated by a leading strand mechanism; moreover HSV infection would not be expected to increase

the components of the cellular replication machinery. Heilbronn *et al.* did observe a substantial additional increase in DNA replication when the genes coding for the HSV polymerase, UL 30/42, were included with the above four genes. Subsequently it was shown in a defined assay that the products of the UL30, 42, and 29 genes were able to support initiation on a duplex AAV genome in a Rep-dependent manner (Ward *et al.*, 2001). Apparently the HSV leading strand replication complex is sufficient to support the initiation of Rep-dependent replication. This assay also demonstrated that the Rep protein is able to function as the replication helicase. Heilbronn and her colleagues have more recently shown that upon co-infection of AAV and HSV, Rep and ICP8 (the UL29 gene product) co-reside in HSV replication domains (Heilbronn *et al.*, 2003). This co-localization was dependent upon the presence of single-stranded AAV genomes. They also showed binding of Rep78 and ICP8 *in vitro*. Single-stranded DNA strongly stimulated this *in vitro* interaction whereas double-stranded DNA did not. This suggested that ICP8, single-stranded AAV and Rep78 form a tripartite complex that can assemble the components of a functional replication complex. AAV DNA is apparently directed to HSV replication compartments where the HSV complex can replicate the AAV genome.

Previously it had been demonstrated that the HSV UL5, 8, 52, and 29 gene products could support the remodeling of the structure of the nucleus that is seen upon HSV infection (Lukonis and Weller, 1997; Uprichard and Knipe, 1997).

Subsequently Weitzman and his colleagues demonstrated by transfecting mutant HSV genes that the helicase-primase activities of the UL5, 8, and 52 gene products were not essential for their AAV helper effect (Stracker *et al.*, 2004). They suggested that these HSV gene products contributed to the replication of AAV by remodeling the nucleus. A tentative conclusion of studies on the HSV helper effect might be that, in cells containing HSV replication factors, the AAV genome can be replicated both by the leading strand HSV replication complex and the host cell replication complex.

In addition to its interaction with ICP8, the Rep protein has been shown to interact with two other single-strand binding proteins, human RPA and adenovirus Ad-DBP (Heilbronn *et al.*, 2003; Stracker *et al.*, 2004). These proteins seem to enhance binding and nicking of the AAV origin by Rep. In addition all three proteins can contribute to AAV DNA replication in extracts from uninfected cells (Ni *et al.*, 1998; Ward *et al.*, 1998; Stracker *et al.*, 2004). Stracker and his colleagues suggested that Rep's ability to interact with various single-strand binding proteins comprises part of the flexibility that allows AAV to replicate under diverse conditions.

### Papilloma virus as helper and replication in keratinocytes

Because AAV had been found to interfere with papilloma virus-induced cellular transformation (Hermonat, 1991),

it seemed logical to ask if human papilloma virus (HPV) might provide a helper effect for AAV. Transfection of HPV-16 DNA into cells containing an integrated AAV genome-induced rescue of the AAV genome, while co-transfection of HPV-16 and AAV2 DNA into human epithelial cells led to AAV2 replication (Walz *et al.*, 1997). In addition, transfection of AAV DNA into immortalized human keratinocytes containing multiple copies of an HPV-16 episome produced infectious AAV (Ogston *et al.*, 2000). The transfection of HPV-16 E2 along with AAV into the episome-containing cell substantially increased the yield of AAV, while transfection of other HPV genes showed no enhancement. The E2 effect required the presence of the HPV-16 episome, indicating that E2 must act by working in concert with other HPV-16 proteins. E2 is known to affect p53 levels but large differences in the level of p53 did not alter AAV replication. It was concluded that E2 stimulates AAV multiplication independently of its effects on p53 levels. Since E2 has been shown to block the G2/M transition (Fournier *et al.*, 1999) these workers used nocodazole to achieve a mitotic block, and found that AAV DNA replication was increased in HeLa cells, but was especially increased in an immortalized keratinocyte line that contained multiple copies of an HPV-16 episome. The yield of infectious virus, however, was not increased. Ogston and her co-workers concluded that its ability to block the G2/M transition may be part of the E2 helper effect.

Interpretation of this data is rendered more complex by the observation that differentiating keratinocytes are at least partially permissive for AAV. It has been shown that AAV can replicate productively without helper viruses or genotoxic agents in an organotypic epithelial raft tissue culture system, which has been developed as a model for normal skin (Meyers *et al.*, 2000). In this model keratinocytes undergo a type of differentiation that closely resembles the pattern observed *in vivo*. In this system production of AAV correlated with epithelial differentiation, and non-differentiating keratinocytes were defective for AAV replication. In addition, apparently pathologic histologic changes were induced in the AAV-infected epithelial tissues. These results led the investigators to propose that keratinocytes might be a natural host for AAV.

Since AAV seemed to be replicating autonomously in keratinocytes, the effect of AAV infection on HPV replication was analyzed. In raft cultures co-infected with both viruses, HPV co-infection was slightly increased at low AAV multiplicity of infection (MOI) but decreased at high AAV MOI. As expected, HPV promoted AAV replication (Meyers *et al.*, 2001). Using plasmid transfections to examine the contribution of individual AAV genes to HPV replication, all four AAV Rep proteins were seen to enhance HPV-31b DNA replication. Surprisingly, Rep40 had the strongest effect (Agrawal *et al.*, 2002).

It has been noted that HeLa cells are particularly well suited for the creation of *rep/cap* expressing cell lines that are capable of giving high titers of rAAV vectors (Chadeuf

*et al.*, 2000). Since HeLa cells carry segments of the HPV genome, the possibility that HPV might be contributing to the production of AAV in HeLa derived *rep/cap* cell lines was addressed. A non-HeLa derived *rep/cap* cell line was produced from A549 cells (a lung carcinoma cell line that has no papilloma virus genes). As mentioned previously, the high yields of some *rep/cap* cell lines are dependent upon adenovirus-induced amplification of the integrated *rep/cap* sequences. However, when compared with a HeLa-derived line, in this new line there was a 10-fold decrease in the adenovirus titer that was needed for maximum amplification of *rep/cap* (Gao *et al.*, 2002). This result is consistent with the notion that an HPV product is not responsible for the high yield of rAAV seen in HeLa based *rep/cap* cell lines.

## Genotoxic agents as helper

The notion that human cells possess all of the factors required for AAV replication was confirmed by the demonstration that, under certain conditions, cultured cells that were not infected with a helper virus could support AAV replication. Treating cultured cells with genotoxic agents was shown to support AAV DNA replication in numerous cases, and to support its complete replicative cycle in some cases. However, this replication was less extensive than that associated with adenovirus or herpes virus infection.

It had been noted that parvoviruses can inhibit oncogenesis (Rommelaere and Tattersall, 1990). While most investigations focused on the autonomous rodent viruses, it was noted that AAV also had an oncosuppressive effect (Kirschstein *et al.*, 1968; Casto and Goodheart, 1972). Oncogenesis can be studied in a model system using cells transformed by SV40, which therefore often contain an SV40 origin of replication. Carcinogen treatment of these cells commonly led to dramatic amplification of the SV40 origin, and AAV infection inhibited this carcinogen-induced SV40 amplification; concurrent with this inhibition, AAV DNA sequences were amplified (Schlehofer *et al.*, 1983; Heilbronn *et al.*, 1984; Schlehofer *et al.*, 1986). A related observation was that AAV5 could selectively kill carcinogen treated cells (Heilbronn *et al.*, 1984).

All early experiments had been performed with cells containing SV40 origins, but subsequently this origin was shown to be unnecessary for carcinogen-induced AAV DNA amplification (Yakinoglu *et al.*, 1988). These workers observed AAV DNA amplification in all cell lines tested, including primary human diploid skin fibroblasts, as well as immortalized and tumorigenic lines. They also observed amplification in response to UV light, heat shock, the carcinogen MNNG, and inhibitors of DNA replication or protein synthesis, although in the latter two cases amplification occurred only upon removal of the agents. Assays were also performed using transfected AAV constructs, to exclude the possibility of adenovirus contamination. With MNNG as a representative inducer, it was determined that only 1–2

percent of the cells were synthesizing AAV DNA. These workers also noted that the time course of AAV DNA amplification in their assays was similar to the kinetics of carcinogen-induced SV40 DNA amplification. In the assays of other workers, however, one could distinguish carcinogen-induced AAV DNA amplification from carcinogen-induced SV40 DNA amplification, both by cell type and by time of induction (Bantel-Schaal and zur Hausen, 1988). In general, DNA amplification occurred more quickly for AAV. In further defining the amplification of AAV DNA by carcinogens, Yakinoglu showed that when cells containing a plasmid in which there was an AAV origin were treated with a carcinogen, DNA synthesis occurred in the origin region (Yakinoglu *et al.*, 1991).

Assays employing UV irradiation as a helper for AAV replication showed that wavelength had a marked effect upon the response. These results suggested that the target for the irradiation was DNA (Yakobson *et al.*, 1989). With UV providing the helper effect, these workers also measured infectious virus production. They saw a substantial helper effect, i.e. 1000-fold increase over the input virus. However, this is still 50- to 300-fold less efficient than adenovirus co-infection, although the disparity depended on the cell type tested. Since only a very small percentage of cells were synthesizing AAV DNA, the efficiency of UV and carcinogen-induced helper effects compare more favorably with adenovirus on a per cell basis.

A separate, possibly related, series of investigations explored factors that enhance the transduction efficiency of rAAV vectors. In addition to being enhanced by adenovirus infection, transduction is also enhanced by some of the same genotoxic agents found to provide a helper effect for AAV DNA replication (Alexander *et al.*, 1994; Russell *et al.*, 1995; Alexander *et al.*, 1996; Ferrari *et al.*, 1996; Fisher *et al.*, 1996). In several of these studies it was shown that enhanced transduction correlated with increased amounts of duplex genomes. The question raised by several of these groups is whether these agents, which affect DNA metabolism generally, might be enhancing transduction and providing a helper effect for wild-type AAV replication by a common pathway. A complicating consideration, however, is that the *Rep* gene is present in the wild-type virus but absent from the recombinant virus.

Adenovirus and herpes virus infection, and the various non-viral methods of rendering cells permissive for AAV replication have in common an attack on the integrity of the cell. It is thought that AAV has evolved so that latent AAV genomes respond to host cell damage by commencing productive replication before the cell dies. Mori and co-workers thus reasoned that cell death itself might provide a helper effect. Specifically, they asked whether apoptosis could render cells permissive for AAV DNA replication (Mori *et al.*, 2002). These workers infected cells with AAV under non-productive conditions and selected two clones that allowed efficient rescue of intact AAV upon adenovirus infection. (A preliminary analysis suggesting that the AAV genomes

in these two clones might not have been integrated at *AAVS1* could be relevant.) Treating these cells with an anti-Fas antibody induced low-level virus production, as well as markers for apoptosis. They next transfected these clones with a Fas-containing construct, and derived new clones that overexpressed Fas. Using the anti-Fas antibody, they were able to induce higher levels of virion production in these Fas-enriched cells. To confirm the role of apoptosis, they employed a caspase-8 inhibitor, which inhibited both DNA fragmentation and AAV production. Interestingly, it has been shown by another group that the Rep78 protein can itself induce apoptosis (Schmidt *et al.*, 2000).

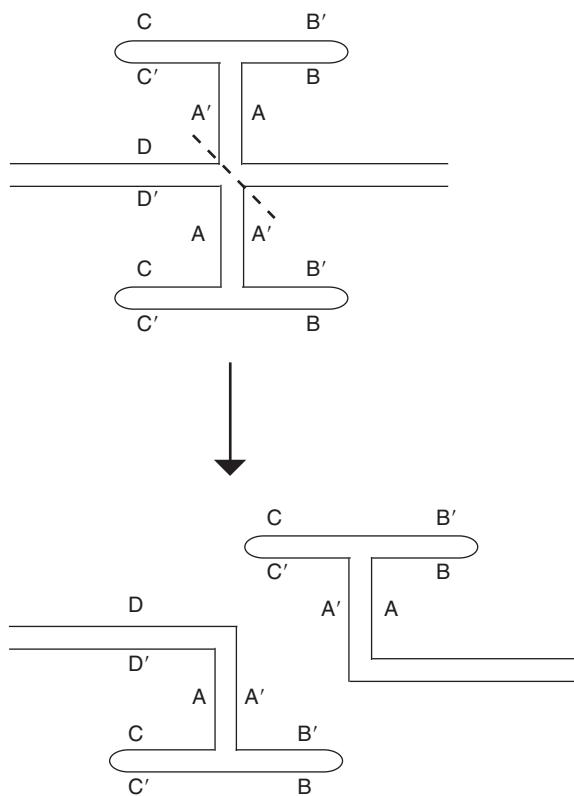
The mechanisms responsible for the helper effects provided by non-viral agents remain unknown. These agents have in common an effect on DNA metabolism, but it is not known if the helper effects of different agents operate through a common pathway. For example, in relation to transduction, there is evidence that UV irradiation and *E4orf6* may induce accumulation of double-stranded forms of rAAV vectors by different pathways (Sanlioglu *et al.*, 1999). It is also unclear why AAV DNA replication is only observed in a small percentage of the treated cells. Cell synchronization by mitotic detachment, a method that uses no drugs and in which there is a minimum of stress to the cells, has demonstrated that there is a small percentage of cells, i.e. 0.1 percent, that are able to synthesize AAV DNA in the absence of outside helper or genotoxic agents (Yakobson *et al.*, 1987). This observation suggests that in a population of cells some small fraction is always able to synthesize AAV DNA, and raises the question of how these differ from the majority of the population. Such a small percentage of cells would seem to preclude the possibility that the permissive cells are simply those at a certain point in the cell cycle. They may represent the small percentage of cells in some specific short-lived metabolic state, for example repairing damaged DNA. In such a scenario 'genotoxic agents' might function by increasing the percentage of cells in this state. The answer to these questions, and an understanding of how the non-viral agents promote AAV replication generally, promises to be revealing for both AAV and the host cell.

## Rescue

As described above, 'rescue' designates the release of the AAV genome from its latent site on chromosome 19 or from a plasmid vector. When AAV was first cloned into a plasmid vector, it was noted that, upon transfection of this construct into an adenovirus-infected cell, the AAV genome was released from the plasmid and productively replicated (Samulski *et al.*, 1982; Laughlin *et al.*, 1983). Since rescue of the viral genome from the human genome probably operates by a mechanism that is similar to rescue from a plasmid construct, the latter has been used as a model for rescue from a chromosomal locus.

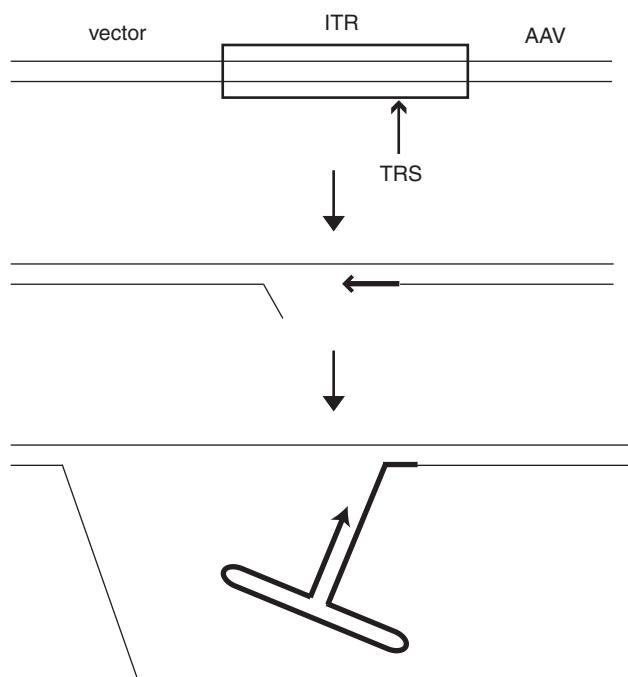
AAV can remain latent in chromosome 19 for many passages, indicating that rescue is greatly downregulated until conditions are appropriate for productive replication. Small numbers of AAV genomes can be detected in latently infected cells after many passages, suggesting that low level rescue is occurring in the cells (Cheung *et al.*, 1980). Nevertheless it seems likely that helper virus infection stimulates rescue, rather than simply amplifying the small number of free AAV genomes. Since genotoxic agents as well as helper virus infection induce cells to commence productive AAV replication, which in some cases involves rescue (Mori *et al.*, 2002), it seems likely that helper virus functions do not directly participate in rescue. Helper virus infection or genotoxic agents probably promote rescue either through stimulating the production of Rep proteins or by altering the cellular environment in other ways.

Two mechanisms have been proposed for rescue: excision by cellular nucleases (Samulski *et al.*, 1982; Gottlieb and Muzyczka, 1988), or rescue as an integral part of DNA replication (Samulski *et al.*, 1983; Ward *et al.*, 1994). One cellular endonuclease with the potential for mediating genome excision is the endo R activity characterized by Gottlieb and Muzyczka (Gottlieb and Muzyczka, 1988), which cleaves GC rich sequences and which, by cleavage in or near the AAV ITR, was shown to release AAV genomic sequences from the plasmid pSM620. While most of the postulated endonuclease mechanisms for rescue involve cellular enzymes, it has been suggested that the AAV Rep protein might itself have a double-stranded endonuclease activity that would allow it to accomplish rescue (Wang *et al.*, 1996). When the AAV ITRs in a plasmid construct melt out and refold so that the potential palindrome on each strand self-basepairs, they create a Holliday structure. The suggestion is that this Holliday conformation might be resolved by Rep acting as a Holliday structure-resolving enzyme, cleaving both strands so as to produce two closed hairpin ends (Wang *et al.*, 1996; Figure 15.4). Rep-dependent cleavage of both strands of AAV constructs that is not through a Holliday intermediate has also been postulated as playing a role in excision of the AAV genome from plasmid constructs (Senapathy *et al.*, 1984). More recently, such an activity has been observed in assays containing only a DNA substrate and the Rep protein. It is possible that this cleavage may have been dependent on cellular enzymes which co-purified with Rep (Li *et al.*, 2003; Ward *et al.*, 2003). In one case it was noted that Rep became attached to the strand opposite the nicking site (Li *et al.*, 2003), but in neither of these assays was the product compatible with that predicted from resolution of a Holliday structure. It has, however, been shown that rescue can occur by a Holliday structure-resolving activity, which is cellular in origin. Such an activity has been observed both in cellular extracts and in cells (Ward and Berns, 1991; Xiao *et al.*, 1997; Figure 15.4). An interesting question raised by this observation is how AAV remains latent in chromosome 19 despite the existence of this activity.



**Figure 15.4** Resolution of a Holliday structure. Shown is the alternate structure which the AAV ITR might assume in a plasmid or chromosome. Cleavage by a Holliday structure-resolving enzyme is indicated by the dashed line. The products are each in a closed hairpin conformation and contain one copy of the palindromic component of the ITR. Only the AAV side contains the D-region sequence.

A replication-based assay has been developed for rescue (Figure 15.5). The assay uses a defined system for the initiation of DNA replication at the AAV origin and a plasmid substrate containing an AAV genome (Ward *et al.*, 2003). The Rep protein and the components of an HSV leading strand replication complex apparently nick the substrate at the ITR and initiate replication from the newly exposed 3' hydroxyl. As the replication complex passes through the ITR the newly synthesized strand is apparently displaced from its template and folds back upon itself, after which replication resumes in the reverse direction. A duplex AAV genome with the rescued ITR in a closed hairpin is produced from the plasmid construct. In this assay, rescue is dependent only on the presence of the Rep protein and a replication complex, factors presumably induced by helper virus infection. Previously a similar phenomenon was seen to occur in extracts from uninfected cells (Ward *et al.*, 1998). In that case rescue by this type of turn-back replication seemed to depend on the presence of adenovirus DNA-binding protein. In initiation supported by the human DNA-binding protein RPA, which was as efficient as the adenovirus DNA-binding



**Figure 15.5** Model for rescue by replication. Shown is an AAV genome cloned into a plasmid vector Rep (not shown) that binds to the RBS, nicks at the TRS, and initiates synthesis in the 5' to 3' direction. When synthesis of the ITR is nearly complete, the newly replicated strand may fold on itself. In such a case, DNA synthesis would be resumed on a structure that is now hairpinned. Therefore replication is directed towards the AAV genome. Bold line indicates newly synthesized DNA. Box at junction between AAV and vector sequences indicates AAV ITR.

protein, replication passed through the ITR without turning back, i.e. rescuing. Interestingly, a mechanism for the resolution of junctions in the autonomous parvovirus minute virus of mice, which is similar to this assay, has recently been reported (Cotmore and Tattersall 2003).

For rescue from a chromosomal location, as opposed to rescue from a plasmid vector, additional factors such as the accessibility of the chromosomal domain and the presence of nearby transcriptional activity must certainly play a role. It may be that the selection of the AAVS1 site on chromosome 19 as the preferred site for Rep-dependent integration in part reflects its suitability for appropriately timed and efficient rescue. This may, for example, be a site that is protected from cellular Holliday structure resolving activity and therefore from inopportune rescue, or it may be a site that allows particularly efficient rescue of the AAV genome at opportune times. Gottlieb and Muzyczka noted that the production of virus from an integrated AAV genome is substantially greater than that obtained from a transfected plasmid at the same copy number (Gottlieb and Muzyczka, 1988). They suggested that the difference in virus production may in part reflect a difference in efficiency of rescue.

## Cellular localization of replication

Studies defining the timing of expression and cellular location of AAV factors and of essential cellular and helper virus factors provide a separate form of insight into the mechanisms of AAV replication. As our knowledge of nuclear processes and architecture increases, studies of the precise location of replicating AAV DNA are likely to be highly informative.

Redemann *et al.* used immunoblotting and immunoprecipitation assays with  $^{35}$ S-methionine-labeled virus to characterize the accumulation of Rep proteins in the infected cell (Redemann *et al.*, 1989). They noted that in the first 10–12 hours after AAV infection of adenovirus infected 293 and KB cells, only very small amounts of duplex AAV DNA or Rep proteins could be detected. Between 12 and 24 hours most of the Rep and capsid proteins were synthesized. During this time there was extensive accumulation of duplex AAV DNA. Rep78 and Rep52 were detected at earlier times than Rep68 and Rep40. Interestingly the relative abundance of the four Rep proteins, once established early in infection, was maintained. By pulse-chase analysis they found that the Rep proteins were stable for at least 15 hours. After 24 hours synthesis of viral proteins was much reduced, the levels of duplex AAV DNA remained constant, and there was an extensive accumulation of single-stranded AAV DNA. Previous studies of single-stranded DNA had shown that its accumulation was dependent upon capsid synthesis. (Myers and Carter, 1981; Hermonat *et al.*, 1984; Tratschin *et al.*, 1984). It may be that single-stranded DNA first becomes detectable at times when it is beginning to be sequestered by capsid protein or preformed capsids.

Using *in situ* hybridization and immunofluorescence, Wistuba *et al.* studied the localization of AAV Rep, capsid proteins, and DNA during AAV/Ad co-infection (Wistuba *et al.*, 1997). At first Rep proteins show a punctate distribution over the nuclei, in a pattern reminiscent of replication foci. At later time points Rep is distributed more homogeneously. Finally the protein is redistributed into clusters that are slightly enriched at the nuclear periphery. At an intermediate time Rep also appears briefly in the interior part of nucleolus, whereas during most of the infectious cycle the nucleolus is Rep negative. AAV2 DNA co-localizes with the Rep proteins. All three capsid proteins are strongly enriched in the nucleolus during a transient stage, during which Rep proteins homogenously fill the nucleus. Assembled capsids, detected by a conformation specific antibody and first seen in the nucleolus, then spread over the whole nucleoplasm. These capsid proteins finally co-localize in the nucleoplasm with Rep clusters and DNA. As yet, this complicated choreography is difficult to interpret. The sequence does however suggest a model in which capsid assembly involves nucleolar components whereas packaging occurs in the nucleoplasm.

Weitzman *et al.* extended this study by examining the relationship between AAV and adenovirus components (Weitzman *et al.*, 1996). Adenovirus reorganizes the infected

nucleus to form specific structures in which adenovirus transcription and replication occur. Weitzman and his colleagues found that in co-infected cells the AAV genome was recruited to adenovirus replication centers.

The same sorts of studies were performed in cells in which HSV was the helper virus (Stracker *et al.*, 2004). AAV and HSV-1 proteins co-localize at discrete intranuclear sites. As mentioned above, these workers showed that remodeling of the nucleus in HSV-infected cells was induced by the products of the HSV *UL5*, *8*, *52*, and *29* genes (Stracker *et al.*, 2004; Uprichard and Knipe, 1997; Lukonis and Weller, 1997), which had previously been shown to provide a helper effect for AAV DNA replication (Weindler and Heilbronn, 1991). Interestingly mutant forms of these genes, which had lost their helicase/primase capacities, were still able to provide some helper effect for AAV DNA replication, emphasizing the importance to AAV of nuclear remodeling (Stracker *et al.*, 2004). Co-localization studies have shown that, upon co-infection with AAV and HSV, Rep and the HSV DNA-binding protein ICP8 co-reside in HSV replication domains (Heilbronn *et al.*, 2003). However, in the absence of AAV DNA the Rep protein did not co-localize with ICP8. Co-localization was restored by introducing single-stranded AAV genomes. These co-localization studies correlate with *in vitro* studies demonstrating the binding of Rep78 and ICP8 (Heilbronn *et al.*, 2003; Stracker *et al.*, 2004). Apparently ICP8 single-stranded AAV DNA and Rep78 form a complex that is able to recruit the AAV genome to HSV replication compartments.

It has been noted that the genomes of adenovirus and HSV-1 associate with nuclear structures termed ND10 or promyelocytic leukemia (PML) nuclear bodies (NBs). PML NB morphology is disrupted by specific viral proteins as replication proceeds. The question arises as to whether AAV genomes associate with these bodies, and whether their disruption is necessary for AAV replication. To answer these questions Fraefel and his colleagues made an rAAV containing multiple copies of the lac repressor-binding site (Fraefel *et al.*, 2004). They could then use a lac repressor-yellow fluorescent protein fusion to visualize these genomes. In adenovirus infected cells AAV replication compartments can associate with modified PML NBs. In HSV-infected cells, they do not do this because the HSV ICP0 protein completely disrupts PML NBs. However, PML NB disruption is apparently not required for AAV replication, as formation of replication compartments was normal when an ICP0 mutant, which does not disrupt PML NBs, was used as helper virus. With the mutant helper virus, AAV replication compartments did not associate with PML NBs.

## Other cellular factors which interact with AAV

Several host cell factors which are not components of the cell's basic replication machinery have been shown to bind

to the AAV genome or to the AAV Rep protein, and may also have a role in AAV DNA replication.

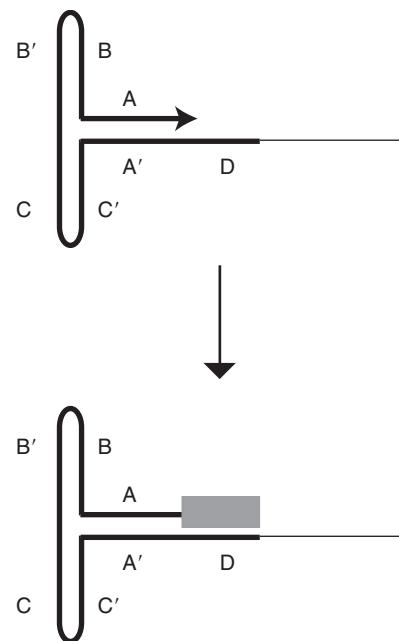
#### D-REGION BINDING PROTEIN

Having found that the D-region sequence was important for the rescue/replication of the AAV genome, Srivastava and his colleagues searched for a protein which might bind to the D-region. By gel shift analysis they found that this sequence interacts with an unknown host cell protein, which they designated the D-region binding protein (D-BP; Wang *et al.*, 1996). The same 10 nucleotides of the 20-nucleotide D-region required for efficient rescue/replication were also necessary and sufficient for interaction with the D-BP (Wang *et al.*, 1997).

D-BP preferentially forms a complex with the single-stranded D-region found at the 3' end of a single-stranded AAV genome. Phosphorylation of the protein at tyrosine residues correlates with a block to second-strand synthesis. Inhibition of cellular protein tyrosine kinases, for example by genistein, correlates with D-BP dephosphorylation at tyrosine residues. This dephosphorylation in turn correlates with enhanced transduction from rAAV vectors and with autonomous replication of AAV genomes, to a level that is comparable to that seen previously with genotoxic agents such as hydroxyurea. Adenovirus infection or the expression of the *E4orf6* gene also correlate with dephosphorylation of D-BP at tyrosine residues (Qing *et al.*, 1997).

Examination of rAAV transduction in several cell lines showed that transduction correlated with the expression level of tyrosine non-phosphorylated D-BP in the cell lines (Qing *et al.*, 1998). (It should be noted that transduction efficiency did not correlate with infectivity in this study.) The same workers also prepared extracts from several mouse tissues that are known to be transduced by rAAV vectors, and observed that D-BP was predominantly in the tyrosine non-phosphorylated form in these tissues. The model they proposed is that phosphorylated D-BP binds the D-region immediately adjacent to the 3' hairpin primer on the single-stranded genome (Figure 15.6). When bound, this protein would be able to block extension of the 3' end, thereby blocking creation of the duplex substrate necessary for transduction. They postulate that the phosphorylated form of D-BP might, in the same manner, block replication of the AAV genome.

Quing *et al.* subsequently identified D-BP as a cellular protein previously observed to bind the immunosuppressive drug FK506 and known as FK506 binding protein 52 or FKBP52 (Qing *et al.*, 2001). To test whether this protein's phosphorylation state correlated with the cell's ability to block second-strand synthesis on an AAV template, they performed *in vitro* replication assays using a single-stranded hairpinned AAV template and the Klenow fragment of pol 1. Incubation with the tyrosine phosphorylated form of FKBP52 before addition of the Klenow fragment blocked second-strand synthesis by more than 90 percent. If the



**Figure 15.5** Inhibition of AAV DNA second strand synthesis by the D-region binding protein (D-BP). Shown is a schematic of the binding of a tyrosine-phosphorylated D-BP to the D-region of the minus strand. Such binding is postulated to block synthesis of the second strand.

protein was phosphorylated at serine or threonine, synthesis was reduced by 40 percent, while if the protein was unphosphorylated it did not block extension. They determined that the unphosphorylated form does not bind the D-region. The phosphorylated form of FKBP52 proved to be a substrate for the T-cell tyrosine phosphatase protein. Overexpression of this phosphatase led to efficient second-strand synthesis (Qing *et al.*, 2003).

Since FKBP52 is known to complex with the cellular heat shock protein 90 (HSP90) and since heat shock treatment enhances transduction, the role of this complex in rAAV transduction was investigated (Zhong *et al.*, 2004). However, these studies suggest that increased expression of HSP90 decreases transduction, and that disruption of the HSP90-FKBP52-D-region complex increases transduction. HSP90 by itself apparently does not bind the D-region. If the HSP90-FKBP52-D-region complex affects second-strand synthesis, it may be that the state of this complex will also affect AAV DNA replication.

#### OTHER PROTEINS

Other cellular factors have been implicated in AAV DNA replication. Costello *et al.* observed that the high mobility group one protein, HMG1, physically interacts with the AAV Rep protein (Costello *et al.*, 1997). HMG1 promotes the formation of Rep-DNA complexes and stimulates Rep nicking and hydrolysis of ATP. Since complexes of Rep

and DNA have similar stabilities in the presence or absence of HMG1, it may be that HMG1 acts by promoting assembly of the Rep-DNA complex in cellular nucleoprotein structures.

Han *et al.* used affinity purification to demonstrate an association between Rep and the epsilon and gamma forms of the 14-3-3 family (Han *et al.*, 2004). Phosphorylation of Rep at Ser 535 allowed Rep to bind 14-3-3 proteins. Binding of Rep to 14-3-3 reduced the affinity of Rep for DNA. A mutant of Rep that was unable to bind 14-3-3 replicated an rAAV genome more efficiently than the wild-type protein, suggesting that Rep's interaction with 14-3-3 may affect AAV DNA replication.

It has been noted that the human genome contains many sequences that closely resemble the AAV Rep-binding site (Wonderling and Owens, 1997). These sequences are mostly located near transcription start sites. The abundance of these sites and their common location suggests the existence of cellular binding factors. It is to be expected that factors binding these genomic sites will also interact with the Rep-binding site in the AAV genome. An analysis of such interactions is likely to reveal a complex interplay between the AAV genomes and the cell. Using a yeast one-hybrid assay, Cathomen *et al.* identified a cellular Rep-recognition sequence binding-protein, the zinc finger 5 protein (ZF5), which binds both *in vivo* and *in vitro* (Cathomen *et al.*, 2001). ZF5 is a highly conserved and ubiquitously expressed transcription factor. Ectopic expression of ZF5 leads to ITR-dependent repression of the p5 promoter and reduces AAV2 replication and production of recombinant AAV2. Studies with mutants demonstrated that different domains of ZF5 are responsible for repression of the p5 promoter and the reduction in replication. These workers speculate that ZF5 represses replication by competing with Rep for binding to the viral ITR and that in this manner ZF5 may be instrumental in maintaining the latent state of AAV.

Finally it is worth remembering that the autonomous parvoviruses have much in common with AAV. In this context it is noteworthy that MVM has been shown to possess internal sequences that bind cellular proteins and that affect MVM DNA replication (Salvino *et al.*, 1991; Tam and Astell, 1993, 1994; Cossions *et al.*, 1996). While such sequence elements have not been demonstrated for AAV, it might be useful to keep an open mind as to their possible existence.

In conclusion it can be said that, though an apparently simple virus by virtue of its limited coding capacity, AAV continues to reveal complexity and subtlety in its replicative processes. If viruses could be described as being more or less clever, perhaps a correlation might be made between viral IQ and the inverse of genome length.

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# Site-specific integration by adeno-associated virus

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The life cycle of adeno-associated virus (AAV) has been investigated for several decades, and its unique viral strategy has several intriguing aspects, one of which will be reviewed below. First, however, a few general comments might be helpful in order to place our analysis into a context that can both underline and put into question possible conclusions that we might be tempted to draw from the observations listed in this chapter. It is noteworthy that even though approximately 80 percent of the American population test seropositive for AAV2 antibodies, we have very little knowledge about the viral life cycle *in vivo* in its natural host. Undoubtedly, this shortfall is correlated to the absence of obvious pathogenic effects resulting from AAV infection. From a virological point of view, however, this absence of pathology together with the extraordinarily wide dissemination might indicate that AAV has evolved a near optimal relationship with its host. Should our model for the AAV lifestyle hold true *in vivo*, this virus might have overcome an apparent contradiction that is inherent to all viruses: on the one hand viruses rely on their host for productive replication and therefore survival, and on the other hand, in most cases, hurt the host to various degrees at some point throughout their life cycle. If our tissue culture observations can be extrapolated to the *in vivo* scenario, AAV has evolved to replicate only in cells that are no longer useful to the host (in the case of adenovirus infection) or that are potentially hazardous to the host (in the case of infections by herpes viruses, possibly papilloma viruses, and tumor transformation). In this, AAV infection could be

seen as protective to the host, a notion that is certainly consistent with the widespread distribution of AAV2 throughout the population. In order to achieve this task AAV is thought to have the choice between two alternative life cycles. In the presence of helper virus co-infection, AAV replicates productively to levels that are extraordinarily high (numbers of  $>10^5$  particles per cell have been suggested). In the absence of helper virus infection (possibly in tissues that are refractory to helper virus infection) AAV is thought to establish latency through genome integration. This model has several implications. With respect to latency it is of note that, in contrast to, for example, retroviruses, this step would not necessarily be required. One can imagine that AAV could propagate exclusively as a ‘satellite virus’ to its helper. Consequently, the mechanisms underlying latency would not necessarily be expected to be as efficient as for example retrovirus cDNA integration. In agreement with this notion is the observation in tissue culture that the establishment of latency is a rather inefficient process. Putting the hypothesis that latency is a byproduct (‘plan B’) of the viral life cycle into question, however, is the observation that the mechanism by which wild-type AAV is thought to secure the persistence of its genome is rather elaborate and therefore unlikely to be accidental. AAV has been shown to integrate its genome site-specifically into the human host genome. To date, this strategy remains unique among eukaryotic viruses and might highlight aspects of the virus–host interactions that are as yet unknown. In addition, this viral

strategy illuminates a potential that has so far been explored to a very limited extent. Understanding the mechanism of site-specific integration might, one day, point us to development of technologies for targeted gene addition for both experimental and therapeutic use.

## LATENT AAV INFECTION *IN VIVO*

Latent AAV infection in human and non-human primates was first proposed in 1972. By screening primary cells for vaccine production, Hoggan and co-workers discovered that 20 percent of African green monkey kidney cells and 1–2 percent of human embryonic kidney cells produced AAV upon adenovirus infection (Hoggan *et al.*, 1972). This observation indicated that latent AAV infection might be common *in vivo*. In support of this notion, epidemiological studies have found that 80 percent of the American population is seropositive for AAV2 antibodies (Mayor *et al.*, 1976). Since seroconversion occurs during early childhood, it was suggested that AAV could be transmitted horizontally. With the more recent advances in molecular biology and the development of sensitive technologies based on polymerase chain reaction (PCR), it became possible to detect AAV DNA sequences in a considerable number of human biopsies. Because these studies are the topic of a different chapter in this book, we will limit our discussion to a few examples. To date, AAV DNA sequences have been detected in human peripheral blood cells (Grossman *et al.*, 1992), in the female and male genital tracts (Tobiasch *et al.*, 1994; Han *et al.*, 1996; Friedman-Einat *et al.*, 1997; Rohde *et al.*, 1999) and more recently in skeletal muscle (Tezak *et al.*, 2000). AAV sequences that had integrated into the human genome have been reported to be present in human testis tissues (Mehrle *et al.*, 2004).

## LATENT AAV INFECTION *IN VITRO*

In order to study the AAV latency and ultimately to dissect the underlying mechanisms, the establishment of *in vitro* model systems had become necessary. Studies emerging from different laboratories demonstrated that latent infection by AAV could be mimicked in tissue culture. To date, almost all of our knowledge on AAV latency is based on such tissue culture systems. Hence, the question as to whether latency represents an integral aspect of the AAV life style in humans remains unanswered.

In the initial experiments, human Detroit 6 cells were infected with wild-type AAV (wtAAV) at a multiplicity of infection (MOI) of 250 infectious units per cell and the cultures were maintained through continuous passages (Hoggan *et al.*, 1972). The infected cells were subsequently cloned after the 39th passage and 30 percent of the clones (18/63) were positive for AAV DNA upon challenge with

adenovirus (Berns *et al.*, 1975). One of the positive clones, clone 7374, was subsequently recloned and remained positive for AAV DNA for more than 100 passages. This study demonstrated that AAV could establish a latent infection in tissue culture. Handa *et al.* extended these studies and showed through DNA reassociation kinetics that AAV DNA had indeed integrated into the cellular genome of latently infected human KB cells (Handa *et al.*, 1977). Initial Southern blot analyses of the 7374 cells indicated that the viral DNA was integrated as head-to-tail tandem repeats and that viral inverted terminal repeats (ITRs) were present at the junction with cellular DNA (Cheung *et al.*, 1980). Further analysis suggested that the integrated viral ITR sequences were unstable and subject to rearrangements. First, comparison of restriction patterns between an early and a late passage of the 7374 line showed significant differences when a restriction enzyme that cuts only the AAV ITR was used, whereas no difference was observed with an enzyme that cuts within the viral coding region. Second, free copies of AAV genomes were detected at a late passage of the clone (118th), but not at passages 9 and 10 (Cheung *et al.*, 1980). These results have since been confirmed and extended to numerous additional cell lines (Laughlin *et al.*, 1986; McLaughlin *et al.*, 1988).

In order to characterize the organization of AAV DNA in latently infected cells at the molecular level, Kotin *et al.* mapped and partially sequenced one viral-viral and two viral-cellular junctions from the 7374 cells (Kotin and Berns, 1989). This study not only confirmed the presence of the viral ITR at the cellular junction but also showed that the AAV ITRs were partially deleted at the integration site. Importantly, AAV sequences had integrated as a tail-to-tail tandem repeat, possibly as a consequence of AAV DNA replication. This finding was in disagreement with previous reports that, based on Southern blot analyses of the same clone, had suggested the presence of head-to-tail tandem repeats. This discrepancy might be explained by the significant number of rearrangements of viral sequences that cannot be identified by Southern blot analysis.

## SITE-SPECIFIC INTEGRATION BY AAV

Based on the early studies it had been assumed that AAV DNA integrates randomly into the host genome. Hybridization to a viral probe had revealed unique restriction patterns for each latently infected cell line examined, which was taken to be indicative of random integration. However, this hypothesis was subsequently ruled out by Kotin and collaborators in 1990. In order to characterize the organization of AAV DNA in latently infected cells, three viral fragments were cloned from the 7374 cell line and sequenced. Two viral-cellular junctions were found (Kotin and Berns, 1989). The cellular sequence flanking the viral DNA was then used as a probe on Southern blots that were derived

from a panel of independent latently infected human cell lines obtained from four laboratories (Kotin *et al.*, 1990). Surprisingly, the cellular probe did not only hybridize to the corresponding cellular locus, but also to additional bands in 78 percent (11 out of 14) and 50 percent (4 out of 8) of the clones infected with wtAAV and recombinant AAV (rAAV) viruses, respectively. These results indicated that this specific cellular sequence had been disrupted through recombination with the viral sequences. This observation led the authors to propose that AAV DNA integrates preferentially into a specific locus (Kotin *et al.*, 1990), which was then called AAVS1 (adeno-associated virus integration site 1; Kotin *et al.*, 1992). To date, AAV remains the only eukaryotic virus capable of site-specific integration. Coinciding with this discovery, AAV has drawn considerable attention as an attractive viral vector for targeted gene delivery. Retrospectively, it must be noted that the latently infected cells used in the study described above had been generated upon either wtAAV or rAAV infection. At this time, the requirements for AAV site-specific integration were unknown and the rAAV viral stocks were often contaminated with wtAAV viruses. It has since been established that AAV site-specific integration requires Rep78/68 in *trans* (Suroskey *et al.*, 1997). It is therefore reasonable to suggest that site-specific integration observed with rAAV DNA was most likely due to a Rep contaminant present within the rAAV virus stocks.

Since the first study by Kotin *et al.*, several latently infected cell lines have been established, including the aneuploid 293, HeLa, Detroit 6, KB, Huh-7, and IB3 cells, and the diploid WI-38 and CD34+ cells (Laughlin *et al.*, 1986; Kotin and Berns, 1989; Kotin *et al.*, 1990; Samulski *et al.*, 1991; Goodman *et al.*, 1994; Yang *et al.*, 1997; Kearns *et al.*, 1996; Pieroni *et al.*, 1998). Through an elegant approach Samulski *et al.* used a protein-DNA binding enrichment assay in order to isolate and characterize proviral AAV sequences (Samulski *et al.*, 1991). The strength of this technique is its potential for isolating a large number of viral-cellular junctions without a bias for the integration site. For this approach, a hybrid virus was generated that contained the lambda operator sequences (OR1-OR2) downstream of the AAV polyadenylation site. HeLa and WI-38 cells were infected with this virus that behaves like the wtAAV in terms of AAV replication and viral production. Genomic DNA from latently infected cells was then digested with a restriction endonuclease that does not recognize AAV sequences. The restricted DNA was incubated with purified lambda repressor protein and then used in filter binding assays. The only fragments retained on the filter contained the OR1-OR2 sequences bound to lambda repressor. Isolation and characterization of the viral-cellular junctions revealed that AAV had integrated its genome into the same region of chromosome 19 previously identified by Kotin *et al.* (AAVS1).

The fact that AAV had been shown to integrate site-specifically in 80 percent of the samples analyzed might indicate the existence of alternative integration sites within the

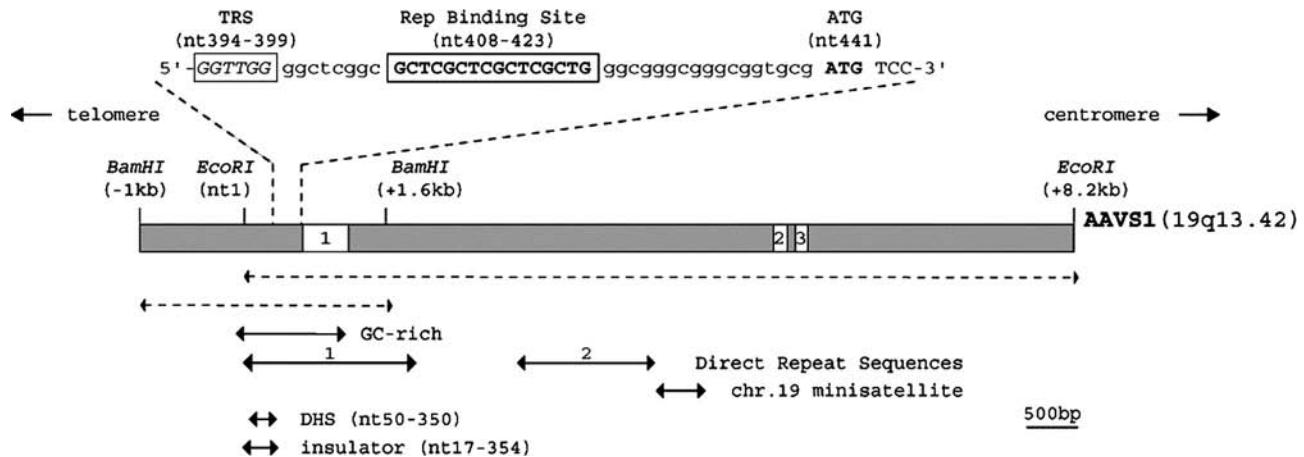
human genome. Indeed, based on fluorescence *in situ* hybridization (FISH) analyses two studies have reported AAV integration into loci other than AAVS1 (chromosome 17 for HA16 cells, a latently infected HeLa cell line [Walz and Schlehofer, 1992] and chromosome 2 for MKrA cells, a latently infected melanoma cell line [Bantel-Schaal, 1995]). However, sequence analysis of the cellular-viral junctions isolated from HA16 cells revealed that AAV DNA had integrated into AAVS1 (Dutheil *et al.*, 2000; Dutheil *et al.*, unpublished results). These conflicting data can be explained by a translocation of AAVS1 to chromosome 17. By analogy, it will be of interest to determine the integration locus in the remaining melanoma cell line at the molecular level. Taken together, to date there is no conclusive evidence of wtAAV integration into human loci other than AAVS1.

## CHARACTERIZATION OF AAVS1

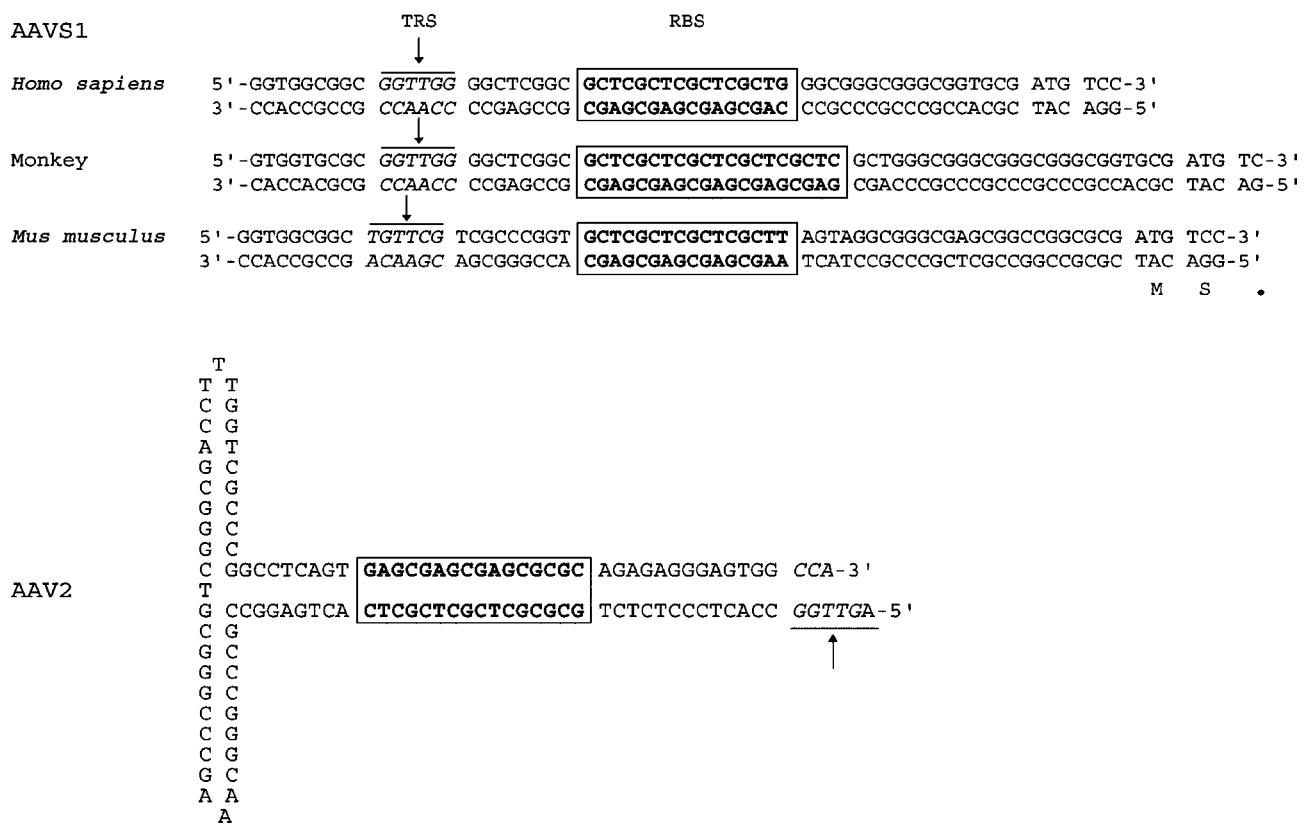
### AAVS1

The pre-integration site, AAVS1 had initially been mapped to the long arm of human chromosome 19 at position 19q13.3-qter by fluorescent *in situ* hybridization of metaphase chromosomes from normal human peripheral lymphocytes (Kotin *et al.*, 1991) and to position 19q13.4-ter by somatic hybrid mapping (Samulski *et al.*, 1991). With the completion of human genome project, AAVS1 has now been assigned to the location 19q13.42.

To better understand the requirements for site-specific integration, Kotin and collaborators dissected AAVS1 at the molecular level. A genomic library of diploid human embryonic lung fibroblasts (WI38) was screened and an 8-kb EcoRI fragment was isolated. Of this fragment the first 4 kb were sequenced (Kotin *et al.*, 1992). It must be noted that Samulski and co-workers had isolated a 2.6-kb BamHI fragment from a cosmid library (Samulski *et al.*, 1991), which partially overlaps the AAVS1 sequence characterized by Kotin *et al.* In this chapter, we will refer to the sequence described by Kotin *et al.* and the EcoRI site will arbitrarily be designated as nucleotide number 1. The AAVS1 sequence, which shares no significant homology with the AAV genome, has several noticeable features (Figure 16.1), including a high number of short repetitive sequences, a mini-satellite sequence present only on the long arm of chromosome 19 and a sequence homologous to the M26 motif. The M26 motif has been characterized as an enhancer element of meiotic gene conversion in fission yeast, and is highly homologous to the cAMP-responsive element (CRE). More interestingly, within the first 500 basepairs of AAVS1 a Rep-binding site (RBS; Weitzman *et al.*, 1994) and a terminal resolution site (TRS; Urcelay *et al.*, 1995) were identified. These motifs are similar to the minimal origin for AAV DNA replication (Smith *et al.*, 1999; Ward *et al.*, 2001; Figure 16.2). The overall GC content of AAVS1 is 65 percent for the first



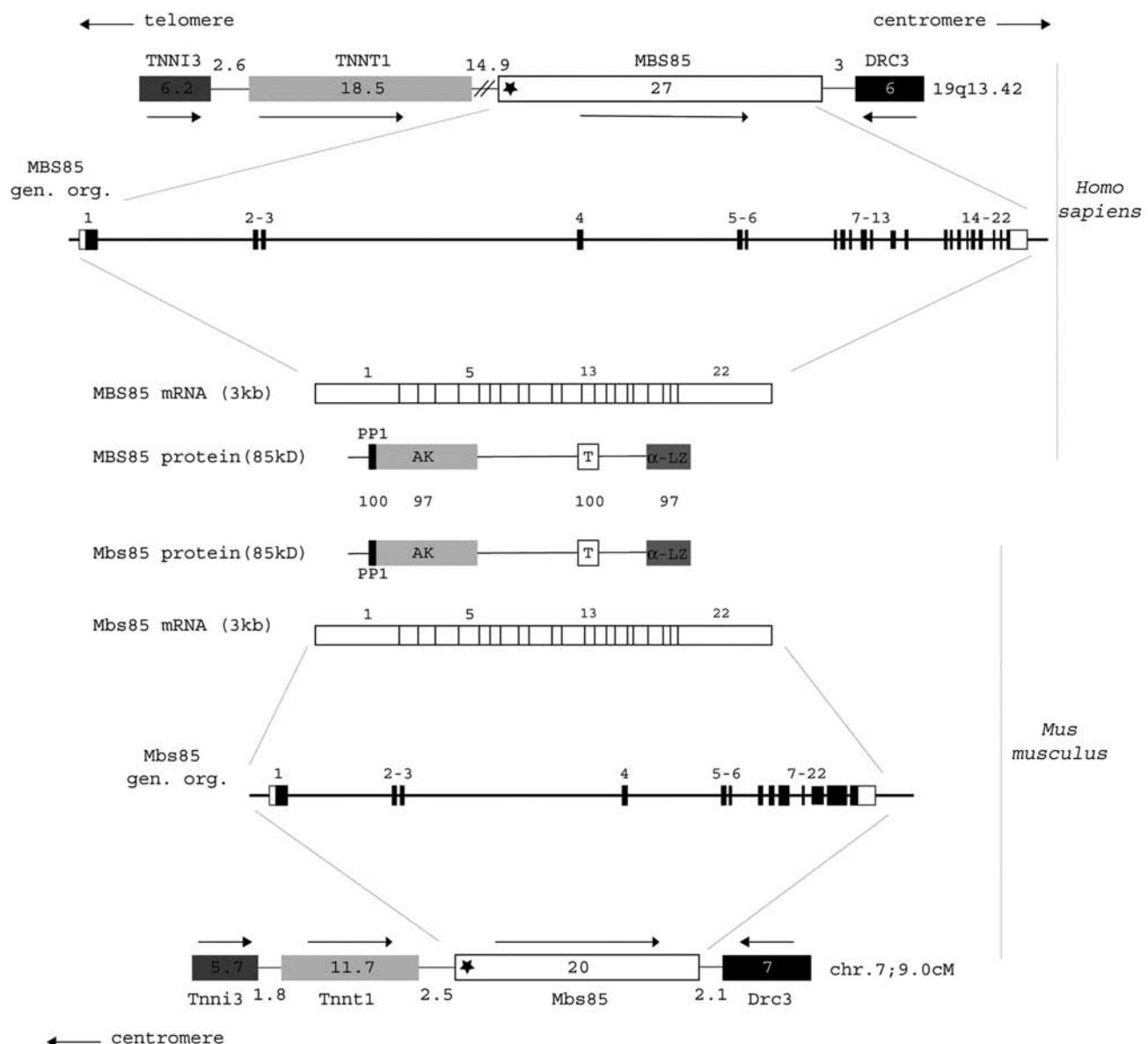
**Figure 16.1** Schematic representation of the AAVS1 region. The gray box represents the AAVS1/MBS85 sequence; the first 3 coding exons (white boxes) are indicated. Dashed arrows indicate the 8.2-kb EcoRI and 2.6-kb BamHI AAVS1 sequences characterized by Kotin et al. (1992) and Samulski et al., (1991) respectively. The EcoRI site is arbitrarily assigned as nucleotide number 1 (nt 1). Sequence and positions of the rep binding site (RBS, in bold), terminal resolution site (TRS, in italic) and MBS85 translation initiation codon (ATG, in bold) are indicated. Locations of the GC rich region, direct repeat sequences (1 and 2), chromosome 19 minisatellite sequence, DNaseI hypersensitive site (DHS) and insulator element are indicated as plain arrows.



**Figure 16.2** Sequence alignment between the human, monkey, and mouse AAVS1 origins and the AAV2 ITR. The RBS is in bold and the TRS is italicized and highlighted by a line. Arrows indicate the nicking site. M and S indicate the translation initiation codon and serine residue, respectively.

900 bases (Kotin *et al.*, 1992). Interestingly, CpG islands have been found in the 5' end of genes and are often associated with the presence of TATA-less promoters (Blake *et al.*, 1990). Strengthening the evidence for the presence of a cellular gene

in AAVS1 was the identification of several putative *cis*-acting DNA elements (i.e. Sp1, CRE, ATF, UBF1; Kotin *et al.*, 1992), the identification of an open reading frame (ORF) downstream of the RBS (Kotin *et al.*, 1992), the mapping of



**Figure 16.3** Schematic representation of the genomic organization of human (top) and mouse (bottom) *MBS85* and the surrounding region. The lengths and distances between the *TNNI3*, *TNNT1*, *MBS85*, and *DRC3* genes are indicated in kilobases. The positions of the TRS-RBS motifs are indicated by an asterisk. Arrows indicate the direction of transcription. **Middle:** Genomic organization of the human (top) and mouse (bottom) *MBS85* genes. Black and white boxes indicate coding regions and non-coding regions, respectively. **Middle center:** Diagram of the spliced mRNAs of human and mouse *MBS85* (the numbers indicate the position of the exons). **Center:** Conservation between the amino acid sequence of the human *MBS85* protein and its mouse homolog. Numbers indicate percent identity within each domain: putative protein phosphatase 1 domain (PP1), ankyrin repeat domain (AK), domain carrying the catalytic threonine 560 residue (T) and  $\alpha$ -leucine zipper domain ( $\alpha$ -LZ).

a DNaseI hypersensitive site (DHS) just upstream of the RBS (Lamartina *et al.*, 2000b), and transient expression of a reporter gene driven by this DHS site (Lamartina *et al.*, 2000b). DHS sites can be considered as open chromatin regions that allow for the access of cellular proteins to bind to *cis*-acting DNA elements (Felsenfeld *et al.*, 1996).

Despite this indirect evidence it was only recently that a gene has been identified within *AAVS1*. During their search for new substrates of the myotonic dystrophy kinase-related

*Cdc42*-binding kinase (MRCK), (Tan and collaborators found that *AAVS1* is embedded in the gene encoding the protein phosphatase I regulatory inhibitor subunit 12C (*PPP1R12C*), also called the myosin-binding subunit 85 gene (*MBS85*; Tan *et al.*, 2001)). Interestingly, the *AAVS1* RBS is located in the 5' UTR of the gene, only 17-nucleotides upstream from the translation initiation start codon.

The human *MBS85* gene spans 27 kb of genomic sequences and is composed of 22 coding exons (Figure 16.3);

Tan *et al.*, 2001). As observed by Northern blot analysis, a unique 3 kb mRNA is ubiquitously expressed in human adult tissues and appears to be highly expressed in the heart (Tan *et al.*, 2001). Further investigations are still required in order to determine the temporal and spatial expression pattern of MBS85 during development. The fact that MBS85 is ubiquitously expressed corroborates the finding of a DHS site upstream from the RBS that allows transcription factors to bind to the promoter. Moreover, this open chromatin conformation might be one of the key requirements for Rep to interact with the AAVS1 RBS and thus might allow AAV to integrate site-specifically in a variety of tissues.

The Mbs85 protein (85 kDa) is composed of 782 amino acids and shares an overall identity of 39 percent with MYPT1 (myosin phosphatase target subunit 1; Tan *et al.*, 2001). To date, four Mbs proteins, encoded by four different genes, have been isolated from human tissues: MYPT1, which has been the most extensively characterized (also referred to as PPP1R12A; Takahashi *et al.*, 1997) and three newly isolated proteins: MYPT2 (PPP1R12B; Fujioka *et al.*, 1998), MBS85 (PPP1R12C; Tan *et al.*, 2001) and TIMAP (PPP1R16B; Cao *et al.*, 2002). An additional MBS protein, MYPT3 (PPP1R16A), has recently been isolated from a mouse adipocyte cDNA library (Skinner and Saltiel, 2001); notably, the gene coding for MYPT3 is also present within the human genome. These myosin-binding subunits are the regulatory subunits of the myosin light chain phosphatase (or myosin phosphatase, MP; for reviews see Cohen, 2002; Ito *et al.*, 2004). MP is composed of 3 subunits: a 38 kDa catalytic subunit of type 1 phosphatase (PP1 $\delta$ ), the regulatory subunit and a 20 kDa subunit of which the function has not yet been completely elucidated. MP is one of the key proteins responsible for regulating the level of myosin phosphorylation and thus for controlling the contractile activity of smooth muscles (Figure 16.4).

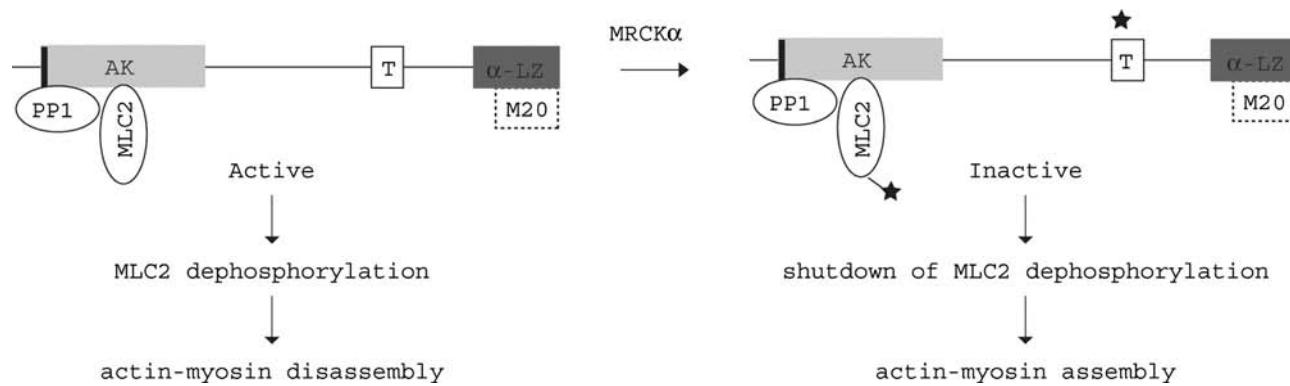
MP binds to the phosphorylated myosin light chains (MLC), through MBS, and then dephosphorylates MLC

through PP1 $\delta$ . MLC phosphorylation, on the other hand is achieved by the myosin light chain kinase (MLCK).

Mbs85 contains several characteristic features that are in common with MYPT1: such as an  $\alpha$ -helical domain with leucine zippers at the C-terminus of the protein and a canonical PP1-c binding motif as well as an ankyrin repeat motif at the N-terminus (Tan *et al.*, 2001). In addition, a conserved phosphorylation inhibitory motif (PIM) containing a threonine at position 560 (T560) is present in the central region (Figure 16.4). Biochemical analyses have demonstrated that the N-terminal region of Mbs85 binds to the catalytic subunit of the myosin phosphatase, (PP1 $\delta$ ), and also to the MLC substrate. Importantly, phosphorylation of T560 by MRCK inhibits the phosphatase catalytic activity of PP1 $\delta$  resulting in a shutdown of MLC2 dephosphorylation. Overexpression of Mbs85 truncation mutants in HeLa cells showed that deletion of the  $\alpha$ -leucine and PIM domains led to various degrees of actin stress fiber disassembly, whereas deletion of the ankyrin domain resulted in an increase in actin polymerization (Tan *et al.*, 2001). The specific role and potential substrates for Mbs85 have not yet been established. The elucidation of the function of Mbs85 *in vivo* will be an essential step towards understanding the consequences of AAV integration into AAVS1.

## Characterization of the region surrounding AAVS1

In the attempt to characterize the extent of the rearrangements induced by AAV integration into AAVS1, the region around MBS85 has been characterized (Dutheil *et al.*, 2000). Surprisingly, mapping of this region has revealed that MBS85 is closely linked to three other genes. Even though, with an average of 26 genes per megabase, chromosome 19 has the highest gene density among the human chromosomes



**Figure 16.4** Model for the regulation of MBS85 phosphorylation. When T560 (T) is not phosphorylated, myosin phosphatase (MP) is in an active form. MBS85 interacts through its ankyrin N-terminal domain (AK) with both PP1 $\delta$  and the MLC2 substrate leading to MLC2 dephosphorylation and actin-myosin disassembly. Phosphorylation of T560 (T asterisk) by MRCK $\alpha$  induces a conformational change in MBS85 leading to MP inactivation. This change results in a shutdown of MLC2 dephosphorylation and actin-myosin assembly. The putative interaction of MBS85  $\alpha$ -leucine domain ( $\alpha$ -LZ) with the M20 subunit (dashed white box) is indicated. (Adapted from Tan, 2001.)

(Grimwood *et al.*, 2004), the region surrounding *AAVS1* contains four genes in less than 100 kb. The *AAVS1* RBS is located 30 kb telomeric to the *EPS8L1* gene (also called *DRC3*) and 14.9 kb and 36 kb centromeric to the slow skeletal troponin T gene (*TNNT1*) and cardiac troponin I gene (*TNNI3*), respectively.

It has recently been shown that targeted AAV integration into *AAVS1* can lead to *TNNT1* disruption and can induce *TNNI3* rearrangements (Dutheil *et al.*, 2000; Dutheil and Linden, unpublished results). In order to highlight the possible consequences of disruption of a gene closely linked to *MBS85/AAVS1*: we will briefly review the function of the TnT1, TnI3, and Eps8L1 proteins as well as the mutations within these genes that are linked to a known disease.

### EPS8L1

Since EPS8-like 1 (EPS8L1) has only recently been discovered, there are few reports about this protein. The EPS8L1 protein belongs to the Eps8 family (epidermal growth factor receptor pathway substrate 8), which plays an important role in signal transduction leading to actin cytoskeleton remodeling (Tocchetti *et al.*, 2003; Offenhauser *et al.*, 2004). As determined by Northern blot analyses and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), Eps8L1 appears to be highly expressed in adult mouse placenta and stomach tissues and to a lower extend in other tissues (i.e. heart, testis, and ovary; Offenhauser *et al.*, 2004).

### TROPONIN

The primary function of the troponin complex is to control the interaction between the actin and tropomyosin filaments during muscle contraction and relaxation (for reviews see Farah and Reinach, 1995; Perry, 1998, 1999). The troponin complex consists of three distinct subunits. Troponin I (TnI) is the inhibitory subunit, which prevents muscle contraction in the absence of calcium. Troponin C (TnC) is the calcium-binding subunit that removes TnI inhibition and regulates the activation of the thin filaments during muscle contraction. Troponin T (TnT) is the tropomyosin-binding subunit. In the absence of calcium, troponin I binds to the actin and tropomyosin filaments and therefore prevents muscle contraction by inhibiting the actomyosin ATPase activity. Upon depolarization of the muscle fibers, intracellular calcium is released and binds to the troponin C subunit. This calcium–troponin C interaction induces a conformational change in the troponin–tropomyosin complex. The actin molecules can therefore interact with myosin resulting in muscle fiber contraction.

Multiple isoforms have been identified for each troponin subunit. There are two troponin C genes, *TNNC1* and *TNNC2*, coding for the cardiac/slow and fast TnC isoforms, respectively. There are three *TNNT* genes, *TNNT1*, *TNNT2*, and *TNNT3*, coding for the slow skeletal, fast, and cardiac TnT isoforms, respectively. There are three *TNNI* genes,

*TNNI1*, *TNNI2*, and *TNNI3*, coding for the slow skeletal, fast, and cardiac TnI isoforms, respectively. The expression of the troponin genes is restricted to specific cell types and is regulated during development.

***TNNT1*.** The human *TNNT1* gene has been mapped on chromosome 19 at position 19q13.4 (Samson *et al.*, 1992) and is located only 14.9 kb upstream of the *AAVS1* RBS. The human *TNNT1* gene spans 18.5 kb of genomic sequences and consists of 14 exons (Barton *et al.*, 1999). It has been shown that alternative splicing occurs in *TNNT1* and gives rise to at least four different isoforms (Gahlmann *et al.*, 1987; Samson *et al.*, 1994). *TNNT1* expression is developmentally regulated and is expressed exclusively in skeletal muscles of adults.

A lethal form of nemaline myopathy (ANM) is directly linked to a non-sense mutation within the *TNNT1* gene (Johnston *et al.*, 2000). This autosomal recessive disease has an incidence of 1 in 500 among the Amish population, and affected children (who have normal muscle power at birth) usually die during the 2nd year of life from respiratory insufficiency due to muscle rigidity or contracture. The mutation was identified within exon 11 and results in a TnT1 protein truncated at amino acid 180. Deletion of the C-terminal 83 amino acids removes the principal site of interaction between TnT and tropomyosin and also with the other two subunits of the troponin complex (TnI, TnC; Johnston *et al.*, 2000). During the development of an ANM-affected fetus, the cardiac TnT isoform that is expressed in fetal skeletal muscles may complement for the loss of the slow skeletal TnT isoform. However, downregulation of the cardiac TnT isoform occurs in skeletal muscle soon after birth leading to a complete depletion of TnT isoforms and thus to the appearance and progression of the disease (Jin *et al.*, 2003).

***TNNI3*.** The human *TNNI3* gene has also been mapped to chromosome 19 at position 19q13.4 (Birmingham *et al.*, 1995; Mogensen *et al.*, 1997) and is closely linked to the *TNNT1* gene (Barton *et al.*, 1999). *TNNI3* comprises eight coding exons contained within 6.2 kb of genomic sequences (Bhavsar *et al.*, 1996). *TNNI3* is exclusively expressed in the heart in adults (Hunkeler *et al.*, 1991).

To date, *TNNI3* mutations have been linked to familial hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), and dilated cardiomyopathy (DCM). It is suggested that *TNNI3* mutations within different domains and also within a specific domain of the protein can result in different clinical symptoms. It is further suggested that the clinical *TNNI3* HCM and RCM phenotypes are more likely multifactorial and therefore additional genetic and environmental factors may influence the expression of the disease (Mogensen *et al.*, 2003).

HCM is considered to be a disease of the cardiac muscle sarcomer. It is one of the most frequently inherited cardiac disorders and the most commonly identified cause of sudden death in young adults (for review see Bonne *et al.*, 1998; Towbin, 2000). *TNNI3* was the seventh gene identified in familial HCM and 5 percent of HCM cases may be linked

to *TNNI3*. So far, seven *TNNI3* HCM dominant missense mutations and one non-sense mutation have been identified, which are located in the C-terminal of the protein, within or in proximity to important domains for the normal function of the troponin complex (Kimura *et al.*, 1997; Kokado *et al.*, 2000; Morner *et al.*, 2000). *TNNI3* mutations have recently also been identified in RCM patients; the six dominant mutations are located in the same domain as are the HCM mutations (Mogensen *et al.*, 2003). RCM is a relatively uncommon heart disease that is characterized by progressive left and right ventricular failure and heart transplantation is often required (for review see Kushwaha *et al.*, 1997). Recently, one recessive *TNNI3* missense mutation was isolated in DCM patients; this mutation is located in the N-terminus of the protein (Murphy *et al.*, 2004). *TNNI3* is the first recessive gene identified in DCM, which is a common cause of heart failure. Previously, all the DCM mutations identified in 11 cardiac sarcomeric contractile proteins showed a dominant inheritance (for a review see Towbin and Bowles, 2001).

In order to understand the functional significance of the HCM *TNNI3* point mutations, transgenic mice carrying an HCM mutation were generated (James *et al.*, 2000). This study suggested that hypertrophy might be due to increased calcium sensitivity and diminished inhibition of the actomyosin ATPase activity of the mutated protein. *TNNI3* knockout mice were also generated in an effort to understand the physiological importance of cardiac TnI expression (Huang *et al.*, 1999). Even though *TNNI3*-deficient mice were born healthy, the mice died 18 days after birth from acute heart failure. The slow skeletal troponin I isoform may initially have compensated for the absence of cTnI, but approximately 15 days after birth, downregulation of slow skeletal TnI occurred, resulting in heart failure.

Despite their close proximity, the *TNNI3*, *TNNT1*, *MBS85* and *EPS8L1* genes have distinct tissue specificity. The specificity of expression of these closely linked genes is likely regulated through the presence of enhancer, silencer, and insulator elements that are as yet little characterized. Intriguingly, an insulator has been identified upstream of the *AAVS1* RBS (Ogata *et al.*, 2003), in a region overlapping the DNasel hypersensitive site and the putative *MBS85* promoter region. More investigation is required in order to characterize the possibly complex mechanisms of gene regulation in the region surrounding *AAVS1*.

### **AAVS1 conserved in the monkey and mouse genomes**

Since to date site-specific integration by AAV has exclusively been studied in transformed human cells *in vitro*, it became of interest to determine whether targeted integration could also occur in primary diploid cells and, more importantly, *in vivo*. Therefore, Samulski and collaborators investigated the extent of phylogenetic conservation of *AAVS1*. The

human *AAVS1* sequence was used as a hybridization probe on genomic DNA isolated from a panel of different species. Southern blot analysis revealed that *AAVS1* was conserved in human and monkey, but not in the other species tested (bovine, canine, mouse, rat, chicken, and yeast; Samulski *et al.*, 1991). Subsequently, a 0.3 kb fragment containing the African green monkey *AAVS1* sequence was characterized (Amiss *et al.*, 2003). With the recent discovery of the *MBS85* gene that overlaps *AAVS1* and with the completion of genome sequencing from different organisms, it became possible to search for an *AAVS1/MBS85* homolog among different species. Thus, it has recently been demonstrated that *AAVS1* is not restricted to primates but is also present in the mouse genome (Dutheil *et al.*, 2004). The reason for the discrepancy between the two studies is probably due to the fact that the human *AAVS1* probe used for Southern blots analyses contained mostly intronic, and therefore less conserved sequences. Interestingly, both the African green monkey and the mouse *AAVS1* sequences contain an RBS and a TRS motif that is homologous to the motifs present in the human *AAVS1* sequence. It remains to be determined how far the homology is conserved during evolution.

In order to investigate the potential effects of AAV mediated integration *in vivo*, it will be important to use an animal model with an *AAVS1* locus that is similar to the human site. It has now been demonstrated that the human and mouse *MBS85* genes display the same overall genomic organization (Dutheil *et al.*, 2004). The mouse gene has been mapped on chromosome 7 in a region previously shown to be syntenic to the human chromosome 19 region containing *AAVS1* (Blake *et al.*, 2000). The *MBS85* gene spans 20 kb of genomic sequence and the TRS and RBS motifs are located 25 nucleotides upstream of the ATG. The deduced protein sequence shares 86 percent identity with its human counterpart and several putative domains are highly conserved between the human and mouse proteins (Figure 16.3, p. 217). Interestingly, the overall genomic organization of the human and mouse loci surrounding *AAVS1* is also remarkably similar, suggesting that the mouse could represent a suitable animal model for the study of site-specific AAV integration *in vivo*. However, the prerequisite for this approach is the demonstration of site-specific integration into the mouse *AAVS1* locus in mouse cells.

### **REQUIREMENTS FOR SITE-SPECIFIC AAV INTEGRATION**

Extensive studies have been performed during the past 10–15 years in order to dissect the requirements for site-specific integration. It is now well established that AAV mediated site-specific integration requires at least three components: the AAV Rep78/68 proteins in *trans*, viral DNA motifs and cellular *cis*-acting sequences. Here, we will describe the different partners involved in this mechanism.

## Viral sequences

It had been suspected for a long time that the Rep proteins and the viral ITR might play a central role in AAV site-specific integration. However, the elucidation of the viral elements required for targeted integration has become possible only recently with the development of transfection systems.

### REP GENE

Shelling *et al.* were first to provide evidence that transfected plasmids containing the *rep* gene and a selectable marker flanked by the AAV ITRs can integrate site-specifically into *AAVS1* in a manner that is similar to the wtAAV virus. Site-specific integration was assessed by Southern blot analysis and defined as the result of *AAVS1* locus disruption. This study also pointed out that the Rep proteins could play an important role in targeted integration (Shelling and Smith, 1994). A second study that was based on FISH analysis demonstrated that a rAAV virus containing the ITRs as the sole AAV sequences (thus devoid of *rep* and *cap* genes) could not integrate in a site-specific manner into chromosome 19 (Kearns *et al.*, 1996). Finally, Surosky *et al.* definitively demonstrated that the large Rep proteins (Rep78/68) are required for targeted integration (Surosky *et al.*, 1997). In these experiments 293 cells were co-transfected with a plasmid that provided Rep in *trans* and an integrating plasmid containing a transgene flanked by the AAV ITRs. Linkage between the vector and *AAVS1* sequences was assessed by PCR on genomic DNA from a pool of transfected cells, using a primer within the AAV ITR and a second primer within *AAVS1*. Site-specific integration was defined as a PCR positive signal upon dot blot hybridization with an *AAVS1* probe. AAV site-specific integration was found only when either of the two large Rep proteins (Rep78/68) were provided in *trans*, whereas no site-specific integration was detected with the two small Reps (Rep52/40).

Delineating a minimal Rep domain that is necessary and sufficient to mediate site-specific integration *in vivo* has not yet been attempted. It may not be trivial to define such a domain since Rep78/68 is a multifunctional protein and many biochemical activities of Rep may be required to achieve site-specific integration *in vivo*. Indeed, the current model for AAV targeted integration predicts that Rep78/68 binds to the viral and cellular RBSs, unwinds the DNA, and then introduces a site-specific nick at the *AAVS1* TRS (Kotin, 1994; Linden *et al.*, 1996a).

Strong evidence indicates that the N-terminal domain of Rep78/68 is required for targeted integration. First, the Rep52/40 proteins, which are missing the N-terminal 224 residues of Rep78/68 are unable to mediate targeted integration into *AAVS1*. Second, the DNA binding and endonuclease domains are both located in the N-terminal region of Rep78/68. Two studies using chimeric proteins containing either the N-terminal 208 or 244 amino acids of Rep78/68 have demonstrated that this region interacts with the viral

RBS both *in vitro* (Yoon *et al.*, 2001) and *in vivo* in a one-yeast hybrid assay (Cathomen *et al.*, 2000). The endonuclease activity of Rep is also contained within the N208-terminal domain (Yoon *et al.*, 2001; Yoon-Robarts and Linden, 2003; Hickman *et al.*, 2002, 2004) that contains a catalytic tyrosine residue at position 156 (Smith and Kotin, 2000). Tyrosine 156 is essential for Rep78 cleavage activity (Smith and Kotin, 2000). Third, charged-to-alanine scanning mutagenesis of the N-terminal half of Rep78/68 demonstrated that almost all the mutations affecting DNA binding and/or nicking activities resulted in a complete loss of targeted integration (as determined by PCR). The mutated residues were located over the entire N-terminal region of the protein, thus suggesting that the whole domain is required for AAV-mediated integration (Urabe *et al.*, 1999).

The central region of Rep78/68 contains the nuclear localization signal (NLS; Horer *et al.*, 1995), a nucleotide triphosphate (NTP)-binding motif (Kyostio and Owens, 1996), and a homo-multimerization domain (Smith *et al.*, 1997; Hermonat and Batchu, 1997); it also retains helicase activity (Im and Muzyczka, 1990; Walker *et al.*, 1997). The structures of both the apoprotein as well as the nucleotide bound form of Rep40 have recently been presented (James *et al.*, 2003, 2004). Most certainly, site-specific integration requires Rep68/78 to be directed to the nucleus, where it binds the viral and *AAVS1* RBS (Weitzman *et al.*, 1994) as a multimer (Hickman *et al.*, 2004), thereby initiating the integration mechanism. Finally, it must be noted that while rAAV vectors (containing the AAV ITRs as the sole viral sequences) retain the potential to integrate into the human genome, these Rep-deficient constructs lack the capacity to integrate into *AAVS1* (Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997; Rutledge and Russell, 1997; Surosky *et al.*, 1997; Miller *et al.*, 2002). These results suggest that integration of rAAV vectors can be achieved by cellular factors. It remains to be evaluated whether site-specific AAV integration requires only the Rep DNA-binding domain, the endonuclease activity, and an NLS motif, i.e. whether cellular proteins could compensate for some of the biochemical activities of Rep.

### CIS-ACTING VIRAL SEQUENCE

To date, few experiments have been performed that are aimed at dissecting the minimal viral *cis*-acting sequences required for site-specific integration. Studies using plasmid transfections have suggested that the RBS is the only *cis*-acting viral element required for targeted integration. Surprisingly, Surosky *et al.* reported that in the presence of Rep an rAAV with no ITR could be targeted into *AAVS1*, even though the frequency of integration based on a PCR assay was 10- to 100-fold lower than for a rAAV vector containing the AAV ITRs (Surosky *et al.*, 1997). The authors suggested that Rep could interact with an RBS-like motif that is present within the backbone of the vector and therefore target the rAAV DNA into *AAVS1*. A second study carried out by Young *et al.* suggested that the viral TRS within the

ITR is not required for AAV site-specific integration. An rAAV plasmid consisting of a reporter gene flanked by a mutated viral ITR was transfected into HeLa cells. The modified ITR vector, which had an 8-bp linker inserted between the RBS and the TRS, displayed restricted nicking activity *in vitro* and failed to replicate in cell lines. However, as determined by Southern blot and PCR analysis, the mutated TRS vector was still able to mediate targeted integration at the same frequency as constructs carrying the wild-type (wt) ITR (Young and Samulski, 2001). Finally, Philpott *et al.* reported that a transfected plasmid containing a reporter gene and the *rep* gene under the p5 promoter could integrate site-specifically into *AAVS1*, as determined by Southern blot analysis (Philpott *et al.*, 2002b). Interestingly, the p5 promoter contains an RBS motif (McCarty *et al.*, 1994) as well as a TRS sequence that can be nicked by Rep *in vitro* (Wang and Srivastava, 1997; Nony *et al.*, 2001). Consistent with this hypothesis, a significant number of recombination junctions between *AAVS1* and the p5 promoter have been described (Giraud *et al.*, 1995; Yang *et al.*, 1997). From these studies, it can be concluded that the only *cis*-acting donor element required for AAV mediated site-specific integration is the RBS, a sequence that through Rep binding can recruit the donor DNA to the *AAVS1* locus in order for the recombination complex to be formed.

Finally, stable HeLa cell lines carrying the *rep* and *cap* genes under their respective promoters have been established for the titration of rAAV virus stocks (Chadeuf *et al.*, 2000; Clark *et al.*, 1996). It has been suggested that the *rep* and *cap* sequences in those cell lines are also integrated within the *AAVS1* locus.

## Cellular sequences

In order to assess whether site-specific integration is determined by the primary *AAVS1* sequence or by a higher order chromatin structure around *AAVS1*, the pre-integration site has been cloned into an Epstein-Barr virus (EBV)-based shuttle vector. EBNA-1 expressing human cell lines (in which this *AAVS1* shuttle vector is maintained in a stable manner as an episome) were infected with wtAAV and the resulting AAV-*AAVS1* recombinants were analyzed. This study established that targeted integration by AAV is directed by the primary sequence of *AAVS1* and that the critical sequences were located within 500 basepairs (Giraud *et al.*, 1994). In addition, Weitzman *et al.* showed that this region of *AAVS1* contains sequences that are similar to the minimal viral origin of replication, i.e. the TRS and RBS motifs (Weitzman *et al.*, 1994). It was further demonstrated that the viral Rep78/68 proteins could mediate the formation of a complex between the cellular and viral RBS in a cell free assay. It was therefore hypothesized that Rep78/68 could act as a bridge to bring the viral and cellular sequences together and thus facilitate AAV DNA integration into *AAVS1* (Weitzman *et al.*, 1994). With these data pointing at the critical role of the

*AAVS1* TRS and RBS motifs for site-specific integration, Linden *et al.* showed that mutations within either the TRS or the RBS sequences completely abrogate AAV site-specific integration into the EBV shuttle vector. Furthermore, they demonstrated that a 33 nucleotide sequence containing both the TRS and RBS was necessary and sufficient for AAV-mediated integration into *AAVS1* (Linden *et al.*, 1996b). Biochemical studies have further supported the critical role of the *AAVS1* TRS. Urcelay *et al.* showed that Rep78 is able to nick the *AAVS1* TRS resulting in a free 3'-OH terminus that can serve as a primer to initiate *AAVS1* DNA replication (Urcelay *et al.*, 1995). It has also been demonstrated that Rep68 can initiate replication on the mouse *AAVS1* TRS motif (Dutheil *et al.*, 2004). Since the endonuclease activity of Rep likely represents the initiating step in the integration mechanism, characterization of the nicking requirements for the *AAVS1* motif was of some interest. First, Lamartina and colleagues demonstrated that a supercoiled plasmid containing the human *AAVS1* TRS-RBS sequence is efficiently cleaved by Rep68, in contrast to a linearized substrate (Lamartina *et al.*, 2000a), indicating that nicking at the human *AAVS1* TRS is affected by DNA topology. Moreover, in order to recruit Rep to the *AAVS1* TRS an appropriately spaced RBS is required. Meneses and colleagues demonstrated that not only are the TRS and RBS motifs necessary but also that the sequence and position of the spacer element separating the TRS and RBS is important (Meneses *et al.*, 2000). Indeed, mutation or alteration of spacer length greatly diminished or, in some cases, completely abrogated AAV integration into the shuttle vector. In support of this notion it has been demonstrated by DNaseI footprinting that Rep protects the entire *AAVS1* RBS, spacer and TRS region in both the human and monkey sequences (Lamartina *et al.*, 2000a, Amiss *et al.*, 2003). In the light of these studies, a model is emerging for the initial steps of AAV-mediated targeted integration. It is likely that Rep targets *AAVS1* by direct contacts with the RBS. In analogy with viral origin activity, the RBS-spacer-TRS sequences are then partially melted, either through a conformational change in Rep or through the Rep helicase function. Once the origin is single-stranded, an appropriately spaced TRS can be brought into the active site of the Rep endonuclease domain, thereby initiating nicking of the TRS. Subsequent steps in this mechanism are less clear to date, although a replication-based model has been put forward (Linden *et al.*, 1996a).

Finally, studies with several transgenic mice carrying different human *AAVS1* sequences confirm the critical role of the *AAVS1* TRS and RBS motifs *in vivo*. The region of human chromosome 19 containing the TRS and RBS motifs was able to mediate integration in mice in a Rep-dependent manner (Rizzuto *et al.*, 1999; Young *et al.*, 2000; Bakowska *et al.*, 2003). Importantly, Rizutto and Bakowska reported frequencies of integration into the human transgenic *AAVS1* locus of 14 percent and 50 percent, respectively, versus 86 percent and 50 percent for non-targeted integration. With respect to those integrants it will be of interest to determine whether

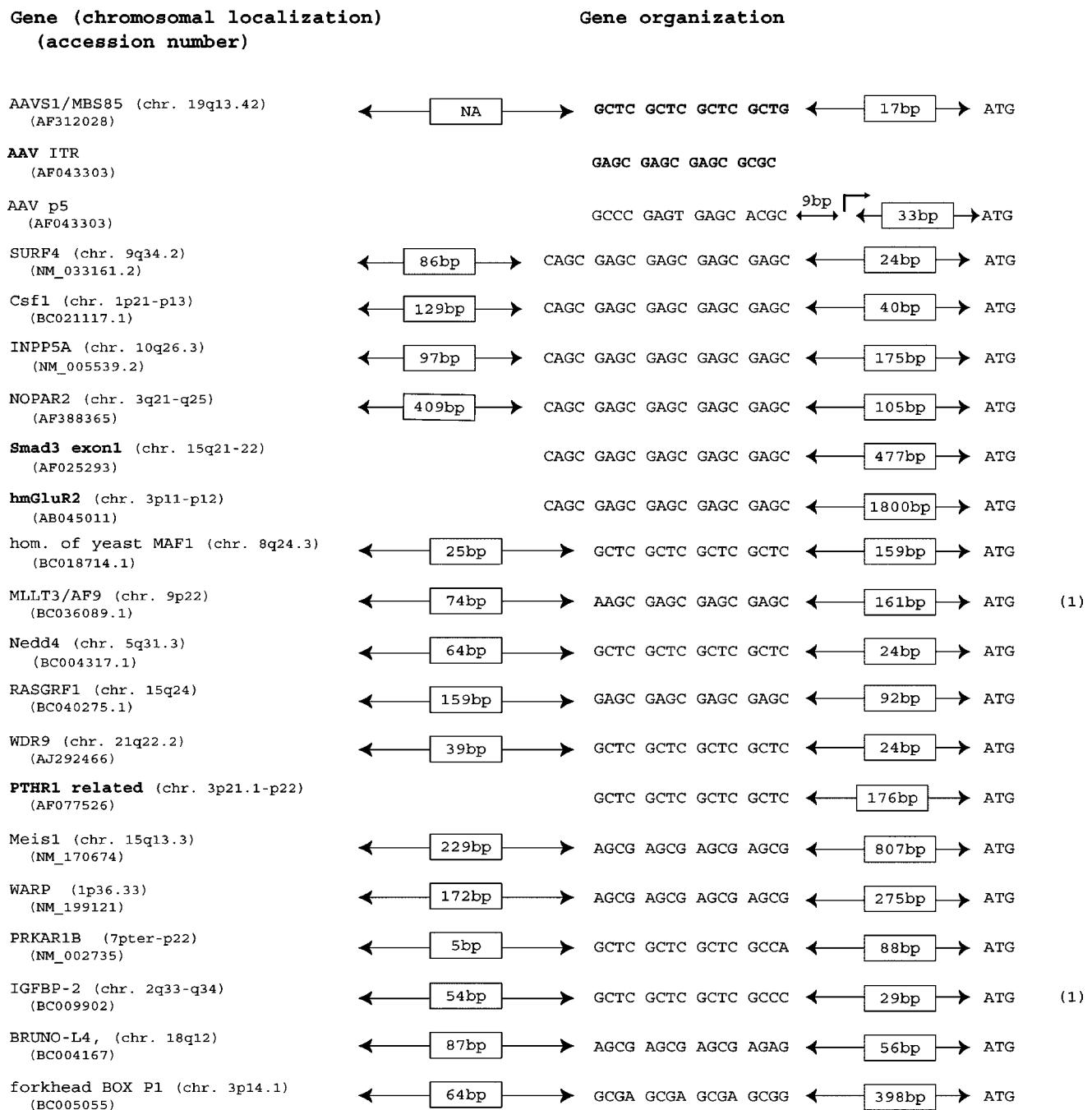
integration had occurred at the mouse *AAVS1* locus or at non-specific sites within the mouse genome.

*AAVS1* remains the only human chromosomal locus identified to date that is able to support AAV integration both *in vitro* and *in vivo*. In order to define the Rep-DNA interactions, Chiorini *et al.* determined the consensus Rep-binding site by using a modified random oligonucleotide selection. Electrophoretic mobility shift assays were performed with purified maltose-binding protein fusions of Rep (MBP-Rep68 or MBP-Rep78) that had been incubated with a pool of random oligonucleotides. After isolation of the oligonucleotides that were shifted by Rep and a further four rounds of selection, the following consensus RBS sequences were determined for Rep78 and Rep68: (A/G)vbGAGCGAGCn (A/C)G and (G/A)nn(T/C)GAG(C/T)GAGCGAG(c/a) (g/a)V, (Chiorini *et al.*, 1995). Subsequently, a BLASTN homology search was performed with the 16-mer core RBS sequence from either *AAVS1*, the viral ITR, or the p5 promoter, in order to identify potential additional RBS motifs within the human genome (Wonderling and Owens, 1996, 1997). Wonderling *et al.* identified 22 such sites in the human genome, localized in or flanking various cellular genes. Batchu *et al.* identified an additional RBS within the c-H-ras promoter (Batchu *et al.*, 1994). Surprisingly, EMSAs performed with 54-mer oligonucleotides containing putative cellular RBS motifs showed that Rep68 binds with equal or higher affinity to these RBS motifs than to the *AAVS1* RBS (Wonderling and Owens, 1997). In contrast, Young *et al.* observed that in the context of their genomic sequences Rep has a higher binding affinity for the *AAVS1* RBS than for the *CSF1* RBS (Young *et al.*, 2000). The authors suggested that in addition to the RBS other cellular sequences within *AAVS1* might influence the interaction of Rep with its target locus. With the human genome project completed, more RBS sequences have now been identified (Figure 16.5). Intriguingly, these motifs are almost all present within the 5' UTR of cellular genes and are located between 17 and 275 nucleotides upstream of the ATG. This observation raises the question of the significance of the RBS *in vivo*. Based on its prominent position within cellular genes it can be speculated that this motif may represent a recognition signal involved in the regulation of either transcription and/or translation. Using a yeast-one hybrid assay, Cathomen and collaborators have shown that the ubiquitously expressed transcription factor zinc finger 5 protein (ZF5) interacts specifically with the AAV RBS and represses the AAV p5 promoter by binding to the viral ITR. In addition, ZF5 and Rep78 have been shown to compete for binding to the AAV ITR in a reporter assay (Cathomen *et al.*, 2001). It will be of interest to determine whether ZF5 can regulate *MBS85* expression through binding to the *AAVS1* RBS and whether Rep and ZF5 can compete for this potential regulatory function. It may be possible that through such competition Rep might interfere with *MBS85* expression. To date, although no information is available for *MBS85* regulation, Rep proteins have been shown to repress or activate a

number of cellular or viral promoters (Rittner *et al.*, 1992; Batchu *et al.*, 1994; Hermonat, 1994; Wonderling and Owens, 1996; Wonderling *et al.*, 1997).

The fact that the Rep proteins can bind to numerous RBS sites *in vitro* raises the question of how Rep can discriminate between the *AAVS1* and others cellular RBS. Using a Rep-dependent DNA filter-binding assay with purified Rep68 proteins, Young *et al.* demonstrated that alternative RBS motifs could efficiently compete with the *AAVS1* RBS for Rep binding. They further estimated that, by 24 hours post infection, Rep78 and Rep68 are expressed at approximately 1000 to 4000 molecules per wtAAV-infected HeLa cell. This suggests that there might be sufficient amounts of Rep in the cell to bind all cellular RBS motifs, indicating that Rep might not be a limiting factor for *AAVS1* targeting.

As discussed above, a TRS motif in close vicinity of the RBS is required for Rep to mediate site-specific integration. To date, *AAVS1* is the only cellular sequence where both motifs are known to be present with appropriate spacing. Furthermore, it is possible that *in vivo* not all RBS motifs are accessible to Rep because of the local chromatin structure. In this context it is of note that the region upstream of the *AAVS1* RBS has an open chromatin structure. In addition to Rep binding to the *AAVS1* RBS, the possibility must be considered that cellular factors are involved in the initial steps of integration. One possible scenario is that Rep78/68 which are the only Rep proteins capable of binding to the RBS (Surosky *et al.*, 1997), compete and displace cellular proteins that are already bound to the *AAVS1* RBS (e.g. ZF5). A second mechanism could involve protein–protein interactions between Rep and cellular factors. Since the *AAVS1* RBS is positioned within a promoter region, it is noteworthy that several cellular proteins that have been shown to interact with Rep either belong to the transcription factor family or are involved in the general transcription machinery, e.g. the TATA-binding protein (TBP; Hermonat *et al.*, 1998). Examples of Rep-interacting proteins include the positive co-activator 4 (PC4; Weger *et al.*, 1999), topors (Weger *et al.*, 2002), Sp1 (Pereira and Muzyczka, 1997), c-jun (Prasad *et al.*, 2003), E2F-1 (Batchu *et al.*, 2001), and p53 (Batchu *et al.*, 1999). Furthermore, Rep can also interact with the cAMP-dependent PKA protein and its homolog PRKX. This interaction has been proposed to result in the inhibition of CREB-dependent transcriptional activation (Chiorini *et al.*, 1998; Di Pasquale and Stacey, 1998). Binding sites for several of these proteins are present within the 5' UTR of *MBS85* (*Sp1*, *ATF* and *CREB*-binding sites). It is not unlikely that either competition with a cellular factor bound to *AAVS1* sequences or the formation of heterodimers or higher order complexes are required for the efficient initiation of Rep-mediated site-specific integration. For example, Rep78/68 interaction with the AAV p19 promoter has been shown to be stabilized through a direct interaction between Rep and Sp1 (Pereira and Muzyczka, 1997). Interestingly, it has further been demonstrated that high mobility group 1 (HMG-1) protein interacts with Rep



**Figure 16.5** Alignment of the human AAVS1 RBS with newly identified RBS within the human genome. A BLASTN search was performed with the human AAVS1 RBS (GCTC GCTC GCTC GCTG) against the homo sapiens database (NCBI). NA indicates that MBS85 transcription initiation site is not yet known. The RBS present in the AAV2 ITR and p5 promoter are indicated. Distances between the RBS and the ATG are indicated as are the distances between the RBS and the first nucleotide of the published sequence. Note that the p5 transcription initiation start (arrow) is located downstream of the RBS. Names of the genes are in bold and mRNAs are in ordinary type. The accession numbers and chromosomal locations are indicated. (1) indicates that the gene or the mRNA has previously been identified by Wonderling and Owens (1997).

and that this interaction can enhance Rep binding and nicking activity at the AAV ITR *in vitro* (Costello *et al.*, 1997). HMG1 is an abundant component of vertebrate cells and has been proposed to play a structural role in chromatin organization. The biological significance and the involvement in integration of this, as well as most other Rep

interactions, remains to be determined. Further studies of the regulation of MBS85 expression and the effects of Rep on this regulation may well contribute to our understanding of the possibly complex mechanism of site-specific integration.

With a different approach, a cell-free integration assay has recently been developed that could allow for the

characterization of cellular factors involved in site-specific integration (Dyall *et al.*, 1999). For example, using this assay it has been suggested that DNA-PK can inhibit AAV integration (Song *et al.*, 2004). Results obtained from both *in vitro* and *in vivo* studies will lead to a better understanding of the unique relationship between AAV and its targeted integration site.

## CHARACTERIZATION OF THE VIRAL-CELLULAR JUNCTIONS

Since the first molecular characterization of the AAV integrant in 7374 cells, many other latently infected cell lines have been established and their cellular-viral junctions characterized. Proviral junctions analyzed thus far vary considerably with respect to their position relative to the *AAVS1* RBS-TRS motif. However, it is difficult to draw general conclusions from comparisons of these junctions. Parameters that might contribute to the actual position of the junction within *AAVS1* vary considerably with the experimental strategies used:

- Studies have been performed using a variety of cellular systems, including human aneuploid cells, diploid cells, EBNA-1 expressing cells that propagate the *AAVS1* shuttle vector as an episome and, more recently, rodent primary diploid cells that are transgenic with the human *AAVS1* sequence.
- The experiments have been performed using different delivery methods, such as infection with wtAAV or Rep-containing recombinant viruses, transfection of plasmids, and the direct delivery of recombinant proteins.
- The purification and titration methods used to produce and titer wtAAV and rAAV viruses differ between laboratories; i.e. the MOI used in these experiments cannot be objectively assessed.
- Rep expression was driven by either the endogenous p5 promoter or by heterologous promoters (strong, inducible, or tissue-specific promoters), resulting in considerably different concentrations of Rep protein.
- Latently infected or transfected cells have been isolated either in the absence or in the presence of selective pressure.
- Characterization of the integration site has been performed at different time points after infection or transfection (from early to late passages).
- Methods to assess site-specific integration vary considerably and include Southern blots analysis, PCR, and FISH.

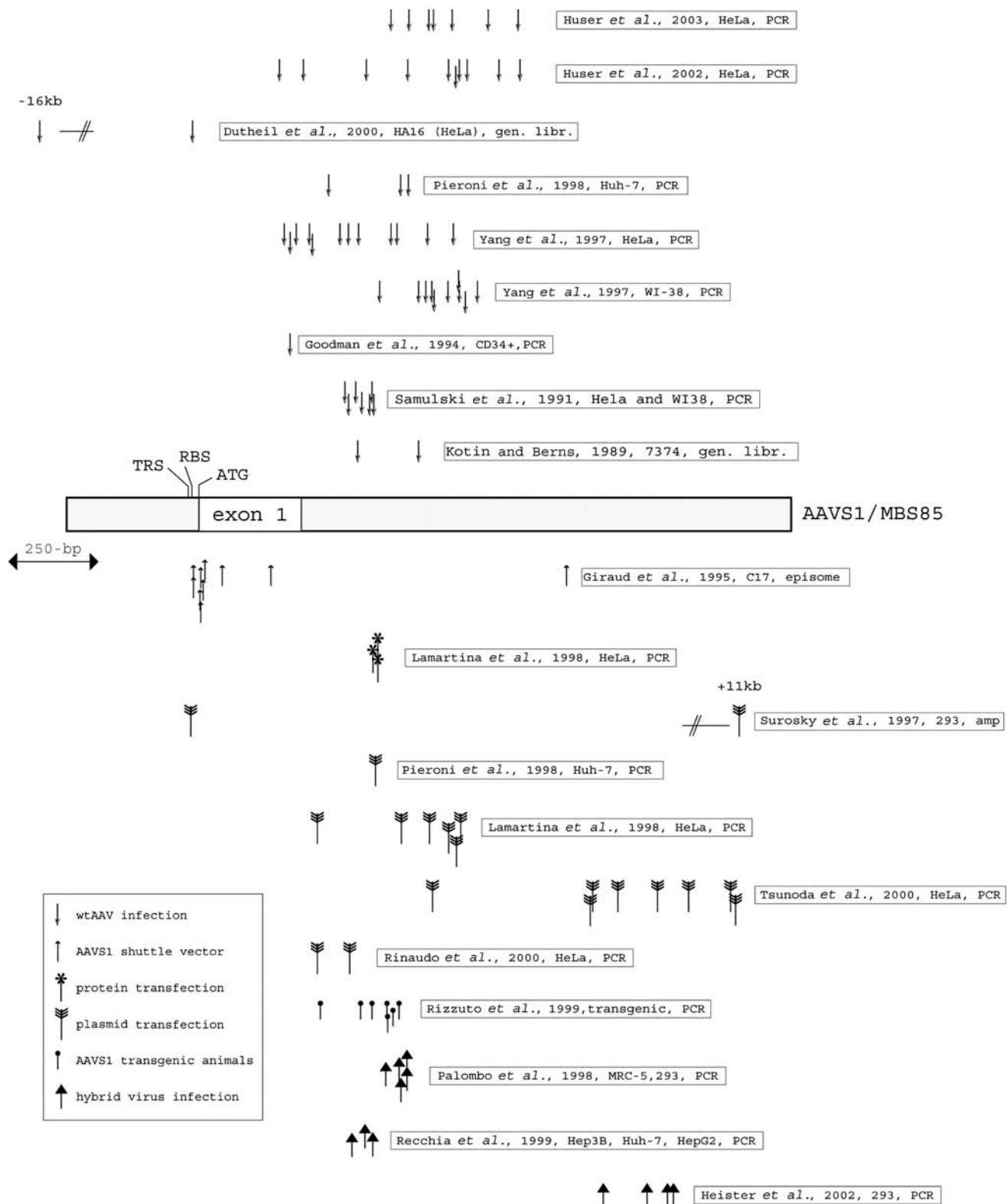
In the following section we will discuss the virus-cell junctions that have been published. A summary of the available junctions is graphically displayed in Figure 16.6.

## Viral-cellular junctions in latently infected cells

Kotin *et al.* characterized the first viral-cellular junctions by screening a subgenomic library of latently infected cells (7374 cells; Kotin and Berns, 1989). The two integration sites identified were located within the first intron of *MBS85*, 540 and 750 basepairs downstream of the RBS. In order to assess integration in diploid cells, Samulski and co-workers isolated recombination sites from a pool of infected WI-38 cells by PCR using AAV and *AAVS1* primers (Samulski *et al.*, 1991), thereby circumventing the requirement for single-cell cloning. In these experiments the junctions were also found in the first intron of the gene, in the same region as identified by Kotin *et al.* (between 510 and 600 nucleotides downstream of the RBS). These early studies demonstrated that, even though AAV integrates its genome site-specifically into *AAVS1*, the cellular-viral junctions are scattered throughout the pre-integration site. Subsequently, many more integration sites have been characterized and also found to lie within this region, i.e. the first coding exon and first intron of *MBS85* (Goodman *et al.*, 1994; Yang *et al.*, 1997; Pieroni *et al.*, 1998; Huser *et al.*, 2002; Huser and Heilbronn, 2003). However, the experimental design of these studies resulted in some bias in favor of this site, since they often employed PCR with primers in a specific position that predetermined the identification of junctions within this particular region. When a genomic library of latently infected HeLa cells was screened, two further recombination sites were identified, one near the *AAVS1* RBS and a second 16 kb upstream of the RBS, within intron 13 of the *TNNT1* gene (Dutheil *et al.*, 2000). To date, molecular characterization of a complete viral genome integrated at the *AAVS1* locus has not been presented. Such studies could provide further insight into the consequence of targeted AAV integration, including an assessment of the extent of rearrangements and deletions within *AAVS1*.

A more detailed look at the pre-integration site, and comparison with the viral-cellular junctions after integration, revealed a homology of one to five nucleotides between the AAV and *AAVS1* sequences at the immediate junctions. The absence of extended homologies suggested that integration occurred via non-homologous recombination (Kotin *et al.*, 1992).

Even though the viral ITR and the p5 promoter are often present at the recombination junction, heterogeneity of the proviral genome has been reported. Most frequently deletions within the ITR sequences have been observed. In support of the hypothesis that integration initiates through recruitment of the viral genome by Rep-RBS interactions, viral RBS motifs (either from the ITR or from p5) are often found close to the viral-cellular junction. Initially many of the viral-cellular junctions that were reported included the right ITR. However, most of these sequences were isolated by PCR using primers located within the right part of the viral genome. Huser *et al.* went on to investigate the hypothesis



**Figure 16.6** Schematic representation of the positions of published AAV-AAVS1 recombination junctions. The gray box represents the first 2.5-kb of AAVS1/MBS85, starting at the EcoRI site. The TRS, RBS, and translation initiation codon are indicated, as is the first coding exon (exon 1). Each arrow represents a recombination site identified between AAVS1 and AAV (or a rAAV). Each type of arrow indicates how latency was established (described in the boxed insert). The cell lines and detection techniques are indicated. Amp, ampicillin; Gen. lib., genomic library.

that one ITR was preferred during formation of the initial junction. They were able to demonstrate, by PCR, that both viral termini could be found at similar frequencies within the viral-cellular junction adjacent to the RBS (Huser and Heilbronn, 2003).

Extensive rearrangements of *AAVS1* are frequently observed as a result of integration. Interestingly, in all of the latently infected cells that have been characterized to date by Southern blot analysis, at least one *AAVS1* allele remains undisrupted. This observation might suggest an essential role for *MBS85* that extends beyond its function in muscle contraction, and consequently, that homozygous loss of this gene could be lethal for cultured cells. As a corollary, it can be hypothesized that the other MBS isoforms (e.g. *MYPT1*) cannot complement for the loss of *Mbs85* in these systems.

Finally, in the absence of helper virus infection in HeLa cells, site-specific integration can take place during the first hours following viral infection and it reaches a plateau at 96 h.p.i, as determined by real time PCR *in vitro* (Huser *et al.*, 2002). However, a recent report suggested that integration might also occur under permissive conditions (i.e. in the presence of adenovirus). During these experiments Huser and colleagues made the interesting observation that AAV-AAVS1 junctions can be detected in purified viral stocks. The authors hypothesized that these junctions had been packaged with the viral genome during virus production (Huser *et al.*, 2003). Although the biological significance of integration under permissive conditions is unclear, the potential for cellular sequences to contaminate recombinant virus stocks needs to be considered.

### Viral-cellular junctions in the *AAVS1* shuttle vector

The *AAVS1* shuttle vector was first developed as a model system to determine the minimal cellular sequences required for AAV site-specific integration. Giraud *et al.* further used this system to map recombination sites (Giraud *et al.*, 1995). Many similarities were found between the integration of AAV DNA into *AAVS1* in the shuttle vector and in latently infected cells. For instance, the AAV sequences at the recombination junctions are mostly from the p5 promoter or from one of the ITRs. As is the case for the junctions in latently infected cells, short stretches of homology between viral and cellular DNA can be found at the immediate junctions. Finally, significant rearrangements of the viral, cellular, and shuttle vector sequences were also found in this system (Dyall and Berns, 1998). In contrast to the recombination sites found in latently infected cells, most of the junctions were clustered around the TRS and therefore mapped to the first exon (90 percent; Giraud *et al.*, 1995). It must be highlighted, however, that the episome system is only of limited use as a tool to assess the extent of rearrangements resulting from AAV integration. As the shuttle vector must be propagated in

*Escherichia coli* subsequent to integration, any recombination events that affect the bacterial sequences of the vector may be lost and are therefore not included in the analysis.

### Viral-cellular junctions in transfected cells

Shelling and collaborators were the first to provide evidence that targeted delivery of a transgene into *AAVS1* could be achieved by transfection-based methods. One intriguing advantage of this system over viral infection is that, in theory, there is no limit to the size of the donor DNA that is integrated. With respect to integration junctions, considerable differences have been noticed both between latently infected and transfected cells as well as within different transfection based studies.

It is possible that these differences can be explained by the fact that different approaches were used to deliver the *rep* gene. Thus, the *rep* gene was either placed inside or outside the ITR flanked transgene, or it was provided in *trans* by a second plasmid (Balague *et al.*, 1997; Shelling and Smith, 1994; Surosky *et al.*, 1997). In a different study the transgene was delivered by transfection while the *rep* gene was delivered by viral infection (Philpott *et al.*, 2002a). *Rep* transfection and rAAV infection to provide the transgene was used by Huttner and colleagues (Huttner *et al.*, 2003). Finally, recombinant Rep proteins were transfected together with a plasmid that contained an ITR flanked expression cassette (Lamartina *et al.*, 1998).

Although it is difficult to draw generalized conclusions from these studies, a unifying theme appears to be that site-specific integration into *AAVS1* occurs at a lower frequency in transfected cells than in cells that are infected (Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998; Rinaudo *et al.*, 2000; Heister *et al.*, 2002). It has been suggested that the viral DNA might integrate more efficiently into *AAVS1* because it has free hairpin structures at its termini. However, the p5 promoter is emerging as an additional parameter that may influence the efficiency of site-specific integration. In some studies significantly higher integration frequencies have been observed when *rep* was expressed from the p5 promoter, independent of its position relative to the ITRs (i.e. inside or outside of the ITRs; Balague *et al.*, 1997; Shelling and Smith, 1994; Tsunoda *et al.*, 2000; Wang *et al.*, 2002; Philpott *et al.*, 2002b; Huttner *et al.*, 2003). In addition, a *cis* acting replicative element (CARE), which overlaps the p5 promoter, has been shown to be involved in AAV replication (Tullis and Shenk, 2000; Nony *et al.*, 2001) and may be also involved in site-specific integration. However, it remains to be determined whether this higher frequency of integration is due to *cis*-acting sequences that are present within the p5 promoter or due to a stringent requirement for appropriate levels of Rep expression.

Targeted integration of AAV-derived plasmids has often been associated with extensive rearrangements in the *AAVS1* sequence, similar to those observed in latently infected cells

(Balague *et al.*, 1997; Surosky *et al.*, 1997; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000; Young and Samulski, 2001; Wang *et al.*, 2002). These *AAVS1* rearrangements are believed to be induced by the Rep proteins and might be independent of the integration process *per se*. Consistent with this notion, high levels of Rep expression appear to increase the frequency of *AAVS1* rearrangements and decrease the frequency of site-specific integration (Urabe *et al.*, 2003). However, it is possible that this observation can be explained by the cellular toxicity of Rep. As pointed out earlier, Rep can affect the expression of numerous cellular and viral promoters (Hermonat, 1991, 1994; Oelze *et al.*, 1994), resulting in a cytopathic effect (Ueno *et al.*, 2000) or even the induction of apoptosis (Schmidt *et al.*, 2000). Consequently, different strategies have been used to limit Rep expression in order to prevent undesired Rep effects. Rinaudo *et al.* constructed a ligand dependent chimeric Rep protein by fusing the N-terminal Rep domain to a truncated hormone-binding domain from the progesterone receptor, which cannot interact with its natural ligand (progesterone) but binds to RU486 (Rinaudo *et al.*, 2000). Using this RU486 inducible system, rAAV vectors integrated site-specifically into *AAVS1* without any *AAVS1* rearrangement. The use of the *Cre/loxP* system for *rep* regulation also resulted in limiting *AAVS1* rearrangements (Ueno *et al.*, 2000; Wang *et al.*, 2002). A further strategy aimed at limiting the amounts of Rep employed the transfection of purified Rep proteins (Lamartina *et al.*, 1998). Under these conditions Rep is only transiently active, since degradation occurs. Finally, in order to avoid Rep activity after integration, Rep can be provided in *trans* on a second plasmid that does not contain ITRs, or the *rep* gene can be inserted outside of the ITR-flanked expression cassette. A prerequisite for this strategy is that rescue of the ITR flanked genome from the plasmid backbone can occur under non-permissive conditions (Samulski *et al.*, 1983; Gottlieb and Muzyczka, 1988; Ward and Berns, 1991). However, there is evidence that excision of rAAV sequences from plasmids under these conditions is not efficient, thus raising the possibility that the *rep*-containing backbone will be integrated into *AAVS1*.

Such integrants have been observed and Surosky *et al.* took advantage of this phenomenon in order to characterize two cellular-viral junctions from such a clone (Surosky *et al.*, 1997). In these experiments the genomic DNA was digested, religated and subsequently used to transform *E. coli* bacteria under ampicillin selection. In this cell line one junction contained lacZ sequences (from the ITR-flanked expression cassette) and the first exon of *MBS85* (2 basepairs downstream the RBS) and the second junction involved the *E. coli* origin of replication and an unknown sequence. With the human genome project completed it is now possible to identify these previously uncharacterized junctions. Surprisingly, the second junction in this clone is located within the third intron of *MBS85*: more than 10 kb downstream of the *AAVS1* RBS (at nucleotide 11245 in *AAVS1*). To our knowledge, this is the first characterization of an

AAV integrant that resulted in the description of both the left and right viral-cellular junctions. This result suggests that significant deletions of the pre-integration sequences may occur as a result of Rep-mediated targeted integration. In most of the other integrants that have been derived from transfection-based strategies, recombination sites have been identified by PCR and localized to the first coding exon or the first intron of *MBS85*, and, not surprisingly, are similar to those described for the latently infected cells (Surosky *et al.*, 1997; Pieroni *et al.*, 1998; Lamartina *et al.*, 1998; Rinaudo *et al.*, 2000; Tsunoda *et al.*, 2000).

## Viral–cellular junctions in transgenic rodents and in human tissues

To date, three transgenic animal models have been established that carry different but overlapping human *AAVS1* sequences (Rizzuto *et al.*, 1999; Young *et al.*, 2000; Bakowska *et al.*, 2003). Studies using these animal models have established that targeted integration into the human *AAVS1* locus can occur *in vitro* in primary and immortalized mouse embryonic fibroblasts, in primary adult rat fibroblasts, primary rat hepatocytes, and also *in vivo* in mouse and rat quadriceps muscle. Rizzuto and co-workers have shown by PCR that recombination sites between human *AAVS1* and viral sequences are similar to those described in latently infected or transfected human cells. Southern blot analyses revealed that integration had also taken place at additional sites within the mouse genome. It will be of interest to determine whether integration had occurred at the endogenous mouse *AAVS1* locus and to compare the integration frequencies at both loci. These studies show that the host factors required for Rep-mediated site-specific integration are present in primary mouse cells and suggest that targeted AAV integration might be achieved *in vivo* in human tissues. In support of this notion, AAV sequences have been found integrated in *AAVS1* in human testis tissues. The recombination sites were located in the first intron of *MBS85*, as determined by unidirectional PCR and genome-walking PCR (Mehrle *et al.*, 2004). Analysis of more samples (there were two individuals in this study) will help to determine the frequency and possible significance of site-specific integration *in vivo*. It will also be of interest to determine whether site-specific AAV integration can occur in other tissues in the human.

## CONSEQUENCES OF LATENT INFECTION

AAV latency is expected to have little effect on the host cell since in tissue culture latent infection has been maintained for more than 100 passages and *in vivo* AAV infection is not associated with any pathology. However, phenotypic changes have been observed in latently infected cells when compared with their uninfected parents. It has been observed that

integration of AAV DNA into the host genome at an unspecified integration site in Chinese hamster embryo cells (CHO) was associated with increased sensitivity to UV-irradiation and loss of the capacity to support SV40 amplification (Winocour *et al.*, 1992). Integration has also been associated with reduced growth rates and enhanced sensitivity to UV- and gamma-irradiation in a HeLa cell line, HA-16, where integration occurred at *AAVS1* (Walz and Schlehofer, 1992), and with a reduced ability to form colonies and diminished saturation densities in the human melanoma cell line MKr-A, where the integration site was unspecified (Bantel-Schaal, 1995). It is still unclear whether these phenotypic changes are directly correlated to disruption of the integration site or, at least to some extent, are the consequences of deregulated Rep expression. In the light of the recent discovery that *MBS85* is conserved between human and mouse genomes, it will be of interest to determine whether *MBS85* with its TRS/RBS motifs is also present in the hamster genome and whether *MBS85* is disrupted in latently infected CHO cells.

A significant issue for AAV latency relates to the consequences of Rep expression. As described earlier, overexpression of Rep proteins can regulate the expression of many cellular and viral genes and may induce apoptosis (Hermonat, 1991, 1994; Oelze *et al.*, 1994; Schmidt *et al.*, 2000). In addition, in the majority of latently infected cells analyzed to date at least one AAV genome remains intact as determined by the observation that infectious viral particles are produced upon adenovirus superinfection (Berns *et al.*, 1975; Samulski *et al.*, 1991; Walz and Schlehofer, 1992; Laughlin *et al.*, 1986). In order to avoid deleterious effects on the host cell during latency, it is likely that expression of proviral genes is highly regulated. It is known that in the absence of a helper virus, Rep78/68 downregulates transcription from the p5 and p19 promoters (Beaton *et al.*, 1989; Kyostio *et al.*, 1994). However, the close proximity of the proviral AAV genome to a cellular promoter (*MBS85*) highlights the possibility that Rep may need to repress the *MBS85* promoter, as well as the viral promoters, in order to limit Rep expression through read-through transcription from *MBS85*. To date, this hypothesis remains to be tested.

Finally, AAV DNA integrates into a ubiquitously transcribed region (Tan *et al.*, 2001). It can now be determined whether altered mRNAs and potentially truncated or chimeric proteins are generated as a result of AAV DNA integration into *MBS85*. Importantly, it has been noted that at least one *AAVS1/MBS85* allele remains intact in all of the latently infected cells analyzed to date (Kotin and Berns, 1989; Kotin *et al.*, 1990; Samulski *et al.*, 1991). This observation raises two possibilities. On one hand, Rep might mediate AAV integration into both alleles, leading to complete loss of *MBS85* proteins. This depletion is probably lethal for the cells, thereby making it impossible to detect such events. On the other hand, AAV may have evolved as an yet unknown mechanism to limit integration to one allele. The elucidation of *MBS85* function and identification of its substrate(s)

*in vivo* will be an essential step towards understanding the unique relationship between AAV and its targeted integration site.

AAV integration into *AAVS1* can occasionally lead to the disruption of the closely linked *TNNT1* gene and can induce rearrangements of the cardiac troponin *TNNI3* gene, as determined *in vitro* in latently infected cells. This raises several related questions, particularly because a lethal form of nemaline myopathy has been directly linked to a recessive *TNNT1* mutation, and cardiomyopathies have been associated with both recessive and dominant *TNNI3* mutations. Are the cardiac and slow skeletal muscles natural target tissues for AAV latency? Can AAV disrupt the *TNNT1* and *TNNI3* genes as a result of site-specific integration *in vivo*? Can AAV be transmitted through the germ line? Can viral infection occur during early development? Finally, can AAV latency be associated with any of these diseases?

## REPLICATION AND INTEGRATION: A MODEL

Many questions remain with respect to integration. For example, what model for the mechanism of integration can take into account the data that has been accumulated thus far and how could this model be tested? At this point we propose that integration occurs through a replication-dependent mechanism (Kotin, 1994; Urcelay *et al.*, 1995; Linden *et al.*, 1996a, 1996b). In general, this model predicts that integration occurs in a manner that is the reverse of the proposed rescue mechanism (Ward *et al.*, 2003). In that system rescue occurs by Rep-initiated replication, resulting in the synthesis of a copy of the integrated genome. Rep-mediated integration would thus be accomplished by replicating a copy of the AAV DNA into the chromosome. This model is therefore quite distinct from other, better defined, recombination systems.

Several observations support the involvement of DNA replication. McLaughlin *et al.* noted that integrated AAV sequences were often present in tandem arrays, suggesting that AAV DNA was replicated either before or after integration (McLaughlin *et al.*, 1988). When cells were infected with two distinguishable variants it was further noted that the tandem arrays seemed composed of only one of the variants, not both. Interestingly, the AAV genomes were found in a head-to-tail conformation, and not as the head-to-head and tail-to-tail structures that are known intermediates of AAV DNA replication.

As pointed out earlier, site-specific integration is dependent on the Rep68/78 protein (Surosky *et al.*, 1997). Moreover, the *AAVS1* origin (RBS-TRS) has been shown to support initiation of Rep-dependent DNA replication in an extract from uninfected HeLa cells (Urcelay *et al.*, 1995). With an episome-based system it has been shown that the *AAVS1* RBS-TRS sequences are necessary and sufficient to mediate AAV integration (Linden *et al.*, 1996b). Numerous

Rep-binding sites have been found in the human genome, yet no integration into these sites has been documented to date. As pointed out above, none of these sites contains an appropriately spaced TRS and therefore cannot act as replication origins. Taken together, these observations suggest that origin activity at the *AAVS1* RBS-TRS is essential for AAV site-specific integration.

Furthermore, a particular feature of site-specific integration is the position of the viral–cellular junctions within *AAVS1*. The only common aspect of these junctions is that they are variable in position and, with the exception of one, are all found downstream of the *AAVS1* origin. Again, this observation is consistent with the unidirectional activity of Rep-dependent RBS-TRS origins.

Basing our observations from cell-free systems, we correlate the spread of the junctions to a particular property of DNA replication. In HeLa cell extracts and in the absence of adenovirus factors the Rep-dependent replication complex has a strong tendency to dissociate from its template. However, after dissociating, this complex remains functional and can resume replication after reassembly at a second locus (Ward *et al.*, 1998). A similar phenomenon has been observed when herpes simplex proteins are used in a reconstituted AAV replication system. In this case the complex dissociates from its template while replicating through the hairpin region of the ITR. Also in this context the complex is able to resume replication after binding the displaced strand (Ward *et al.*, 2003). In a replication model of integration such displacement and reassembly would play a key role. Rep-dependent replication initiates at the *AAVS1* RBS-TRS using the cellular machinery. At some point downstream, the replication complex may dissociate from its template and resume replication on any nearby DNA element. It has previously been demonstrated that Rep can simultaneously bind more than one DNA fragment that contains an RBS (Weitzman *et al.*, 1994), thereby providing a bridge between *AAVS1* and AAV DNA. This bridge would allow for the displaced replication complex to reassemble on AAV DNA and thus, after continuing replication, form a viral–cellular junction.

This model makes two predictions.

- Rep-dependent disruption of *AAVS1* should be observed even in the absence of AAV genomes, since *AAVS1* disruption has been observed (Balague *et al.*, 1997; Kotin *et al.*, 1991; Samulski *et al.*, 1991; Surosky *et al.*, 1997) and might, in fact, occur more frequently than integration. Indirect support for this suggestion comes from the finding that use of inducible Rep expression results in efficient integration while the frequency and extent of *AAVS1* rearrangements are reduced (Rinaudo *et al.*, 2000). This might suggest that Rep-induced rearrangements are not only a result of integration but can occur at any time that Rep is present.
- No AAV DNA replication is necessary for integration to occur and consequently that only an RBS (and no TRS) is required on the donor (viral) DNA. This

prediction was supported by Young *et al.*, who observed integration with a construct that lacks functional TRS (Young and Samulski, 2001).

The question arises whether AAV genomes also replicate as replication is initiated on *AAVS1*. Although this question has not been answered to date it must be noted that the viral origin is different from that of *AAVS1*: making selective initiation of replication possible. In contrast to the viral origin, the *AAVS1* origin is double-stranded and does not contain a D-region. On the viral genome both of these characteristics (the requirement for second-strand synthesis and the D-region) have been implicated in the regulation of replication initiation. It is reasonable to hypothesize, based on these differences, that preferential replication would occur on *AAVS1* under the latency conditions.

An additional consideration is the state of the donor DNA during integration. Frequently, the viral–cellular junctions were found to involve p5 promoter sequences. As determined by the episome-based assay, the sequences most likely to be retained in the integrated copy were located between the p5 promoter and the left ITR, followed by CAP and finally the Rep sequences (Giraud *et al.*, 1994, 1995). These observations are consistent with the hypothesis that the donor AAV DNA is in a circular form, that replicative integration begins in the p5 promoter region and that replication proceeds leftwards through the ITR into the CAP region. In addition, the number of integrated tandem copies (head-to-tail) would reflect the extent of this ‘rolling-circle’ replication. Evidence for the presence of circular AAV molecules in tissue culture has been provided by several investigators, including Engelhardt and co-workers, who observed circular (rAAV) molecules in cells that were not infected with a helper virus (Duan *et al.*, 1997).

The study of the integration mechanism has been plagued by the absence of additional suitable assays with which to test the various parameters of the model for replicative integration. While the circumstantial evidence listed above is in support of this model, only the requirement for replication initiation has directly been tested using the episome system. All other aspects of this model, however, remain untested to date. In addition to mechanistic details, more general questions remain untested. If replication mediates integration, which components of the cellular machinery are involved? With the possible biological role of integration taken into account, it could reasonably be assumed that cellular repair functions, rather than the replication machinery, would be involved. As a corollary, it has not yet been shown if integration occurs more efficiently in dividing or in arrested, differentiated cells.

In summary, our understanding of the mechanism of integration remains limited, and alternative models have yet to surface. Only the development of innovative assays that can test specific aspects of this, or competing models, will be able to shed light on this unique and potentially quite useful viral strategy.

## AAV RESCUE FROM THE PROVIRAL STATE

As described, AAV latency is one important phase of the viral life cycle, but a more crucial phase is the virus propagation cycle. AAV has to ensure the rescue of its genome from chromosomal DNA, establish a replicative life cycle, produce infectious viral particles and reinfect new cellular targets before it can re-establish viral latency. Efficient viral rescue from latently infected cells is observed only after helper viral superinfection (Berns *et al.*, 1975; Samulski *et al.*, 1991; Walz and Schlehofer, 1992; Laughlin *et al.*, 1986). An implication of this observation is that at least one entire copy of the viral genome must be integrated in the host genome. In an attempt to dissect the underlying mechanisms involved in AAV rescue, an *in vitro* system has been developed. In this model, the AAV genome is rescued from a recombinant plasmid in human cell lines after superinfection with a helper virus (Samulski *et al.*, 1982). While AAV integration results in partial deletion of the ITRs at the recombination site, rescue of the proviral genome requires one complete ITR (Ward *et al.*, 2003). In order to overcome this potential contradiction, AAV may have developed a strategy that relies on integrating viral concatemers, which contain one intact ITR between two AAV genomes. This hypothesis is supported by the identification of such concatemers in latently infected cells (Berns *et al.*, 1975; Laughlin *et al.*, 1986; Cheung *et al.*, 1980).

A central question with regard to site-specific integration has been the evolutionary benefit that targeted integration might have over random genome insertion as seen in various other viruses. Although no information is available to date that would shed light onto this question, it might be reasonable to hypothesize that the rescue of functional AAV genomes might require components from the cellular integration locus. A possible requirement that comes to mind is the Rep-dependent *AAVS1* replication origin, i.e. the TRS-RBS. This origin would allow rescue through replication to initiate outside of the proviral genome. This replication would proceed into the AAV genome and, in effect, 'peel off' a single-stranded copy of the viral DNA that could serve as a template for further replication (Ward and Linden, 2000). However, it is quite possible that additional, as yet unidentified, *AAVS1* components are also involved.

## AAV AS A VECTOR FOR TARGETED GENE DELIVERY

Since recombinant AAV vectors for gene transfer are the topic of several different chapters in this book, we will limit our discussion to targeted gene delivery to *AAVS1* and describe some of the more recent technologies.

During the last decade, AAV has become one of the most promising viral vectors for gene delivery. This enthusiasm is due to several interesting features of wtAAV; its ability to

infect a broad range of cells including non-dividing cells, its low immunogenicity, its apparent lack of pathogenicity, and its ability to integrate site-specifically into the human genome. Although AAV-based vectors have shown great utility for gene transfer, only a few studies have addressed what is possibly the most intriguing characteristic of the wild-type virus, namely site-specific integration. In consequence, many central questions relating to this mode of delivery have yet to be explored. For example, under what circumstances is gene targeting advantageous? What are the effects of targeting transgenes on *AAVS1*? Can genes be expressed or even regulated at this locus, and if so, in which tissues? In the final section we will not attempt to address these questions but rather will limit ourselves to reviewing those approaches that have already entered the literature.

One potential disadvantage of rAAV vectors for certain applications is the limited size of the DNA sequence that can be packaged into the viral particle (<5 kb). This problem becomes even more significant if Rep is included in order to achieve gene targeting. Several approaches have been proposed to overcome this problem. One of these takes advantage of the larger transgene capacities of other viral delivery systems, such as herpes simplex virus 1, adenovirus, and baculovirus. Such hybrid viruses, containing the *rep* gene and a transgene flanked by AAV ITRs, can efficiently target *AAVS1* (Palombo *et al.*, 1998; Recchia *et al.*, 1999; Heister *et al.*, 2002). It must be pointed out, however, that AAV has evolved rather complex mechanisms to control interactions with its helper viruses. For example, Rep significantly interferes with the replication of adenovirus, thus putting into question the usefulness of adenoviruses that express Rep, a protein which must inevitably limit their own replication.

As discussed above, another potential problem concerning targeted gene transfer is the cytotoxicity of Rep. Ideally, Rep should be expressed transiently in order to provide sufficient protein to achieve site-specific integration. Some promising preliminary attempts have been made to control the level of Rep expression using inducible promoters and regulated Rep proteins, as discussed above.

One of the most appealing features of targeted gene delivery is that, possibly, the risk of insertional mutagenesis can be reduced. Only further study of site-specific integration and its effects on cellular metabolism can provide us with the necessary insight into the potential of this unique and intriguing strategy.

## WEB SITES AND ACCESSION NUMBERS

*AAVS1* location: ([http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list\\_uids=54776](http://www.ncbi.nlm.nih.gov:80/entrez/query.fcgi?db=gene&cmd=Retrieve&dopt=Graphics&list_uids=54776))

Gene Bank accession numbers:  
AAV2: AF043303

*Homo sapiens* chromosome 19 clone CTD-2587H24: AC010327; AAVS1: S51329; MBS85 cDNA: AF312028; MBS85 protein: AF312028\_1  
*Homo sapiens* *TNNT1* gene: AJ011712/AJ011713; *TNNT1* cDNA: NM\_003283; *TNNT1* protein: NP\_003274; *TNNI3* gene: X90780; *TNNI3* cDNA: NM\_000363; *TNNI3* protein: NP\_000354; *DRC3* (*EPS8L1*) gene: AF282168; *DRC3* cDNA: NM\_133180; *DRC3* protein: NP\_631943  
*Mus musculus* BAC clone RP23-313M20: AC079521; *Mus musculus* *Tnnt1* gene: U92882; *Tnni3* gene: Z22784

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# Latent infection of the host cell by AAV and its disruption by helper viruses

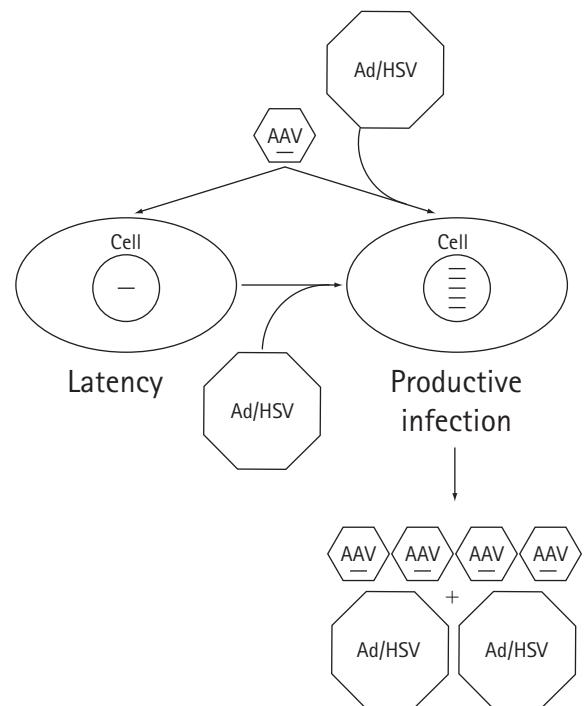
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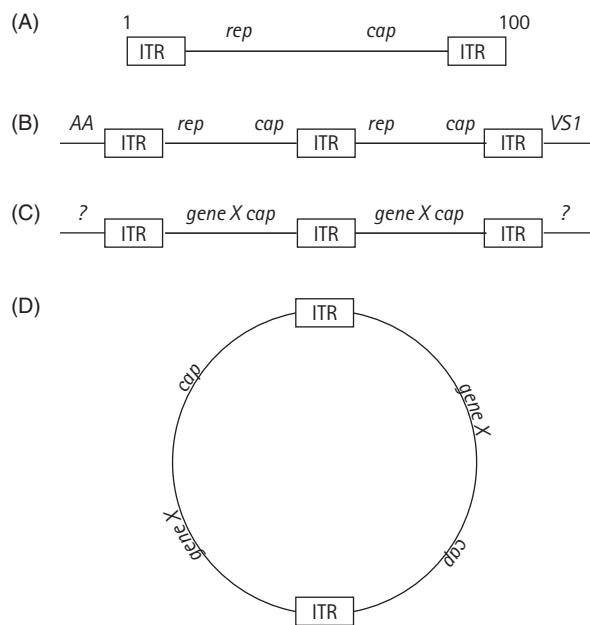
Adeno-associated viruses (AAVs) are helper-dependent members of the *Dependovirus* genus of parvoviruses (Siegl *et al.*, 1985). AAVs contain linear, single-stranded DNA genomes of approximately 4.7 kilobases (kb; Srivastava *et al.*, 1983; Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997, 1999b; Rutledge *et al.*, 1998; Xiao *et al.*, 1999; Bossis and Chiorini 2003). A typical AAV life cycle is shown in Figure 17.1. They usually require a helper virus, such as an adenovirus or herpes virus for productive infection (Carter, 1990). Their name is derived from the fact that several members of this genus were discovered as contaminants in human and simian adenovirus preparations (Hoggan *et al.*, 1966; Blacklow *et al.*, 1967). In the absence of helper virus, AAVs can establish a latent infection in many cell types. AAVs can be rescued if a latently infected cell is subsequently infected with a helper virus, allowing production of infectious AAV particles. Most studies of AAV persistence, rescue and helper virus functions have involved AAV serotype 2 (AAV2), primarily because it was the first AAV whose genome was cloned and sequenced (Samulski *et al.*, 1982; Laughlin *et al.*, 1983; Srivastava *et al.*, 1983). One interesting component of AAV2 latency is its ability to integrate its genome preferentially into a 4 kb locus on the q-arm of human chromosome 19 (19q13-qter), designated AAVS1 (Kotin and Berns, 1989; Kotin *et al.*, 1990, 1991, 1992; Samulski *et al.*, 1991; Shelling and Smith, 1994).

## AAV GENES AND PROTEINS

By convention, the AAV genome is divided into 100 map units, and is oriented as shown in Figure 17.2. AAV DNA



**Figure 17.1** The AAV life cycle. AAVs normally require a helper virus, such as an adenovirus (Ad) or herpesvirus (HSV) for productive infection. In the absence of helper virus, AAVs can establish a latent infection. Subsequent infection by helper virus leads to rescue and productive infection. The hexagons represent AAV particles. The line segment represents the AAV genome that is amplified in the presence of helper virus. The octagons represent Ad or HSV particles. Drawings are not to scale.



**Figure 17.2** Persistent forms of AAV2 DNA. **(A)** The AAV genome. The inverted terminal repeats (ITR) and rep and cap genes are indicated. **(B)** AAV( $\text{rep}^+$ ) integrated as a head-to-tail dimer into AAVS1. **(C)** Recombinant AAV in which the rep gene has been replaced with another gene (gene X) randomly integrated as a head-to-tail dimer into unknown host DNA(?). **(D)** Recombinant AAV in which the rep gene has been replaced with another gene (gene X) as a circular, head-to-tail episomal dimer. Drawings are not to scale.

contains inverted terminal repeats (ITRs) that include the origins of replication (Straus *et al.*, 1976). The AAV2 ITRs are 145 bases in length (Srivastava *et al.*, 1983). The ITRs also function in AAV gene regulation (Flotte *et al.*, 1992; Pereira *et al.*, 1997), persistence (Philip *et al.*, 1994), integration (Balagué *et al.*, 1997) and rescue (Samulski *et al.*, 1982; Ward and Berns, 1991). A recombinant AAV (rAAV) or rAAV vector may be loosely defined as any DNA sequence flanked by AAV ITRs. In rAAV, some or all of the internal AAV sequences may be replaced with heterologous DNA.

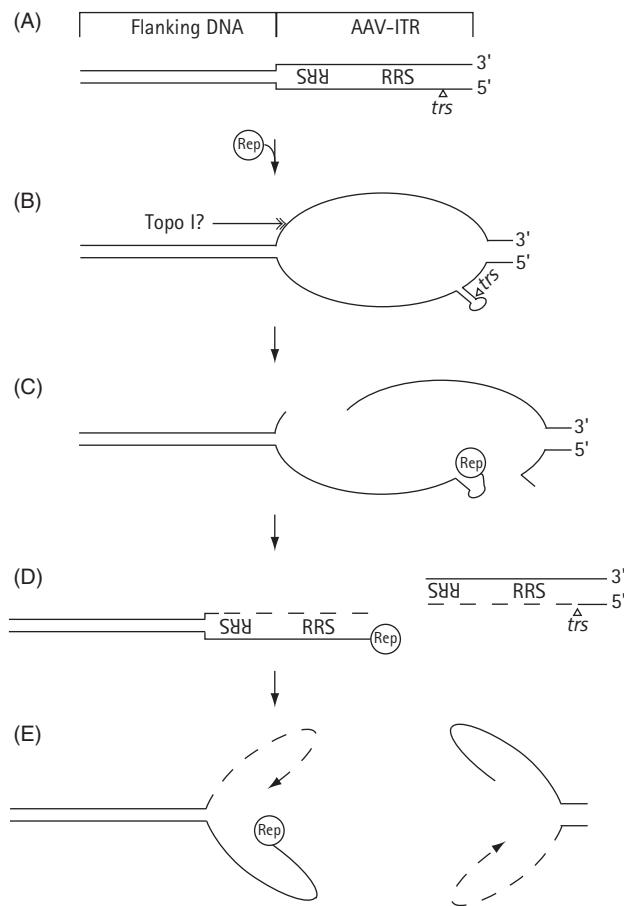
AAV genomes contain two major open reading frames (ORFs). The *cap* ORF encodes three capsid proteins, usually referred to as VP1, VP2, and VP3 (Carter *et al.*, 1990b). The AAV2 capsid proteins are encoded by spliced transcripts initiated at the promoter at map position 40 (p40; Srivastava *et al.*, 1983; Becerra *et al.*, 1985, 1988).

The *rep* ORF encodes four Rep proteins that are involved in AAV replication (Snyder *et al.*, 1990a), gene regulation (Labow *et al.*, 1986; Tratschin *et al.*, 1986; Beaton *et al.*, 1989; Kyöstiö *et al.*, 1994), integration (Shelling and Smith, 1994; Weitzman *et al.*, 1994; Urcelay *et al.*, 1995; Linden *et al.*, 1996; Balagué *et al.*, 1997), packaging (Chejanovsky and Carter, 1989; King *et al.*, 2001) and rescue (Hermonat *et al.*, 1984; Tratschin *et al.*, 1984). For AAV2, these proteins are called Rep78, Rep68, Rep52, and Rep40, because of their apparent

molecular sizes (Mendelson *et al.*, 1986). Rep40 and Rep52 are encoded by spliced and unspliced mRNA, respectively, initiated at the promoter at map position 19 (p19) of the AAV2 genome (Srivastava *et al.*, 1983; Mendelson *et al.*, 1986; Chejanovsky and Carter, 1989).

For purposes of integration and rescue, the key AAV2 proteins are Rep68 and Rep78 (Shelling and Smith, 1994; Ward *et al.*, 1994; Weitzman *et al.*, 1994; Urcelay *et al.*, 1995; Linden *et al.*, 1996; Balagué *et al.*, 1997). Rep68 and Rep78 are encoded by spliced and unspliced mRNA, respectively, initiated at the promoter at map position 5 (p5) of the AAV2 genome (Srivastava *et al.*, 1983; Mendelson *et al.*, 1986). Since they are made from the same ORF, their first 529 amino acids (out of 621 for Rep78 and 536 for Rep68) are identical and they are nearly identical in their functions (Mendelson *et al.*, 1986; Im and Muzyczka, 1992; McCarty *et al.*, 1992; Owens *et al.*, 1993). For this reason, they will be referred to collectively as Rep68/78 for the remainder of this chapter. Rep68/78 are DNA-binding proteins that specifically recognize double-stranded DNA containing imperfect repeats (usually four) of 5'-GCTC-3' and its complement 5'-GAGC-3' (Chiorini *et al.*, 1994; McCarty *et al.*, 1994a,b; Weitzman *et al.*, 1994). These repeats are referred to as Rep recognition sequences (RRSs; Chiorini *et al.*, 1994; Weitzman *et al.*, 1994), Rep-binding sites (RBSs) (McCarty *et al.*, 1994a,b) or Rep-binding elements (RBEs; Young *et al.*, 2000). RRSs have been identified within the ITRs of all AAV serotypes for which the ITRs have been sequenced (Chiorini *et al.*, 1999b; Xiao *et al.*, 1999; Bossis and Chiorini, 2003), in the promoter for Rep68/78 in multiple AAV serotypes (Chiorini *et al.*, 1999b; Xiao *et al.*, 1999; Bossis and Chiorini, 2003), and within numerous cellular genes (Batchu *et al.*, 1994; Wonderling and Owens, 1996, 1997).

Rep68/78 also have a nucleoside triphosphate (NTP)-dependent DNA helicase activity that has both a non-specific and a sequence-specific aspect. Rep68/78 can unwind non-specific DNA with a 3' single-stranded overhang and are therefore believed to process in a 3' to 5' direction relative to the overhanging strand (Wu *et al.*, 1999; Zhou *et al.*, 1999b). Rep68 (and it is presumed that Rep78 can also) can unwind double-stranded DNA without a single-stranded overhang, if it contains an RRS (Zhou *et al.*, 1999b). This appears to give Rep68/78 the ability to form bubbles of unwound DNA within the AAV ITRs. Rep68/78 also have an NTP-dependent strand-specific, site-specific endonuclease or nicking activity that is believed to be involved in replication, integration, and rescue of AAV genomes (Snyder *et al.*, 1990a; Ward *et al.*, 1994; Urcelay *et al.*, 1995; Linden *et al.*, 1996; Urabe *et al.*, 1999). Rep68/78 nicking sites have been identified near RRSs within the AAV ITRs (Chiorini *et al.*, 1999a; Im and Muzyczka, 1990) and within AAVS1 (Urcelay *et al.*, 1995). A Rep68/78 nicking site is referred to as a terminal resolution site (TRS), because of its role in resolving the hairpin form of an AAV ITR into linear, double-stranded DNA (Snyder *et al.*, 1990a, b). The nicking sites are believed to be bounded by a stable secondary structure (see Figure 17.3).



**Figure 17.3** AAV Rescue model. (A) Schematic of one AAV-ITR/cellular DNA junction. The positions of the two RRSs (inverted relative to one another) and the TRS are indicated. (B) Rep68/78 (Rep) unwinds the RRS-containing DNA, allowing a hairpin to form at the TRS. A hypothetical cellular nicking enzyme (Topo I?) cleavage site is indicated by the double-headed arrow. (C) Cleavage at the TRS by Rep68/78, in conjunction with Topo I cleavage produces a staggered double-stranded break, with 5' overhangs, within the AAV-ITR. Rep68/78 covalently attaches to the 5' side of the nick. (D) Cellular DNA polymerase fills in the 5' overhangs. (E) Rep68/78 unwinds the newly formed RRSs, allowing the complementary sequences to form intrastrand hairpins that can prime the next round of replication (arrowheads indicate 3' hydroxyls). Drawings are not to scale.

formed by intrastrand interactions after the two DNA strands are separated by Rep68/78 (Brister and Muzyczka, 1999).

## LATENT/PERSISTENT INFECTION

### Definition

The definition of AAV latency can vary from laboratory to laboratory, but the general working definition drawn from the literature is the lack of production of infectious AAV

particles, but the persistence of detectable viral DNA or virus-encoded proteins two months or more after infection, transduction, or transfection of the cells with wild-type or rAAV (Berns *et al.*, 1975; Afione *et al.*, 1996; McCown *et al.*, 1996; Herzog *et al.*, 1997; Murphy *et al.*, 1997; Duan *et al.*, 1998; Lalwani *et al.*, 1998; Wang *et al.*, 1999). The encoded proteins can be either proteins normally made by AAV or the products of reporter genes placed into a rAAV genome. Wild-type AAV and/or rAAVs have been reported to persist for many months (a year or longer in some cases) in both whole animal and tissue culture systems (Kotin and Berns, 1989; Xiao *et al.*, 1996; Omori *et al.*, 1999; Snyder *et al.*, 1999; Nakai *et al.*, 2001; Song *et al.*, 2001). Since many of the experiments described below involve rAAVs, it is often more appropriate to use the more general term, persistence, rather than latent infection.

## Distribution of AAV

The tissue distribution of AAVs is probably dependent on their route of entry, the cell tropism of the specific AAV type (Rabinowitz *et al.*, 2002) and the absence, presence, or type of accompanying helper virus. Wild-type AAVs have been isolated from human anal and throat specimens (Blacklow *et al.*, 1967) and a penile lesion (Bantel-Schaal and zur Hausen, 1984). Using Southern blots or polymerase chain reaction (PCR)-based methods, AAV2 DNA sequences have been detected in the genital tract of female patients (Friedman-Einat *et al.*, 1997), human amniotic fluid (Burguete *et al.*, 1999), established cell lines derived from human trophoblast and amniotic cells (Dutheil *et al.*, 1997), and human peripheral blood leukocytes (Grossman *et al.*, 1992). Using real-time PCR, AAV genomes could be detected in the brain, heart, lung, liver, spleen, duodenum, kidney, testes, and lymph node tissues of rhesus macaques (Gao *et al.*, 2003).

AAV1 could be detected in the lungs and kidneys of fetuses and newborns after pregnant mice were injected with wild-type AAV1 plus a mouse adenovirus (Lipps and Mayor, 1980). Stable transduction of mouse, dog, rabbit, non-human primate, and rat tissues in intact animals has been demonstrated with rAAV vectors with capsids of multiple AAV serotypes in the absence of helper viruses (Flotte *et al.*, 1993; Kaplitt *et al.*, 1994; Afione *et al.*, 1996; Herzog *et al.*, 1997; Snyder *et al.*, 1997; Monahan *et al.*, 1998; Snyder *et al.*, 1999; Weber *et al.*, 2003). rAAVs containing marker or therapeutic genes have been shown to persist in many tissues, including muscle, brain, retina, liver, and nasal and lung epithelium (Kaplitt *et al.*, 1994; Afione *et al.*, 1996; Bartlett *et al.*, 1996; Xiao *et al.*, 1996; Koeberl *et al.*, 1997; Ali *et al.*, 1998; Weber *et al.*, 2003).

## Modes of persistence

There appear to be at least three mechanisms by which wild-type AAV or rAAV genomes can persist in the host cells

(Figure 17.2). The first is site-specific integration into the host DNA at *AAVS1* (Kotin and Berns, 1989; Kotin *et al.*, 1990, 1991, 1992; Samulski *et al.*, 1991; Weitzman *et al.*, 1994; Urcelay *et al.*, 1995). Site-specific integration, first demonstrated for AAV2, requires RRSs within the target and insert, the Rep68/78 proteins, and a Rep68/78 nicking site within the target (Giraud *et al.*, 1994; Weitzman *et al.*, 1994; Urcelay *et al.*, 1995; Linden *et al.*, 1996; Balagué *et al.*, 1997; Young and Samulski, 2001; Philpott *et al.*, 2002; Steigerwald *et al.*, 2003). The second mode of persistence is non-specific integration, which is Rep68/78-independent but appears to have a bias towards actively transcribed genes (Nakai *et al.*, 2003b). AAV integration does not require cell division (Wu *et al.*, 1998). The third mechanism involves the persistence of AAV DNA as episomal concatemers (Afione *et al.*, 1996; Duan *et al.*, 1998). AAV integration is examined in depth elsewhere in this volume (Chapter 16). However, several aspects of integration are discussed here, because it is important for the understanding of complexities involved in studying AAV latency.

## METHODS OF DETECTION

Each method for detecting persistence of wild-type or recombinant AAV has its own strengths and weaknesses. Gene expression can be the simplest if one includes a gene encoding a selectable marker, such as neomycin/G418 resistance, or a protein, such as green fluorescent protein, that can be easily detected in live cells. Gene expression, however, provides no data with regards to the state of the viral DNA (e.g. integrated versus episomal). In the absence of helper virus infection (helper virus usually kills the cells), the proteins of wild-type AAV are not easily detected because of their low expression levels (Berns *et al.*, 1975; Mendelson *et al.*, 1986). Southern blotting (Southern, 1975) of either total DNA or low molecular weight DNA can also be used to determine persistence. PCR, using primers within the AAV or rAAV genome, can be used to enhance the sensitivity of Southern blot analysis. The presence of AAV genomes in low molecular weight DNA is a clear indication of episomal persistence. Detection of AAV genomes in high molecular weight DNA (>10 kb) does not, however, guarantee that the AAV DNA is integrated into host DNA (Cheung *et al.*, 1980; Duan *et al.*, 1998). One method for distinguishing between high molecular weight episomal concatemers and integrated AAV DNA is to digest the DNA with a restriction enzyme that cuts once within AAV. This digestion will produce junction DNA fragments with distinct sizes.

Once it is determined that AAV is integrated, it is often desirable to know where it is integrated. If integration occurs at *AAVS1*, a band of the same size will be detected using either an AAV probe or an *AAVS1* probe on the same sample (Kotin *et al.*, 1990). Detection of integration at other sites is more problematic. This often involves isolation and amplification of the junction fragment so that it can be sequenced (Kotin and Berns, 1989; Yang *et al.*, 1997; Miller *et al.*, 2002),

or fluorescence *in situ* hybridization (FISH) analysis (Kotin *et al.*, 1991; Kearns *et al.*, 1996). Identification of the chromosomal location by sequencing has only become practical since the recent completion of the human genome sequence. It can still produce ambiguous results if AAV integrates into repetitive DNA. FISH analysis, although it provides direct visualization of integrated DNA, does not provide precise locations and can sometimes give false-negative results if only a single or partial copy of AAV is integrated at a particular locus (Kearns *et al.*, 1996).

## INTEGRATION VERSUS EPISOMAL PERSISTENCE

An early characterization of AAV latency was reported by Berns *et al.* (1975). They examined a human cell line, Detroit 6, which had been infected with AAV2 at a multiplicity of infection (MOI) of 250. Even after 47 passages, AAV2 capsid protein production could be detected in these cells upon infection with adenovirus. They also demonstrated the persistence of AAV2 DNA by showing that unlabeled DNA isolated from these latently infected cells blocked self-annealing of radiolabeled AAV2 DNA much more effectively than control DNA from uninfected cells (Berns *et al.*, 1975).

In the absence of data indicating that AAV DNA could replicate without helper virus, for decades it was assumed that persistence equaled integration. This hypothesis was supported by the observation that AAV DNA in persistently infected or transduced cells was often associated with high molecular weight DNA. Southern blot analyses of cells persistently infected with either wild-type AAV2 or rAAV2 often showed bands with apparent sizes of >12 kb (Cheung *et al.*, 1980; Mendelson *et al.*, 1988a; Duan *et al.*, 1998; Nakai *et al.*, 2003c). Integration in the presence or absence of the *rep* gene was assumed to be random since the size of the fragments containing the AAV genome varied between clonal isolates (Mendelson *et al.*, 1988a). By cloning the cellular flanking sequence from a persistently infected Detroit 6 cell line subclone and using it as a probe, Kotin *et al.* were able to show that the majority of integration events with wild-type (*rep*<sup>+</sup>) DNA occur in the same locus, *AAVS1*, but that the precise integration junctions can vary (Kotin and Berns, 1989; Kotin *et al.*, 1990). It was later discovered that *rep*<sup>-</sup> AAV vectors do not integrate into *AAVS1* at any significant frequency (Xiao *et al.*, 1993). Depending on the cell type examined, persistence of these *rep*<sup>-</sup> AAV vectors was often comparable to that of wild-type AAV (Mendelson *et al.*, 1988b). Many researchers therefore continued to believe that persistence of *rep*<sup>-</sup> AAV vectors was entirely due to random integration. This belief began to be questioned when FISH analyses found that a *rep*<sup>-</sup> rAAV2 vector persisted episomally both in tissue culture cells (Kearns *et al.*, 1996) and in the bronchial epithelial cells of rhesus macaques (Afione *et al.*, 1996). The *rep*<sup>-</sup> rAAV2 vector tested by Kearns *et al.* appeared to have a 10-fold lower frequency of integration than wild-type AAV2, but this difference could be partly due to the sensitivity of FISH to the length of the target

sequence hybridized. FISH can more readily detect the presence of tandem copies of a DNA sequence, which occur commonly with AAV2 integration into *AAVS1*, than a single copy integrant (Kearns *et al.*, 1996).

### EPISOMAL AAV GENOMES

The formation of high molecular weight circular and linear episomal AAV DNA does not seem to require Rep68/78 (Duan *et al.*, 1998). Rep68/78 may actually destabilize such concatemers through their excision function (see below). Circular and linear concatemerization may, however, require elements of the cellular double-stranded DNA break repair machinery. The hypothesis that linear AAV genomes are recognized as double-stranded DNA breaks is supported by the observation that cells containing a mutated version of the double-stranded break signaling protein, DNA-dependent protein kinase (DNAPKcs), show a lower ratio of circular to linear AAV episomal genomes when transduced with *rep*<sup>-</sup> rAAV (Song *et al.*, 2001). There is evidence for both DNAPKcs-dependent and DNAPKcs-independent circularization of *rep*<sup>-</sup> rAAV genomes (Nakai *et al.*, 2003c). In addition, cellular proteins Ku86 and Rad52, which are known to bind double-stranded DNA breaks, have been shown by co-immunoprecipitation assays to associate directly with rAAV DNA (Zentilin *et al.*, 2001).

There are two major mechanisms by which cells repair double-stranded breaks; homologous recombination and non-homologous end joining. DNAPKcs and Ku86 are believed to be associated primarily with non-homologous end joining, while Rad52 is associated with homologous recombination (van Dyck *et al.*, 1999). It is therefore likely that both pathways are acting on AAV DNA (Zentilin *et al.*, 2001; Nakai *et al.*, 2003c). Many of the episomal and integrated AAV concatemers are in a head-to-tail configuration (see Figure 17.2). However, dimers created by productive AAV replication are either head-to-head or tail-to-tail (Carter *et al.*, 1990a). In theory, head-to-tail concatemers can be formed by homologous recombination between the ITRs, which would place a single, modified fused ITR at the AAV-AAV junction (see Figure 17.2) or by non-homologous end joining, which would result in two complete ITRs at the AAV-AAV junction. Head-to-tail concatemers can be easily distinguished from head-to-head or tail-to-tail concatemers by restriction endonuclease mapping (Kotin and Berns, 1989). However, due to the palindromic nature of the ITRs, it is often quite difficult to get sufficient sequence information to distinguish between a single fused ITR and two tandem ITRs (Duan *et al.*, 1999). Each AAV2 ITR contains 125 bases of almost perfectly self-complementary sequence (Srivastava *et al.*, 1983). This can result in stalling of the polymerases used for sequencing, as well as aberrant migration of sequencing reaction products. However, an artificially constructed fused ITR has been shown to function in AAV replication (Xiao *et al.*, 1997). A more recent chemical sequencing analysis (as opposed to polymerase-based

sequencing) of ITRs within circular rAAV DNA formed *in vivo*, found predominantly single fused ITRs, but also several AAV-AAV junctions consistent with non-homologous end joining of partially deleted ITRs (Duan *et al.*, 1999). It is therefore possible that AAV has been selected to survive both arms of the double-stranded DNA repair system, with either result being rescuable.

It is suspected that circularization of *rep*<sup>-</sup> rAAV DNA represents a dead end with regards to integration (Song *et al.*, 2001; Nakai *et al.*, 2003a). This suspicion is in part based on the observation that, in mouse cells lacking DNAPKcs, integrated rAAV DNA could be easily detected after intramuscular injection of a *rep*<sup>-</sup> rAAV (Song *et al.*, 2001). Integration was defined as the presence of rAAV-containing DNA that showed a high molecular weight in Southern blot analyses, whose migration could be affected by predigestion with a restriction endonuclease that does not cut within the rAAV (Song *et al.*, 2001). The DNAPKcs-deficient cells contained dramatically lower amounts of persistent circular rAAV genomes, and higher amounts of linear episomal concatemers of rAAV than wild-type mouse muscle injected with the same rAAV (Song *et al.*, 2001). Comparison of samples taken at 18 weeks and 52 weeks after transduction suggested that linear episomal concatemers are the precursors for integrated rAAV (Song *et al.*, 2001). Although side-by-side comparisons were not presented, these analyses also implied that integration of rAAV DNA was higher in the DNAPKcs-deficient muscle cells than in wild-type muscle (Song *et al.*, 2001).

If circularization is part of a host defense mechanism against viral DNA integration, this may explain part of the need for high MOIs to achieve efficient persistence of wild-type AAV or rAAV in rapidly dividing cells (Berns *et al.*, 1975; Flotte *et al.*, 1992). It should be noted that there was no significant difference in expression of the gene delivered by the rAAV between the wild-type and DNAPKcs-deficient muscle cells, out to 52 weeks post-transduction (Song *et al.*, 2001).

As stated above, there also appears to be a DNAPKcs-independent mechanism for circularizing rAAV (Duan *et al.*, 2003; Nakai *et al.*, 2003c), consistent with the hypothesis that AAV genomes can form concatemers by either non-homologous end joining or homologous recombination. Analyses of transfections of mouse hepatocytes with linear or circular DNA suggest that circular *rep*<sup>-</sup> rAAV genomes, at least those containing a fused single ITR, represent a dead end for both concatemerization and integration (Nakai *et al.*, 2003a).

Since persistent forms of AAV DNA are predominantly double-stranded, second-strand synthesis appears to be critical for the persistence of AAV DNA that is delivered in the single-stranded packaged form (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). Pretreatment of cells with DNA damaging agents, such as ionizing radiation, UV light and topoisomerase inhibitors (which tend to result in nicked DNA) or DNA synthesis inhibitors, such as hydroxyurea, enhance the transduction efficiency and/or integration of *rep*<sup>-</sup> AAV

vectors (Alexander *et al.*, 1994; Russell *et al.*, 1995; Ferrari *et al.*, 1996). It has been shown that second-strand synthesis of rAAV and helper-independent replication of wild-type AAV2 are also stimulated by some of these agents (Yalkinoglu *et al.*, 1988, 1991; Yakobson *et al.*, 1989; Ferrari *et al.*, 1996). The mechanism of this stimulation is not yet understood, but is presumed to involve the induction or redistribution of DNA repair polymerases.

Another major question is whether or not integration is required for AAV persistence in actively dividing cells. The ability of AAV DNA to be excised after integration (discussed below), even in the absence of Rep proteins, makes this a very difficult question to answer, since some episomal AAV genomes could be the result of excision of formerly integrated genomes (Cheung *et al.*, 1980). Nakai *et al.* performed partial hepatectomies on mice transduced with a *rep*<sup>-</sup> rAAV vector expressing the human factor IX protein (Nakai *et al.*, 2001). They found that upon regrowth of the liver, the serum factor IX levels, as well as the average number of rAAV vector genomes per cell, dropped to about 10 percent of the prehepatectomized levels. Their interpretation was that the 90 percent loss was due to episomal genomes that could not replicate (Nakai *et al.*, 2001).

## Consequences of latent infection

Latent AAVs are under two, often opposing, selection pressures. AAV genes must be repressed in the absence of helper virus, but they must be able to be activated in the presence of helper virus. There is therefore a selection for the ability to integrate into chromosomal regions that are generally transcriptionally active. AAV2 has redundant mechanisms for achieving this balance. In the presence of Rep68/78, AAV2 DNA integrates preferentially into the *AAVS1* locus on human chromosome 19. This locus has been shown to contain a DNase I hypersensitive site (Lamartina *et al.*, 2000), normally believed to represent a region of open chromatin configuration, which is associated with gene expression. A cDNA was isolated which hybridizes to this region, suggesting that there is RNA expression within *AAVS1* (Kotin *et al.*, 1992) and a gene encoding the slow skeletal troponin T gene is nearby (Dutheil *et al.*, 2000). Even in the absence of Rep proteins, rAAV2 integration preferentially occurs in regions containing active genes (Nakai *et al.*, 2003b).

Since the Rep proteins of AAV2 inhibit cell division (Yang *et al.*, 1994) and may even cause cell death (Schmidt *et al.*, 2000) when expressed at high levels, minimizing expression of the *rep* gene is critical to persistence of a wild-type AAV genome. This minimization is achieved by multiple mechanisms. First, the Rep proteins (Rep78, Rep68, Rep52, and Rep40) repress *rep* gene expression (Beaton *et al.*, 1989; Kyöstiö *et al.*, 1994, 1995; Hörer *et al.*, 1995). Cellular transcription factors YY1, PC4, and ZF5 have also been shown to repress AAV2 promoters in the absence of helper

virus (Chang *et al.*, 1989; Shi *et al.*, 1991; Weger *et al.*, 1999; Cathomen *et al.*, 2001).

*AAVS1* also contains an insulator element upstream of the section where most AAV2 integrations occur (Ogata *et al.*, 2003). This insulator should prevent activation of AAV genes by upstream cellular enhancers and also appears to block the extension of heterochromatin, which could lead to inactivation of AAV genes in a way that might not be reversible by a helper virus (Ogata *et al.*, 2003). It has not yet been determined if there is a similar insulator on the downstream side of the preferred integration locus.

The consequences to the cell of having an AAV provirus can be affected by the site of integration (if any), environmental factors, and the transcription factors expressed within the cell. Since persistence of *rep*<sup>-</sup> rAAV vectors appears to be generally without consequences to the cell (Flotte *et al.*, 1993), it is presumed that many of the negative events seen occasionally with wild-type proviral AAV are due to the Rep proteins (Winocour *et al.*, 1992; Yang *et al.*, 1994; Zhou *et al.*, 1999a). For example, HeLa cells (a human cervical carcinoma cell line) containing AAV2 proviruses integrated on the long arm of chromosome 17 had a slower growth rate and were more sensitive to heat, ultraviolet light, gamma irradiation, and several genotoxic chemicals than uninfected cells (Walz and Schlehofer, 1992). Human 293 cells constitutively express the E1a gene of adenovirus type 5 (Graham *et al.*, 1977), whose products stimulate *rep* gene expression (Chang *et al.*, 1989). Integrated proviruses resulting from infection of 293 cells with wild-type AAV2 or *rep*<sup>+</sup> rAAV2 frequently show rearrangements or become deleted (Mendelson *et al.*, 1988a; Giraud *et al.*, 1995; Dyall and Berns, 1998; Urabe *et al.*, 2003). This may be due to a combination of the imprecise repair of DNA damage initiated by Rep68/78 nicking and selection against cells containing an intact *rep* gene under conditions when that gene would be highly expressed. In proviral cell lines with relatively low Rep68/78 expression in the absence of helper virus, such as Detroit 6, HeLa, or KB cells, the integrated AAV genomes show much less gross rearrangement (Berns *et al.*, 1975; Laughlin *et al.*, 1986; Mendelson *et al.*, 1988a; Kotin and Berns, 1989). Such rearrangement can have a negative impact on the ability of the provirus to be rescued by subsequent infection of the cells with helper virus (Mendelson *et al.*, 1988a).

## RESCUE

### Definitions

Rescue can be defined as the induction of replication and packaging of wild-type AAV or rAAV by supplying the appropriate helper functions to a cell containing AAV genomes in either a chromosomally integrated or episomal state. Rescue is defined functionally by the ability to produce encapsidated AAV genomes and/or infectious AAV particles

from either cells latently infected with AAV or cells transfected with plasmids containing a wild-type or rAAV genome. As with AAV2 productive infection, efficient rescue requires the AAV2 ITRs, the AAV *rep* and *cap* gene products, and factors provided by the helper virus and cell.

## Early studies of rescue

The aforementioned study of Berns *et al.* (1975) was also one of the earlier studies of AAV rescue. They showed that infection of Detroit 6 cells with adenovirus type 2, 67 passages after infection with AAV2, resulted in the production of infectious AAV2 particles.

The ability of AAVs to be rescued, in particular their ability to be rescued from an integrated state, allowed the generation of plasmids containing infectious AAV clones (Samulski *et al.*, 1982, 1983; Laughlin *et al.*, 1983; Muramatsu *et al.*, 1996; Rutledge *et al.*, 1998; Xiao *et al.*, 1999; Bossis and Chiorini 2003). Since AAV2 was the first AAV cloned (Samulski *et al.*, 1982; Laughlin *et al.*, 1983), most rescue studies have been performed using this serotype.

## Phases of rescue

Rescue can be divided into four phases:

- The first phase is activation of AAV gene expression, which results in the production of critical quantities of the AAV Rep and capsid proteins.
- The second phase is the generation of free AAV genomes that are not covalently attached to flanking DNA, otherwise known as excision.
- The third phase is replication of AAV DNA.
- The fourth phase is packaging of AAV DNA into capsids.

Several of these phases may occur simultaneously within a given cell.

## Helper functions

AAV rescue can be triggered by either helper virus superinfection, a wide variety of DNA damaging agents, or by transient inhibition of DNA replication (Carter, 1990). Reported helper viruses for AAVs include adenoviruses (Atchison *et al.*, 1965), herpes viruses (Atchison, 1970; Schlehofer *et al.*, 1986), vaccinia virus (Schlehofer *et al.*, 1986), and human papilloma viruses (Walz *et al.*, 1997). DNA damaging agents and DNA synthesis inhibitors capable of rescuing AAVs include UV light and hydroxyurea (Yakobson *et al.*, 1987, 1989; Yalkinoglu *et al.*, 1988). Helper functions are required for the efficient accumulation, splicing (all AAV capsid proteins are translated from spliced mRNAs), and translation of AAV RNA, as well as

AAV DNA replication (Buller *et al.*, 1979; Laughlin *et al.*, 1982; Trempe and Carter, 1988; Carter, 1990). Although it is not necessary for rescue, recent evidence suggests that adenovirus capsid proteins can facilitate the transport of infecting wild-type AAV particles into the nucleus (Xiao *et al.*, 2002).

One interesting aspect of the ability of AAVs to use multiple helpers is that a particular AAV type will often have a much broader host range than any individual helper virus (Carter, 1990). For example, AAV1 was first isolated from rhesus monkey kidney cells infected with the adenovirus, simian virus 15 (Atchison *et al.*, 1965). AAV1 will also replicate in mouse kidney cells, if a mouse adenovirus is used as a helper (Casto *et al.*, 1967).

The diverse nature of these rescue triggers has led to the general belief that the major role of the helper virus is to establish a cellular environment that is conducive to AAV replication and packaging, as opposed to the AAVs using the helper virus-encoded replication machinery (Carter, 1990; Xiao *et al.*, 1998). *In vitro* replication assays using crude or partially purified cell extracts also suggest that all cellular factors absolutely required for AAV DNA replication are present in uninfected cells (Ward *et al.*, 1994; Ni *et al.*, 1998). It is therefore possible that the helpers cause a redistribution of either the cellular factors and/or the AAV DNA. In support of this hypothesis, immunofluorescence microscopy studies of cells individually or jointly infected with wild-type or rAAV2 and adenovirus suggest that AAV2 DNA is positioned differently within the nucleus, depending on the absence or presence of adenovirus (Weitzman *et al.*, 1996). These same studies also demonstrated the co-localization of wild-type or rAAV2 DNA with adenovirus replication centers (Weitzman *et al.*, 1996). Recent data show that the herpes simplex virus type 1 (HSV-1) replication protein, ICP8, is able to relocate AAV DNA to HSV replication domains, through an interaction with Rep78 bound to AAV DNA (Heilbronn *et al.*, 2003).

## Cellular functions

Overexpression of the cellular transcription factor, PC4, has been shown to enhance rescue of AAV3 (Muramatsu *et al.*, 1998). Cellular transcription factors SP1, YY1, and MLTF have also been implicated in the activation of AAV2 genes in the presence of helper virus (Chang *et al.*, 1989; Seto *et al.*, 1991, 1993; Shi *et al.*, 1991; Pereira and Muzyczka, 1997a,b). *In vitro* replication studies indicate that human DNA replication factors, replication protein A (a single-stranded DNA-binding protein), replication factor C (a primer-binding complex) and proliferating cell nuclear antigen (a processivity enhancing factor) are required for AAV2 replication (Ni *et al.*, 1998). The cellular DNA polymerase used for AAV replication has not yet been identified; however, human DNA polymerases  $\alpha$ ,  $\beta$  and  $\gamma$  have been ruled out by inhibitor studies (Ni *et al.*, 1998).

## Adenovirus helper functions

Although the genes, and in some cases the proteins, involved in providing the viral helper functions have been identified, the precise mechanisms of all aspects of the helper functions have not yet been elucidated. Some functions are inferred from the functions these proteins perform in the helper virus life cycle. The best-studied helpers are the adenoviruses, in particular serotypes 2 and 5 (Carter, 1990). An excellent review of early studies on adenovirus helper functions has already been published (Carter, 1990). These results are briefly summarized and more recent data discussed. Studies of adenovirus helper functions have generally used human or non-human primate cells that were either infected with AAV (usually AAV2) or transfected with an AAV infectious clone. These cells were then either infected with mutant adenoviruses, microinjected with crude or purified preparations of RNAs transcribed from various adenovirus genes, or co-transfected with DNA containing specific portions of the adenovirus genome (Carter, 1990).

The adenovirus type 5 *E1a*, *E1b*, *E2a*, *E4*, and VA RNA genes are sufficient to provide the AAV helper functions and can be supplied in plasmid form to promote rescue of an AAV2 genome from a second plasmid *in vivo* (Xiao *et al.*, 1998). Co-infection experiments have shown that mutation of any of these genes, within the context of an adenovirus genome, can result in a dramatic decrease in AAV infectious particle yield (Myers *et al.*, 1980; Ostrove and Berns, 1980; Janik *et al.*, 1981, 1989; Laughlin *et al.*, 1982; Carter *et al.*, 1983; Carter, 1990). Neither adenovirus late genes nor adenovirus DNA replication are required for efficient AAV productive infection (Ito and Suzuki, 1970; Ishibashi and Ito, 1971; Carter, 1990). The adenovirus *E1a* gene encodes proteins that positively regulate AAV promoters (Laughlin *et al.*, 1982; Chang *et al.*, 1989; Carter, 1990). *E1a* gene products appear to override inhibition of the AAV2 p5 promoter by cellular transcription factor YY1, allowing increased production of Rep68/78 (Shi *et al.*, 1991; Lewis *et al.*, 1995). The mechanism of positive regulation of the p19 and p40 promoters by *E1a* gene products may be indirect since, in the presence of adenovirus, AAV2 Rep proteins positively regulate the p19 and p40 promoters (McCarty *et al.*, 1991).

During an adenovirus infection, products of the *E1b* gene block p53-mediated apoptosis that would otherwise be induced by *E1a* gene products (Debbas and White, 1993), and are also involved in the inhibition of host protein synthesis (Babiss and Ginsberg, 1984; Babiss *et al.*, 1985). Mutational analyses suggest that the 55 kilodalton (kDa) protein encoded by *E1b* (also referred to as the E1b 58 kDa protein) may play a role (indirect or direct) in mRNA transport, translation, and stability (Babiss and Ginsberg, 1984; Pilder *et al.*, 1986; Carter, 1990). The E1b 55 kDa protein and a protein encoded by open reading frame 6 (ORF-6) of the *E4* gene interact with each other, as well as the apoptosis-regulating cellular transcription factor, p53

(Sarnow *et al.*, 1982, 1984; Carter, 1990; Leppard, 1997). A product of ORF-6 of the *E4* gene also stimulates second-strand synthesis of *rep*<sup>-</sup> rAAV DNA that enters the cell in a single-stranded state (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). This stimulation is enhanced by *E1* gene products (Fisher *et al.*, 1996) and may be mediated by regulation of dephosphorylation of a cellular protein that binds to the single-stranded portion of the ITR (Qing *et al.*, 1998). In addition, an adenovirus type 2 mutant with a deletion that includes part of the *E4* gene is defective in promoting DNA replication of wild-type AAV2 (Carter *et al.*, 1983).

The single-stranded DNA-binding protein encoded by the adenovirus *E2a* gene stimulates the processivity of AAV2 DNA replication *in vitro*; however, the human single-stranded DNA-binding protein, replication protein A, can substitute, but does not stimulate, replication as effectively as the *E2a* protein (Ward *et al.*, 1998). The *E2a* gene product may have additional functions, since mutations in this gene are also associated with defects in splicing of AAV mRNAs, capsid protein synthesis, and the accumulation of single-stranded AAV progeny DNA (Myers *et al.*, 1980; Jay *et al.*, 1981; Myers and Carter, 1981; Carter, 1990). Since single-stranded AAV progeny DNA is inserted into pre-formed capsids (Myers and Carter, 1980), it is possible that part of the defect in progeny DNA accumulation is due to the lack of capsid protein.

Adenovirus VA RNAs are not believed to encode polypeptides (Horwitz, 1985). The 5.5 s VA<sub>I</sub> RNA is believed to be the key VA gene product that provides an AAV helper function (Carter, 1990). VA<sub>I</sub> RNA is required for the efficient translation of adenovirus late mRNAs (Thimmappaya *et al.*, 1982). Co-transfection studies suggest that VA<sub>I</sub> RNA can stimulate translation of AAV RNA (West *et al.*, 1987). This stimulation is highly dependent on the host cell type (West *et al.*, 1987).

## Herpes virus helper functions

In spite of their name, several lines of evidence suggest that AAVs have evolved to function more intimately with herpes viruses than with adenoviruses. Although no extensive sequence homology has been found between AAV and adenovirus proteins, a subtype of human herpes virus 6 (HHV-6) encodes a Rep68/78 homologue (Thomson *et al.*, 1991). This homologue can actually substitute for Rep68/78 in AAV2 replication (Thomson *et al.*, 1994). It is hypothesized that the presence of this homolog in HHV-6 is the result of a recombination event between HHV-6 and an AAV during a co-infection. The genes of HSV-1 that provide AAV helper functions have been determined (Weindler and Heilbronn, 1991). Unlike adenoviruses, all of the HSV-1 genes that provide AAV helper functions encode proteins that are directly involved in HSV-1 DNA replication (Challberg, 1986; Wu *et al.*, 1988; Weindler and Heilbronn, 1991). These genes encode the helicase-primase complex (UL5, UL8, and UL52)

and the single-stranded DNA-binding protein (UL29) (Weindler and Heilbronn, 1991; Lehman and Boehmer, 1999). It should be recognized that these proteins may have additional functions. For example, as stated above, the UL29 gene product, ICP8, interacts directly with Rep78 and can reposition AAV DNA to herpes virus replication centers (Heilbronn *et al.*, 2003). *In vitro* studies suggest that the HSV-1 DNA polymerase (UL30 gene product) can use AAV2 DNA as a template, although it is not clear what proportion of AAV2 DNA replication is mediated by cellular polymerases in an AAV2/HSV-1 co-infection (Handa and Carter, 1979; Ward *et al.*, 2001).

## Proposed mechanisms of excision

In theory, there are two methods by which free AAV DNA can be generated from integrated AAV DNA. A copy of the integrated AAV genome could be made, using the integrated AAV genome as a template, or the actual integrated AAV DNA could be excised. Data from several systems is consistent with the latter method. For example, rescue of AAV2 DNA from a plasmid is associated with the generation of a linear plasmid backbone fragment containing only part of the AAV ITRs, but missing the central portion of the AAV genome (Ward and Berns, 1991). The ITRs do, however, appear to be at least partially duplicated during the excision process (Ward and Berns, 1991). Furthermore, inclusion of the AAV2 *rep* gene in a hybrid virus, containing a rAAV genome embedded within a baculovirus genome, can result in the packaging of baculovirus genomes with the region between the AAV2 ITRs deleted (Palombo *et al.*, 1998).

One early model of AAV2 genome excision invoked the formation of hairpins by spontaneous isomerization of the palindromic sequences of the ITRs, which were then recognized and cleaved by a combination of AAV2 Rep68/78 and cellular Holliday junction resolution enzymes to release the AAV2 genome (Ward and Berns, 1991). The requirement for hairpin formation was in part driven by the fact that it was not yet known that Rep68 and Rep78 could bind and nick linear DNA (Chiorini *et al.*, 1994; McCarty *et al.*, 1994b; Weitzman *et al.*, 1994; Urcelay *et al.*, 1995). A simpler model for Rep68/78-mediated excision is here proposed (Figure 17.3, p. 239). In this new model, Rep68/78 nick the linear duplex form of both ITRs.

Rep68/78 have the ability to unwind DNA that contains RRSs (Zhou *et al.*, 1999b). This unwinding appears to be a prerequisite for nicking at the TRS (Snyder *et al.*, 1993; Brister and Muzyczka, 1999; Davis *et al.*, 2000). It allows the region around the TRS to form a putative stem-loop structure that is recognized by Rep68/78 and cleaved (Brister and Muzyczka, 1999). Since Rep68/78 can covalently attach to the DNA at the nicking site, such a nick would not be easily repaired (Snyder *et al.*, 1990a; Urcelay *et al.*, 1995). Nearby spontaneous nicking by cellular enzymes, such as type 1 topoisomerases (Zhu and Schiestl, 1996) or endo R (Gottlieb

and Muzyczka, 1988), could make the other two strand breaks required for complete excision. It should also be noted that AAV2 Rep68/78 have been shown to interact with Topors, a topoisomerase I-binding protein (Weger *et al.*, 2002), so it is possible that Rep68/78 attract topoisomerase molecules to the ITR regions. It has also been reported that Rep protein-independent initiation of replication at an AAV2 ITR by N-methyl-N'-nitro-N-nitrosoguanidine, a DNA damaging agent, is highly sensitive to coumermycin A<sub>1</sub>: a topoisomerase I inhibitor (Yalkinoglu *et al.*, 1991). Eukaryotic type I topoisomerases cleave at a wide variety of sites with a consensus sequence of 5'-(A/T)(G/C)(A/T)T-3', in which cleavage would occur at the 3' side of the 3'-most T residue (Champoux, 2001). Several potential type I topoisomerase nicking sites have been found within AAVS1 and the AAV2 ITRs, near known AAVS1-AAV2 integration junctions (Kotin *et al.*, 1992).

The DNA unwinding mentioned above would tend to make the broken ends fall apart (Im and Muzyczka, 1990). The hypothesized 5' overhangs would then be filled in by a cellular DNA polymerase, in a fashion that would mimic the terminal resolution process during AAV productive infection (Snyder *et al.*, 1990a,b).

Since the newly replicated ends could each contain as many as two RRSs, the ends of each DNA would be separated by Rep68/78 (Zhou *et al.*, 1999b). Once the strands are separated, it is presumed that the affinity of Rep68/78 for the ITR would be reduced, since they only recognize the RRS within double-stranded DNA (Smith and Kotin, 2000). Given the palindromic nature of the ITRs (Srivastava *et al.*, 1983), and the relative lack of restraints on the ends, each strand of the ITR would fold into a hairpin (Ni *et al.*, 1998). One hairpin on each end of the liberated AAV genome would have a 3' end that could function as a primer for DNA replication in a fashion similar to that proposed for AAV productive infection (Snyder *et al.*, 1990b; Ni *et al.*, 1998). One round of replication of the flanking DNA might also occur, but since it would not contain a TRS, it would not be able to initiate a second round of replication as easily as the TRS-containing AAV DNA (Snyder *et al.*, 1990b).

This model correctly predicts the hairpin structures seen in both released AAV DNA and residual plasmid backbone after Rep68/78-mediated excision of AAV (Ward and Berns, 1991). This model also predicts the observation that excision can occasionally occur in the absence of Rep proteins if, by random chance, cellular enzymes make the four cleavage events required for excision. Such excision has been observed, mediated by endonuclease R (Gottlieb and Muzyczka, 1988), which cuts G-rich sequences similar to those found within the ITRs (Gottlieb and Muzyczka, 1990). Such excision has limited potential for replication without the Rep proteins.

In addition, this model explains why AAVS1-AAV junction sequences are sometimes found in packaged AAV2 (Hüser *et al.*, 2003). During a standard 48–72 hours co-infection of AAV2 with a helper virus, there would be time

for the AAV2 genome to integrate into *AAVS1* and be rescued (Hüser *et al.*, 2002). If the cellular enzymes cut within the flanking *AAVS1* region, a portion of *AAVS1* would be excised along with the AAV genome. Even though such a hybrid virus may not replicate efficiently, it may still be packaged (Zhou and Muzyczka, 1998; King *et al.*, 2001).

## Questions to be resolved

A major question in AAV biology is: what principles of latency and rescue, derived primarily from studies on AAV2, will be true for most or all AAVs? Over 30 AAV types have been identified in humans, non-human primates, and birds (Atchison *et al.*, 1965; Blacklow *et al.*, 1967; Yates *et al.*, 1973; Bantel-Schaal and zur Hausen, 1984; Gao *et al.*, 2002, 2003). For example, do all AAVs, even those whose Rep proteins recognize a different nicking site, integrate preferentially?

Expression of the AAV4 Rep68 protein, which recognizes a sequence very similar to the AAV2 RRS/TRS complex, can direct preferential integration of rAAV2 DNA into the monkey homolog of *AAVS1* (Amiss *et al.*, 2003). On the other hand, AAV5 Rep68/78 nicks at a different sequence to AAV2 Rep68/78, so it is unlikely that wild-type AAV5 integrates at *AAVS1* (Chiorini *et al.*, 1999a). It is possible that there is no human sequence into which AAV5 can integrate preferentially. It is unclear whether the hypothetical lack of a preferred integration locus would favor dispersed integration or episomal persistence of wild-type AAV5.

It should also be noted that an early study showed that AAV1 could be rescued by helper virus (SV15) infection up to six cell passages (approximately 10 days per passage) after infection of LLC-MK<sub>2</sub> cells (a monkey kidney cell line) with AAV1, but not after seven passages (Casto *et al.*, 1967). Their results were consistent with the dilution of input AAV1 with cell division (Casto *et al.*, 1967). Since the latently infected cells were not clonally isolated, such dilution could have resulted from either a lack of integration or slower cell division by cells carrying AAV1 proviruses, resulting in overgrowth by uninfected cells.

The genome of minute virus of mice, a distantly related parvovirus, will integrate preferentially into an episome containing a binding and nicking site for its homolog of the AAV Rep68/78 proteins (Corsini *et al.*, 1997). It is therefore not too great a leap to speculate that any *rep*<sup>+</sup> AAV will preferentially integrate into any chromosomal location that has a good binding and nicking site for its Rep68/78 proteins.

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# Parvovirus RNA processing strategies

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Over the past few years, the transcription profiles of many of the parvoviruses have been determined at a detailed level. In general, these profiles are similar, but also display many variations on a theme. In each case, parvoviruses have adapted a complex pattern of alternative splicing and polyadenylation to maximize the information from their small genomes. These complex patterns of expression generally result in a set of mRNAs that express two to three capsid proteins from overlapping open reading frames (ORF), and one large and two to three small non-structural proteins. In addition, adeno-associated virus (AAV) gene expression has evolved to be dependent on helper-virus functions.

Most alternative processing of parvovirus pre-mRNAs is constitutive, and even minor variations in the levels of the various final products can have dramatic effects on virus replication. However, there have been reported examples, primarily with the human parvovirus B19, in which these events are regulated in a cell-type specific manner and have been implicated in viral tropism.

Thus far the only parvovirus protein that has been shown to be required for efficient RNA processing is the AAV2 Rep78/68 protein. Whether this role is direct, or facilitates the function of Ad gene products in this regard is not known.

Although the characterization of parvovirus alternative splicing and polyadenylation has been extensive, very little is known about the export of parvovirus RNAs. Two interesting cases highlight the importance of this process to parvovirus biology. The cytoplasmic export of single-spliced NS1-encoding MVM RNAs prevents their further splicing to NS2-encoding R2, and thus export helps govern the relative ratio of the two non-structural proteins. In the case of AAV2, two critical Rep proteins (Rep78 and Rep52), are generated from completely unspliced RNAs, which are exported to the cytoplasm at high efficiency.

Parvovirus RNA processing is an excellent model to study alternative RNA processing of complex overlapping transcription units in a small but complete biological system. These investigations have also yielded insight into the basic relationship between splicing and polyadenylation, and into the co-transcriptional nature of these processes.

## **PARVOVIRUSES OF NON-PRIMATE MAMMALS (*PARVOVIRUS* AND *AMDOVIRUS*)**

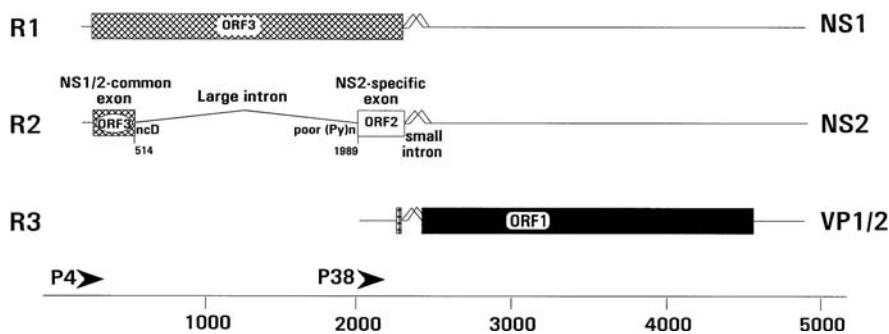
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### ***Rodent parvoviruses (genus Parvovirus)***

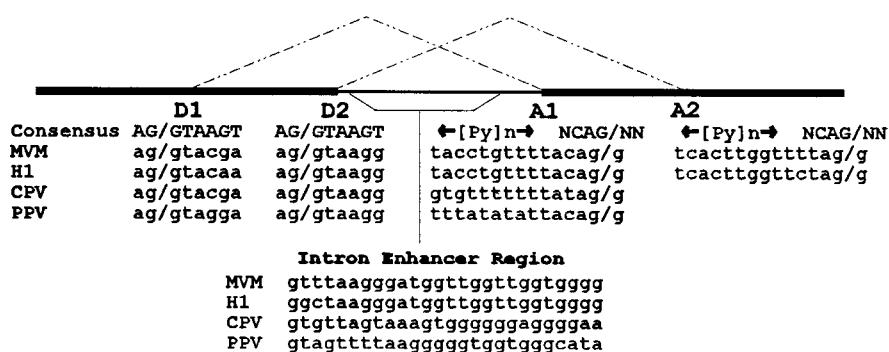
The rodent parvoviruses minute virus of mice (MVM) and H1 have very similar transcription profiles. The most extensive work in this area has been done for MVM (previously reviewed in Pintel *et al.*, 1995), which we will describe in detail (see Figure 18.1). The transcription maps of feline/canine parvovirus (FPV/CPV), porcine parvovirus (PPV) and Aleutian mink disease parvovirus (AMDV) show differences from the MVM model and will be discussed below.

### **GENERAL TRANSCRIPTION ORGANIZATION OF MINUTE VIRUS OF MICE**

MVM RNAs are generated from two promoters, one at map unit 4 (the RNA initiation site is at approximately nucleotide 201) and one at map unit 38 (the RNA initiation site is at approximately nucleotides 2005–2010; reviewed in Pintel *et al.*, 1995). (Nucleotide numbers refer to GenBank accession number NC\_001510.) All MVM RNAs are polyadenylated at the far right-hand end of the genome, approximately 16–20 nucleotides downstream of the final AAUAAA motif that lies at nucleotide 4885 for



**Figure 18.1** Transcription map of MVM. The three major transcript classes (R1, R2, R3) are shown relative to the 5 kb genome diagrammed below. The proteins that they encode are listed on the right of each transcript, and the open reading frames (ORFs) used for each are indicated. The small intron in the center of the genome is spliced using two donors and two acceptors as discussed in the text. The locations of the promoters (P4 and P38) are indicated. The large intron is excised using a non-consensus donor (ncD), and a poor polypyrimidine tract ([Py]n) at its 3' splice site. The different ORFs that are used are shown in different shading patterns.



**Figure 18.2** Nucleotide sequence of the small intron donors, acceptors, and intron splicing enhancer motif (IES) of various parvoviruses. The nucleotide sequences of the donor and acceptor sites for the small intron of MVM H1, CPV, and PPV are shown, relative to published consensus sequences. Also shown is the homology to the experimentally defined IES of the MVM small intron. CPV and PPV use a single acceptor for small intron excision. (Py)n indicates the polypyrimidine region of the 3' splice sites.

MVMp and nucleotide 4821 for MVMi (Clemens and Pintel, 1987). All MVM pre-mRNAs contain a small intron in the center of the genome that is alternatively spliced using two donors and two acceptors (Jongeneel *et al.*, 1986; Morgan and Ward, 1986; Cotmore and Tattersall, 1990; Clemens *et al.*, 1990) and a portion of the P4-generated R1 RNAs are additionally spliced between nucleotides 514 and 1989 to generate R2 (Cotmore *et al.*, 1983; Pintel *et al.*, 1983; Jongeneel *et al.*, 1986). Polyadenylation of MVM RNAs precedes splicing in the nucleus (unspliced polyadenylated molecules can be detected; Clemens and Pintel, 1988) and there is no detectable accumulation of unspliced MVM RNAs in the cytoplasm of infected cells (Clemens and Pintel, 1988). There is temporal phasing to the production of MVM RNA. Products of the P4 promoter, R1 and R2, which encode the non-structural proteins NS1 and NS2, are generated prior to the R3 RNAs produced from P38, which encode the capsid proteins VP1 and VP2 (Clemens and Pintel, 1988). MVM mRNAs are very stable (>6 hours) in infected cells (Schoborg and Pintel, 1991) and can comprise close to

25 percent of the total mRNA at late times following infection (Pintel *et al.*, 1983).

#### P38-GENERATED RNAs (R3)

The P38-generated pre-mRNAs (R3) are alternatively spliced to generate two mRNAs, which separately encode the two capsid proteins VP1 and VP2 (Jongeneel *et al.*, 1986; Labieniec-Pintel and Pintel, 1986; Clemens *et al.*, 1990). Therefore, alternative splicing of the P38-generated pre-mRNA governs the relative ratio of accumulation of the capsid proteins during infection (see Figure 18.2). RNAs that splice using the first donor and first acceptor (between nucleotides 2280 and 2377; D1-A1) are predominant and have been given the M (major) designation (in this case R3M; Clemens and Pintel, 1988; Schoborg and Pintel, 1991). The VP2 protein is translated from this message, beginning at an AUG codon at nucleotide 2794 (Labieniec-Pintel and Pintel, 1986). P38-generated pre-mRNAs are also spliced between the second donor and second acceptor

(between nucleotides 2317 and 2399; D2-A2; Jongeneel *et al.*, 1986; Morgan and Ward, 1986; Clemens *et al.*, 1990). These RNAs have been given the m (minor) designation (R3m). This RNA encodes the VP1 protein from an AUG codon at nucleotide 2286 (Labieniec-Pintel and Pintel, 1986). R3 pre-mRNAs that splice using D1 and A2 have also been identified (Morgan and Ward, 1986). This species, designated R3r (rare), would be predicted to encode VP2. The major D1-A1, minor D2-A2, and rare D1-A2 splicing patterns are found, respectively, in approximately 70 percent, 25 percent and 5 percent of all classes of MVM RNAs (Morgan and Ward, 1986; Schoborg and Pintel, 1991). No splicing is detected between D2 and A1 for any MVM-generated pre-mRNA, probably, at least in part, because the intron is very small (60 nucleotides; Morgan and Ward, 1986; Schoborg and Pintel, 1991).

#### **REGULATION OF SPLICING OF THE SMALL INTRON FROM P38-GENERATED PRE-mRNAs**

The determinants that govern the alternative excision of the small intron from P38-generated pre-mRNAs are complex (Haut and Pintel, 1998, 1999). Factors that govern splicing of the small intron include similarity to consensus of the donors and acceptors, the position of the two donors relative to each other, the position of the two acceptors relative to each other, the size of the intron, and the presence of an intronic splicing enhancer sequence (Haut and Pintel, 1998). R3M + R3r, which both use D1 and are predicted to encode VP2, and R3m, which uses D2 and encodes VP1, comprise approximately 75 percent and 25 percent of P38-generated mRNA, respectively (Schoborg and Pintel, 1991). Interestingly, while VP1 comprises approximately 25 percent of the total capsid protein in infected cells, reflecting the relative levels of the R3m mRNA, it only makes up approximately 10 percent of the total protein in viral capsids (Tattersall *et al.*, 1977), suggesting there may exist an additional constraint on the morphogenesis of the MVM virion.

The two donor sites of the MVM small intron compete for the splicing machinery: experiments in which the donor sites were exchanged have shown that the position of D1 favors its usage, while the primary sequence of D2 must be more like the consensus sequence than the upstream donor to be used efficiently (Haut and Pintel, 1998). However, recognition of the elements comprising the small intron acceptors is likely to be the dominant determinant in alternative small intron excision (Haut and Pintel, 1998, 1999). Although the downstream acceptor position seems to be preferred, the splicing machinery preferentially uses A1, which has a better polypyrimidine tract (PPT), two functional potential branch points, and a more consensus 3' acceptor (Haut and Pintel, 1998). Experiments in which the acceptor signals have been switched have shown that the sequence of A1 must be stronger than that of A2 for it to be used more efficiently than A2 (Haut and Pintel, 1998). Recognition of A1 by splicing factors is a critical determinant

that leads to preferential use of D1 and generation of the major D1/A1 spliced form, since D2 is too close to be efficiently spliced to A1 (Haut and Pintel, 1998, 1999).

Splicing of the minor spliced form D2/A2 requires the presence of an ISE downstream of D2 (Haut and Pintel, 1998, 1999). The relatively greater strength of A1 seems to impose a requirement for the IES on A2, and consequently, on production of the minor spliced form. This was concluded because, when the sequence of A2 was improved, usage of A1 was decreased and usage of the downstream acceptor no longer required the IES (Haut and Pintel, 1998). Similarly, debilitation of A1 also rendered usage of the downstream acceptor independent of the IES (Haut and Pintel, 1998).

The small intron appears to be defined primarily by an 'intron definition' model, in which all the elements that control its excision are contained wholly within the intron (Haut and Pintel, 1998). Consistent with this hypothesis, increasing the size of the intron to a length no longer compatible with intron-defined excision was shown to change the relative use of the donors and acceptors (Haut and Pintel, 1998, 1999). Because the small intron defines the 3' border of the NS2-specific exon, it also plays an important role in definition of the NS2-specific exon, and hence excision of the large intron (Haut and Pintel, 1999). Increasing the size of the small intron diminishes its help in this regard (Haut and Pintel, 1999).

#### **NS2-EXON DEFINITION AND EXCISION OF THE LARGE UPSTREAM INTRON**

Splicing of the large intron from P4-generated pre-mRNAs is a complicated process that involves small intron excision, NS2-specific exon definition, and selective RNA export (Zhao *et al.*, 1994, 1995a,b; Gersappe and Pintel, 1999; Haut and Pintel, 1999). The relative rates that these RNAs are processed help determine the relative ratio of the non-structural proteins NS1 and NS2. At steady-state levels, there is approximately twice the amount of R2 as R1 in MVMP-infected murine cells (Schoborg and Pintel, 1991). Alternative splicing of the small intron does not affect expression of NS1, which terminates prior to D1. However, alternative splicing of R2 RNAs at the small intron generates three NS2 isoforms that vary at their COOH termini (Clemens *et al.*, 1990; Cotmore and Tattersall, 1990).

All P4-generated RNAs that are found in the cytoplasm are spliced at the small intron; no unspliced P4-generated RNAs are exported from the nucleus of infected cells (Clemens and Pintel, 1988). Following small intron splicing, a portion of the P4-generated RNAs are exported from the nucleus (comprising the R1 class of MVM RNAs); however, a portion is spliced again, removing the large intron before export. These are the R2 mRNAs. Export of R1 from the nucleus thus prevents further splicing to R2. The determinants that govern export of R1 versus its nuclear retention and further splicing to R2 – a critical feature in determining

the ultimate accumulated levels of NS1 and NS2 – is not known. However, it seems at least in part to be related to the efficiency of excision of the large intron. When splicing of the large intron is artificially made more efficient, less mature R1 accumulates in the cytoplasm (Choi and Pintel, unpublished). The large intron has both a non-consensus 5' donor site and a poor 3' acceptor. If either of these signals is improved, large intron excision is considerably more efficient (Zhao *et al.*, 1995a). Excision of the wild-type large intron, however, can best be understood based on exon definition models (see Figure 18.3).

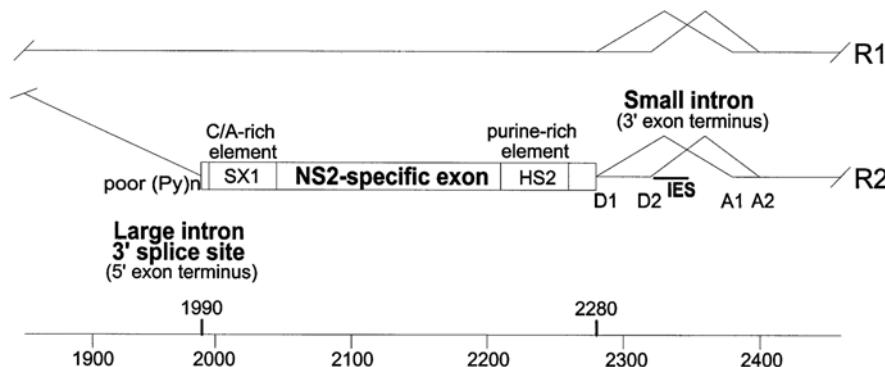
As mentioned above, the first step in splicing of the P4-generated pre-mRNAs is likely to be splicesomal engagement and excision of the small intron to generate R1. This has been inferred because no molecules in which only the large intron has been excised are detected in infected cells (Clemens and Pintel, 1988). Subsequent splicing of the large intron, from a subset of spliced R1 molecules, requires the definition of the NS2-specific exon, which is flanked by a poor 3' splice site at its upstream border (Zhao *et al.*, 1995b; Gersappe and Pintel, 1999). Efficient recognition of this 3' splice site by the splicing machinery requires an exon splicing enhancer (ESE) that lies within the NS2 specific exon (Zhao *et al.*, 1995b; Gersappe and Pintel, 1999). The NS2-specific ESE is bipartite: mutation of either element alone has only a minor effect on excision of the upstream large intron (Gersappe and Pintel, 1999). Mutation of both elements, however, leads to loss of definition of the NS2-specific exon with its subsequent skipping: almost all of the P4-generated RNA in such ESE mutants is spliced between the large intron donor and the small intron acceptor A1 (Gersappe and Pintel, 1999). That these mutations can be suppressed by improvement of the polypyrimidine tract of the large intron 3' splice site suggests that the ESE acts to strengthen this site (Zhao *et al.*, 1995b; Gersappe and Pintel, 1999). The sequence within the NS2-specific exon is tightly constrained. In addition to a sequence-specific ESE, this region

is translated in two ORFs. The splicing factor(s) that bind(s) to the NS2-specific ESE has not yet been identified.

As well as defining differences in their capsid sequence, the two allotropic variants of MVM, MVMP and MVMi, have different 3' splice sites, which affect the splicing of the large intron (Gardiner and Tattersall, 1988). The 3' splice site of MVMP is much more efficient in mouse fibroblasts than the 3' splice site of MVMi. This difference can be attributed to an A residue at nucleotide 1970 in MVMP, which may aid branch point selection, rather than the G residue found in MVMi (Choi, Burger and Pintel, unpublished). The 3' splice site of MVMi is as efficient in murine lymphocyte cell lines, as is the 3' splice site of MVMP in murine fibroblasts (Choi, Burger and Pintel, unpublished).

Definition of exons also requires a strong downstream 5' donor site. In the case of the NS2-specific exon, the strength, or efficiency, of usage of the small intron donors (which constitute the 3' end of the NS2-specific exon), is dependent on the complex regulation of the small intron (Haut and Pintel, 1999). Mutation of the small intron ISE, or expansion of the small intron in such a way that small intron excision is altered, results in loss of NS2-specific exon definition and so P4-generated mRNAs are spliced from the large intron donor to the small intron acceptor A1, skipping the NS2-specific exon (Haut and Pintel, 1999).

Although MVM (and in fact all the rodent parvoviruses) use a non-consensus large intron 5' donor site, each has a strongly consensus 5' donor site four to five nucleotides downstream of this site that is not used. Use of this downstream donor would join the NS2-specific exon out of frame, and an understanding of the molecular constraints that prevent the use of this seemingly more favorable site will be of general interest. Recent evidence has suggested that both an ESE in the upstream NS1/NS2-shared exon as well as intronic sequences downstream of the donor governs usage of the non-consensus large intron donor (Choi and Pintel, unpublished).



**Figure 18.3** Detailed map of the MVM NS2-specific exon. The region of the NS2-specific exon in both R1 and R2 is shown, relative to the genome below. The large intron 3' splice site, and the small intron donors, which comprise the 5' and 3' termini of the NS2-specific exon, respectively, are shown. Also shown are the SX1 C/A-rich and HS2 purine-rich elements of the NS2-specific exon bipartite exonic splicing enhancer. In addition, the small intron intronic splicing enhancer (IES), as well as the location of the poor polypyrimidine tract (Py)n of the large intron 3' splice site are shown.

## **Porcine (PPV) and canine/feline parvoviruses (CPV/FPV; genus Parvovirus)**

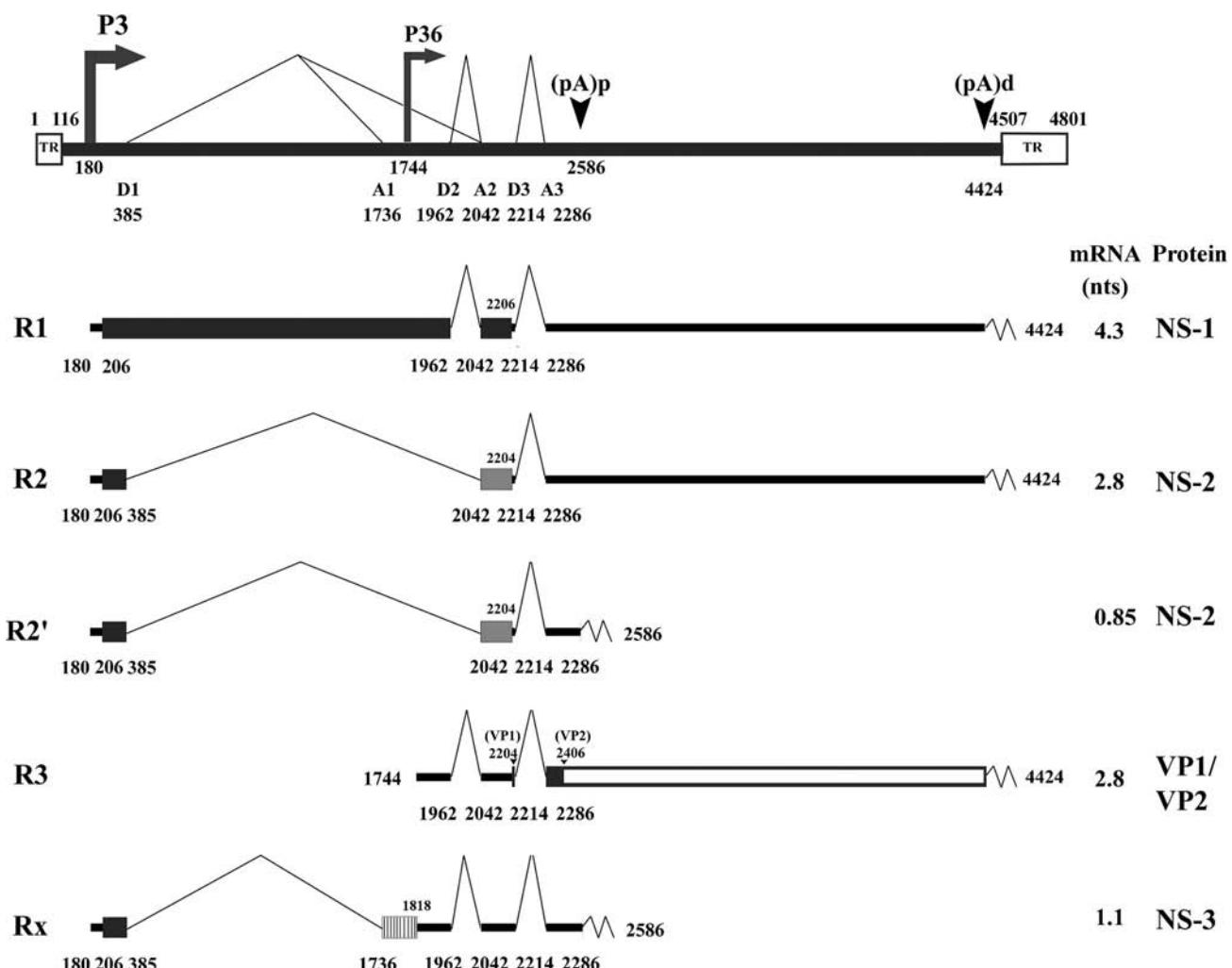
A detailed picture of the primary structure of the mRNAs generated by PPV has been obtained (Bergeron *et al.*, 1993); however, the relative steady-state abundances of these mRNAs has not yet been fully determined. In contrast to MVM, the PPV small intron has two donor sites and only a single acceptor site (see Figure 18.2). Both the NS1 and NS2 coding regions of PPV terminate prior to the small intron splice sites and so alternative splicing of the small intron affects only production of the capsid proteins. Perhaps the most striking difference between the splicing pattern of MVM and PPV, however, is that PPV has been shown to encode an mRNA (R3), which joins the large intron donor to the single small intron acceptor, skipping the NS2-specific exon. The small non-structural protein NS3, which is encoded by the PPV R3 mRNA, may be required to compensate for the absence of multiple isoforms of PPV NS2 (Bergeron *et al.*, 1993). Since an mRNA analogous to the PPV R3 message is not produced by other parvoviruses in this group and because its only identification has been by the cloning of PCR-generated fragments, it bears additional validation by other laboratories. For MVM, an mRNA in which the NS2-specific exon is skipped is not seen during infection, but as described above, is generated after mutagenesis of the small intron donors or the NS2-specific exon itself. Both the PPV large intron donor (AC/GGCAAG) and 3' polypyrimidine tract (TAAATACACCAACAG/AC) are further from consensus than the comparable MVM sequences and, as is the case for MVM, the PPV small intron acceptor appears significantly stronger than the PPV large intron acceptor. Finally, the PPV NS2-specific exon is only 75 percent the size of the MVM NS2-specific exon and computer-aided structural predictions (Jacobson and Zuker, 1993) of the sequences within this exon are quite different from the other RV-like parvoviruses. Some or all of these features probably favor the skipping of the NS2-specific exon of PPV.

CPV is similar to PPV in that its small intron uses two donors and a single acceptor (Wang *et al.*, 1998). The CPV donors are identical to MVM; however, in contrast to MVM, the polypyrimidine tract of the CPV 3' small intron splice site is very strong, relative to the 3' splice site of the large intron. Therefore, while MVM, H1, CPV, and PPV have similarly consensus small intron donor sites, their small intron acceptor sites – and thus the relative strength of these sites compared with their large intron 3' splice sites – vary significantly. Interestingly, although R2-like molecules can be detected, a role for the NS2 protein during CPV infection has not yet been demonstrated (Wang *et al.*, 1998). Accumulation of RNAs generated by the related mink enteritis virus (MEV) during a highly synchronous infection of Crandell feline kidney (CRFK) cells is qualitatively and quantitatively similar to the profile seen for MVM (Storgaard *et al.*, 1997).

## **Aleutian mink disease parvovirus (AMDV; genus Amdovirus)**

Aleutian mink disease parvovirus has a more complex transcription profile than other parvoviruses of non-human mammals (Alexandersen *et al.*, 1988; Storgaard *et al.*, 1997; see Figure 18.4). Two promoters have been identified, at map unit 3 and map unit 36; however, the splicing and polyadenylation of AMDV RNA is quite different from other autonomous parvoviruses (Alexandersen *et al.*, 1988; Storgaard *et al.*, 1997). P3- and P36-generated transcripts, which terminate near the right hand end of the molecule (AMDV also uses an internal polyadenylation site as described below), are spliced in a complex manner. The P3-generated 4.3 kb RNAs, which encode the NS1 protein, excise two small introns and hence use an additional internal exon, in the center of the genome (Alexandersen *et al.*, 1988; Storgaard *et al.*, 1997). The P3-generated 2.8 kb RNAs, which have been reported to encode the AMDV NS2 protein, join a donor at nucleotide 384 to the first small intron acceptor (nucleotide number refers to GenBank accession number M20036). The small internal exon is translated in different reading frames for NS1 (frame 1) relative to NS2 (frame 3). The RNAs reported to encode the capsid proteins are initiated at P36 prior to the two small introns, both of which are spliced from these RNAs. VP1 is initiated at nucleotide 2204, which would be from the 8th AUG in the R3 mRNA in the small internal exon and VP2 is initiated from an AUG further downstream. The mechanism that governs the relative use of these initiation codons, and thus determines the relative levels of these capsid proteins, has not been characterized (Alexandersen *et al.*, 1988). The P3-generated 2.8 kb mRNAs make up the majority of the viral RNA in infected CRFK cells and these RNAs could also theoretically encode the AMDV capsid proteins. AMDV does not exhibit the dramatic increase in levels of P36-encoding RNAs as infection proceeds, typical of the other autonomous parvoviruses. This observation is consistent with analysis of the relative poor strength of the AMDV P36 promoter and its relative inability to be trans-activated and thus has been suggested to contribute to the latent pathogenesis of AMDV infection (Alexandersen *et al.*, 1988; Storgaard *et al.*, 1993; Storgaard *et al.*, 1997).

In addition to these transcripts, two additional AMDV-generated RNAs, which use a proximal polyadenylation site at nucleotide 2586, have been identified. These RNAs are spliced between the large intron donor at nucleotide 385 and an acceptor prior to the first small acceptor at nucleotide 1736 (the 1.1 kb RX RNA), or between the nucleotide 385 donor and the acceptor of the first small intron at nucleotide 2042 (the 0.85 R2' RNA; Alexandersen *et al.*, 1988; Storgaard *et al.*, 1997). The R2' and RX RNAs were found to be quite abundant and R2' is predicted to encode an NS2 molecule identical to that encoded by the R2 RNA. This NS2 molecule is smaller than NS2 molecules of the rodent viruses (Alexandersen *et al.*, 1988). The RX molecule is translated



**Figure 18.4** Transcription map of Aleutian mink disease parvovirus (AMDV). The AMDV genome is depicted on the top of the figure. The promoters (P3 and P36), splice donors (D) and acceptors (A), polyadenylation sites ([pA]*p* and [pA]*d*), translation initiation sites and terminal palindromes (TR) are shown. The individual RNAs that have been detected, and the proteins they are predicted to encode are also shown. The different open reading frames (ORFs) that are used are shown in different shading patterns.

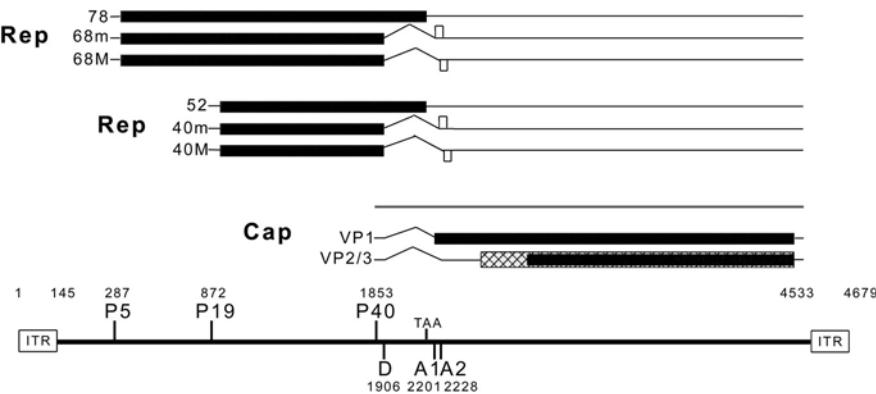
in ORF 1 prior to the first small intron (NS1 is encoded in ORF 3 in this region) and would be predicted to encode a unique small non-structural protein (NS3; Alexandersen *et al.*, 1988). In contrast to AAV5 and B19 (see below), unspliced RNAs from P3 and P36 are apparently not polyadenylated at the proximal site, even though this polyadenylation signal is consensus and is used efficiently for the R2' and RX RNAs.

## PARVOVIRUSES OF HUMANS AND NON-HUMAN PRIMATES

### Dependoviruses

AAVs have been isolated from multiple species including humans and other primates (Parks *et al.*, 1967; Blacklow

*et al.*, 1968; Bantel-Schaal and zur Hausen, 1984; Georg-Fries *et al.*, 1984; Gao *et al.*, 2002), birds (Yates *et al.*, 1973), bovine (Coria and Lehmkuhl, 1978), sheep (Clarke *et al.*, 1979), and snakes (Farkas *et al.*, 2004). Survey of the transcriptional profiles of human AAV serotypes 1–6 has shown that, with respect to their transcription profiles, these viruses fall into two main groups (Qiu and Pintel, unpublished). The first group, for which AAV2 can be taken as the prototype, includes AAV1, AAV2, AAV3, AAV4, and AAV6. They all possess similar large (approximately 320 nucleotides) introns and a non-consensus donor and acceptor site. The RNAs generated from these viruses all polyadenylate only at a site at the right-hand end of the genome (although they all contain a cryptic AAUAAA site within their intron), and they show a variable dependence on helper viruses. AAV2 is very dependent on both helper virus and Rep for proper RNA processing and high levels of



**Figure 18.5** Transcription map of AAV2. The AAV2 genome is depicted at the bottom of the figure, and includes the location of the viral promoters ( $P_5$ ,  $P_{19}$ ,  $P_{40}$ ), the small intron donor ( $D$ ) and acceptors ( $A_1$  and  $A_2$ ), the termination site for the Rep proteins, and the inverted terminal repeats (ITRs). The major transcript classes, and the proteins that they encode are shown above. The different open reading frames (ORFs) that are used are shown in different shading patterns.

transcription initiation and AAV3 and AAV4 are similar. AAV1 and AAV6, however, generate relatively low levels of spliced RNA products and are significantly less responsive to Ad5 helper functions for enhancement of RNA processing. The second main group, comprising AAV5, Avian-AAV and goose parvovirus (GPV), use an internal polyadenylation site within the central viral intron. Plasmid clones of these viruses express constitutively higher levels of spliced RNAs and so are less dependent on Ad5 or HSV helper virus, or Rep, for these aspects of gene expression. They will be described in detail below. Although portions of the genomes of AAV7 and AAV8 have been isolated from monkey tissue (Gao *et al.*, 2002), infectious virus has not yet been characterized.

#### AAV2-LIKE ADENO-ASSOCIATED VIRUSES

**General transcription organization of AAV2.** The transcriptional organization of AAV2 has been previously reviewed (Carter *et al.*, 1990; Muzychka and Berns, 2001). The AAV2 genome has three different promoters identified by their map positions of  $P_5$ ,  $P_{19}$ , and  $P_{40}$  (Green and Roeder, 1980; Lusby and Berns, 1982; see Figure 18.5). All of the AAV2 transcripts contain a single intron, which uses a 5' splice donor at nucleotide 1906, and one of either two splice acceptors at nucleotide 2201 ( $A_1$ ) or nucleotide 2228 ( $A_2$ ) (see Carter *et al.*, 1990). Unspliced  $P_5$ - and  $P_{19}$ -generated RNAs encode Rep78 and Rep52, respectively, while spliced  $P_5$ - and  $P_{19}$ -generated RNAs encode Rep68 and Rep40. Alternative splicing to either  $A_1$  or  $A_2$  generates two isoforms each for Rep68 and Rep40; however, individual functions for the different isoforms generated from RNAs using these different acceptors has not yet been reported. Spliced  $P_{40}$ -generated RNAs using the  $A_1$  acceptor encode the VP1 capsid protein, while spliced RNAs using the  $A_2$  acceptor encode VP2 and VP3 (Trempe and Carter, 1988a). Unspliced  $P_{40}$  mRNAs express very little capsid proteins even though they contain the entire VP1 ORF (Becerra *et al.*, 1988). This may be because there are a number of short

ORFs within the unspliced  $P_{40}$  transcripts that are available and may be used instead (Carter *et al.*, 1990). Recombinant AAV vectors have been shown to express transcripts generated from the AAV2 ITR (Flotte *et al.*, 1992, 1993; Haberman *et al.*, 2000) and a similar set of ITR-initiated transcripts has been detected at low levels during AAV2 infection (Nayak, Tullis and Pintel, unpublished). The biological function of these ITR-initiated transcripts remains to be defined. AAV2 gene expression is tightly controlled by both the AAV-encoded Rep proteins (Qiu and Pintel, 2002) and co-infecting helper virus (see Carter *et al.*, 1990).

Similar to the autonomous parvovirus MVM, there is a temporal order to the appearance of AAV2 RNAs during infection (Labow *et al.*, 1986; Trempe and Carter, 1988a; Mouw and Pintel, 2000). Unspliced  $P_5$ -generated transcripts are detectable prior to the significant accumulation of other AAV2 RNAs. Ultimately,  $P_{19}$ -generated RNAs accumulate to levels greater than those generated from  $P_5$ , and  $P_{40}$ -generated transcripts come to predominate in the total RNA pool. In addition, the percentage of each class of AAV2 RNA that is spliced increases during infection (Mouw and Pintel, 2000). The degree of this increase is different for the  $P_5$  and  $P_{19}$  RNA compared with those generated by  $P_{40}$ . At late times post infection, 90 percent of  $P_{40}$  RNAs, but only 50 percent of RNA generated by  $P_{19}$ , and <10 percent of RNAs generated by  $P_5$ , are spliced. Surprisingly, therefore, the same AAV2 intron is excised to different final levels from those different RNA species. In addition, it has been observed that the ratio of splicing to  $A_2$  relative to  $A_1$  also increases as AAV2 infection progresses; however, as noted above, the functional relevance of this is not clear (Mouw and Pintel, 2000). All AAV2 RNAs have been shown to be quite stable during infection (Mouw and Pintel, 2000).

#### AAV2 RNA SPLICING

Pre-mRNA splicing is a critical determinant for productive AAV infection. Although unspliced RNAs migrate to the cytoplasm and unspliced  $P_5$ - and  $P_{19}$ -generated RNAs are

translated into essential viral proteins (see below), the ratio of the viral capsid proteins is determined by alternative splicing and the ratio of Rep78 and Rep52 versus Rep68 and Rep40 proteins depends upon the level of splicing of the P5 and P19-generated RNAs (Carter *et al.*, 1990; Muzyczka and Berns, 2001). Splicing of AAV2 pre-mRNAs requires the participation of both the helper virus (e.g. adenovirus; Myers *et al.*, 1980; Trempe and Carter, 1988b; Berns and Giraud, 1996; Mouw and Pintel, 2000), or herpes virus (Buller *et al.*, 1981; Mishra and Rose, 1990; Mouw and Pintel, 2000), and the large Rep proteins (Qiu and Pintel, 2002). As mentioned above, the majority of P40-generated RNAs are spliced in the presence of adenovirus; however, the steady-state ratios of spliced to unspliced RNAs generated by the P5 and P19 promoters are much less.

**Helper virus function in AAV2 RNA splicing.** Co-infection of cells with AAV2 and either adenovirus or herpes virus stimulates the splicing of AAV2 RNA, but this is manifest only in the presence of Rep. In the absence of helper virus, splicing of both P40- and P19-generated transcripts produced following plasmid transfection is very poor (Carter, 1990; Mouw and Pintel, 2000). Adenovirus clearly has multiple roles in supporting AAV2 infection (Carter, 1990; Berns, 1996; Berns and Giraud, 1996). Five adenovirus gene products (E1A, E1B, E2a, E4orf6, and VA RNA) have been shown to be required for productive AAV2 replication and recombinant virus production (Janik *et al.*, 1981; Richardson and Westphal, 1981; Samulski and Shenk, 1988; Seto *et al.*, 1991; Ferrari *et al.*, 1996; Ward *et al.*, 1998); 293 cells, which express the adenovirus *E1A* and *E1B* genes, provide the necessary helper environment for AAV2 replication when transfected with a plasmid that expresses the adenovirus gene products E2A, E4, and VA (e.g. pXX6; Carter, 1990; Xiao and Samulski, 1998). Transfection of 293 cells with plasmid pXX6 is as effective as adenovirus co-infection in stimulating the splicing of AAV2 RNAs generated by co-transfection of either an infectious clone, or an ITR-minus AAV2 RepCap plasmid (Qiu and Pintel, unpublished). Expression of either the E2A, E4, or VA genes individually does not enhance splicing of AAV2 RNA to the same levels reached by adenovirus infection or pXX6 expression in 293 cells (Qiu and Pintel, unpublished). How these adenovirus gene products function, in conjunction with Rep, to enhance AAV RNA splicing is not clear. The herpes virus UL5, UL8, UL52, and UL29 gene products can fully support AAV2 replication (Weindler and Heilbronn, 1991). That successful recombinant AAV production can be achieved using those herpes gene products suggests that they can, along with Rep, efficiently enhance splicing of the P40-generated RNA. However, a direct role for these gene products in enhancement of AAV2 RNA splicing has not yet been shown. Comparison of the role of adenovirus and herpes gene products in AAV2 RNA splicing should reveal interesting insights into this aspect of the helper function.

It is interesting that certain features of the AAV2 transcription unit, including the intron and the polyadenylation site, need to be present for the splicing of AAV2 RNA to be

responsive to helper virus gene products. Specifically, when the AAV2 capsid-gene transcription unit is driven by the HIV or cytomegalovirus (CMV) promoters, or when the AAV2 intron within the P40-driven transcription unit is replaced with an HIV intron, enhancement by Ad5 is diminished (Qiu and Pintel, 2002). The AAV2 intron is unusually large (322 nucleotides) and has a non-consensus donor and poor 3' splice site. Neither exonic nor intronic splicing enhancer signals have yet been identified. How helper virus gene products function to enhance the splicing of AAV2 RNA in the presence of Rep has not yet been characterized.

**The role of the large Rep proteins in AAV2 RNA splicing.** AAV2 large Rep proteins (Rep78/68) can act to increase the ratio of spliced to unspliced AAV RNA when they are targeted to the transcription template via a Rep-binding element (RBE; Qiu and Pintel, 2002). An extended RBE provided either by the P5 promoter or the viral ITR can function to target Rep in a way that sustains full enhancement of the splicing of P40-generated RNA. This effect requires the presence of adenovirus gene products, and if the transcription template lacks an extended Rep-binding element, neither Ad5 nor Rep have significant detectable effects on AAV2 RNA splicing. It has so far not been possible to separate Rep enhancement of splicing from Rep activation of transcription initiation. In addition, the required RBE is both location and orientation independent, similar to the classically defined transcription enhancer-factor binding sites, which suggested that Rep might be acting co-transcriptionally to effect RNA processing.

Although the Rep effect on AAV2 RNA processing appears to be linked to its transcriptional transactivation activity, transactivation of transcription initiation is not sufficient for enhancement of RNA processing (Qiu and Pintel, 2002). Activation of the P40 transcription unit by other activators targeted to transcription template (such as Gal-VP16 and MVM NS1) does not enhance AAV2 RNA splicing, although transcription of the P40 promoter is strongly transactivated by these proteins.

Rep enhancement of the relative levels of spliced RNA decreases as the distance between the promoter and the intron of the affected transcription unit increases (Qiu and Pintel, 2002). This is consistent with a co-transcriptional model of enhancement of RNA processing by Rep, and explains why the same AAV2 intron is excised to a much greater extent from the proximal P40-generated transcripts than from upstream P5 and P19 transcripts.

It is not yet clear how Rep and adenovirus affect AAV RNA processing; indeed, it is not yet clear whether the combined effect of adenovirus and Rep is directly on splicing or other RNA processing mechanisms that ultimately determine the steady-state levels of spliced AAV RNA. Together these factors may alter the composition of the elongating RNA polymerase II complex, specifically, by altering levels or types of RNA processing factors associated with the RNA polymerase II carboxy terminal domain (CTD). Alternatively, a direct interaction of Rep with cellular splicing factors could

be possible. The recent demonstration of Rep co-localization in replication centers with helper adenovirus, or HSV, single-strand DNA-binding proteins (ssDBP and ICP8, respectively), following co-transfection (Stracker *et al.*, 2004), suggests it is also possible that Rep functions to enhance AAV2 RNA splicing by targeting AAV2 to these replication centers, which normally are active for splicing (Bridge *et al.*, 1993; Pombo *et al.*, 1994; Bridge *et al.*, 1996).

As described above, the ratio of spliced to unspliced P5- and P19-generated RNAs increases over the course of infection. While the reason for this is not clear; conceivably, this might be due to either an increase in Rep production, or perhaps, differences in viral localization as infection proceeds.

**Export of AAV2 RNA.** An unusual feature of AAV2 gene expression is that unspliced RNAs from P5, P19, and P40 promoter accumulate in the cytoplasm at high levels. The P5- and P19-generated unspliced RNAs are translated to generate the non-structural proteins, Rep78 and Rep50, respectively. In general, unspliced cellular and viral RNAs remain in the nucleus. Their export typically requires a regulated pathway including a specific *cis*-acting export signal, such as the CTE on the HIV rev RNA (Malim *et al.*, 1989). The export and translation of unspliced P5- and P19-generated RNAs may compensate for the poor splicing of P5- and P19-generated RNAs, impaired because of their distance from the intron (see above).

During adenovirus infection, the adenovirus E1B55K-E4orf6 complex both inhibits the export of cellular mRNAs and is also required for efficient cytoplasmic accumulation of adenovirus mRNAs. The E1B55K-E4orf6 complex has also been suggested to be necessary for the timely cytoplasmic accumulation of AAV RNAs during adenovirus co-infection (Samulski and Shenk, 1988). These experiments were performed using adenovirus mutants that individually did not generate either the E1B 55K or E4orf6 proteins. Because of recent experiments highlighting the critical importance of these proteins in multiple steps during the replication and expression of AAV2, their role in export of AAV2 RNA probably needs to be re-evaluated. Following transfection of an infectious clone of AAV2 into 293 cells in the absence of Ad5, unspliced AAV2 RNAs can be efficiently exported to the cytoplasm (Mouw and Pintel, 2000), suggesting that their export can be achieved in the absence of additional adenovirus gene products.

Whether the export of unspliced AAV2 RNAs requires a particular *cis*-acting signal that directs it through a regulated export pathway, or, alternatively, whether these RNAs are exported via the TAP/Aly (Stutz *et al.*, 2000; Zhou *et al.*, 2000) cellular pathway will be interesting to determine.

#### RNA SPLICING OF OTHER AAV2-LIKE SEROTYPES OF AAV

AAV1, 2, 3, 4, and 6 share a substantial homology within the intron region and exhibit identical donor sites and highly similar acceptor sites (see Figure 18.6). Splicing of AAV3 and AAV4 is basically similar to that of AAV2 with regards

to their dependence on helper virus; however, the splicing of AAV1 and AAV6 appears to be less dependent on helper virus (Qiu, Farris and Pintel, unpublished). Most of the P40-generated transcripts generated by AAV1 and AAV6 RepCap plasmids following transfection of 293 cells remain unspliced even in the presence of Ad5. Lower levels of splicing of P40-generated products are also seen during AAV1 and AAV6 viral infection. The levels of splicing seem to be governed by intron sequences. The AAV2 intron, when placed in an AAV1 background, splices at high efficiency, while the AAV1 intron still splices poorly when inserted into AAV2 (Qiu, Farris and Pintel, unpublished). These results suggest that it may be possible to identify *cis*-acting sequences within the AAV2 intron that confer splicing responsiveness to adenovirus and Rep.

#### AAV5-LIKE ADENO-ASSOCIATED VIRUSES

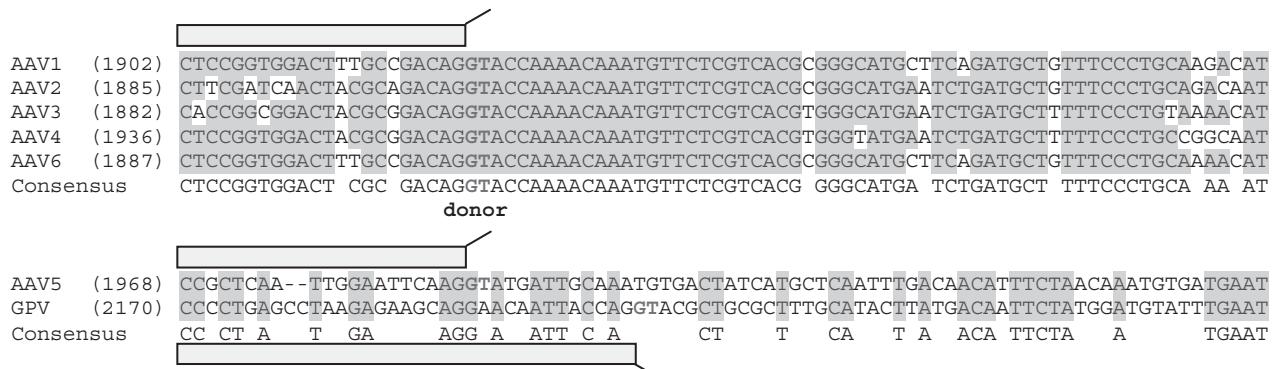
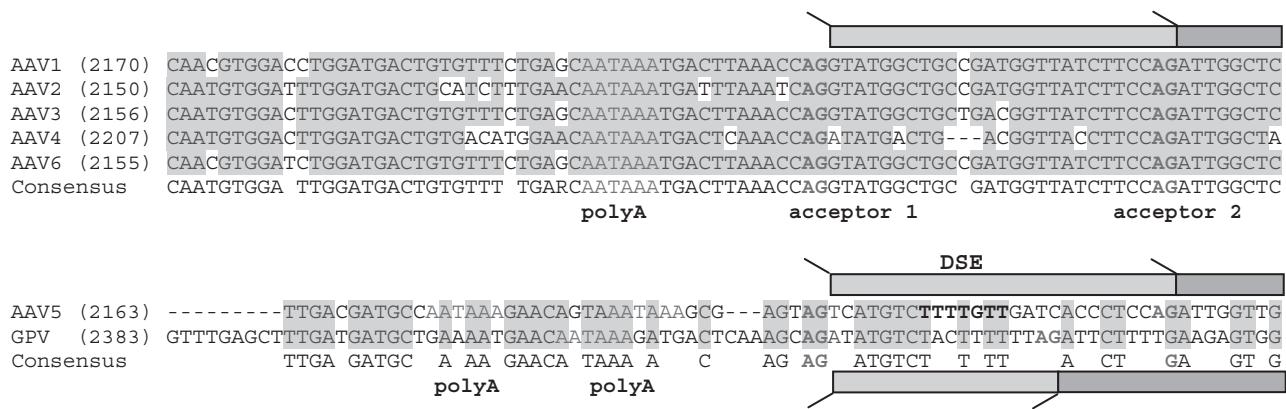
##### GENERAL FEATURES OF AAV5 RNA TRANSCRIPTION

While the basic transcription profile of AAV5 has similarities to that of AAV2, there are also significant differences (see Figure 18.7). Most surprisingly, RNAs generated from both the AAV5 P7 and P19 promoters are efficiently polyadenylated at a site ([pA]p, at nucleotide 2193) lying within the intronic region in the center of the genome (Qiu *et al.*, 2002; nucleotide numbers refer to GenBank accession number AF085716). Because P7- and P19-generated transcripts are polyadenylated at this site and not spliced, Rep78 and Rep52 are the only Rep proteins detected during AAV5 infection of 293 cells. Four different species of P41-generated RNAs have been detected in cells infected with AAV5 or transfected with full-length AAV5 clones. Two unspliced species are detected, one polyadenylated at (pA)p and one polyadenylated at the distal site (pA)d at nucleotide 4434. P41-generated RNAs polyadenylated at (pA)d are also spliced using the A1 or A2 acceptor and generate VP1 and VP2/VP3, respectively. The function of the unspliced P41-generated RNAs is unknown.

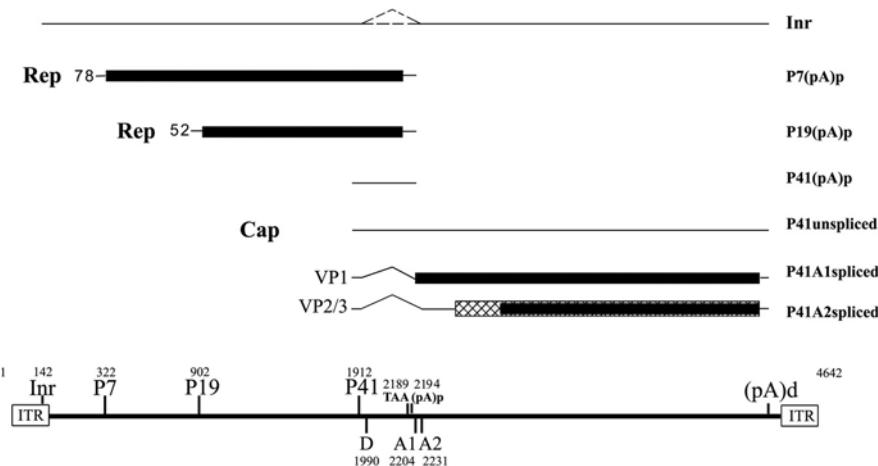
Mapping of the AAV5 transcripts also demonstrated a transcription initiation site within the AAV5 inverted terminal repeat (ITR; Qiu *et al.*, 2002). A similar transcript generated from the ITR of recombinant AAV2 has also been identified (Flotte *et al.*, 1992, 1993). The initiation site of the AAV5 ITR-generated transcripts has been mapped to the terminal resolution site in the hairpin, and are unspliced 4.7 kb RNAs that polyadenylate at (pA)d. The function of the ITR-initiated RNA remains unknown; however, these molecules are relatively more abundant during viral infection, or following transfection of a replicating, compared with non-replicating, plasmid clones of AAV5 (Qiu *et al.*, 2002).

##### SPLICING AND POLYADENYLATION OF AAV5 RNA

The AAV5 intron, which is 240 nucleotides, is considerably smaller than the 322 nucleotides AAV2 intron. It has a non-consensus 5' splice site (AAG/GTATGA) at nucleotide 1990, and the 3' acceptor site A1 is at nucleotide 2204, and site A2 is at 2231 (Qiu *et al.*, 2002). The polypyrimidine tract in the

**AAV DONOR SITES:****AAV ACCEPTOR AND POLYADENYLATION SITES:**

**Figure 18.6** Nucleotide sequence of the AAV and GPV 5' donor and 3' acceptor sites. The nucleotide sequence of the 5' splice donor region, and the 3' splice acceptor and polyadenylation site region are aligned for AAV1, AAV2, AAV3, AAV4, AAV6, and compared with AAV5 and GPV. A consensus sequence is also presented. The intron bordering nucleotides and potential polyadenylation signals are shown in red. The downstream element (DSE) required for AAV5 polyadenylation at (pA)p is also shown.



**Figure 18.7** Transcription map of AAV5. The AAV5 genome is depicted at the bottom of the figure, and includes the location of the viral promoters (Inr, P7, P19, P41), the small intron donor (D) and acceptors (A1 and A2), the termination site for the Rep proteins, the internal polyadenylation site (pA)p, and the inverted terminal repeats (ITRs). The major transcript classes, and the proteins that they encode are shown above. The different open reading frames (ORFs) that are used are shown in different shading patterns. It is not clear whether a portion of Inr-initiated RNA is spliced or not; this is indicated by a dashed line. See also Color Plate 18.7.

region is pyrimidine-rich and splicing of AAV5 intron is constitutively relatively high even in the absence of adenovirus (Qiu *et al.*, 2002). Interestingly, splicing at the A1 site is dependent on the A2 site; mutation of the A2 AG motif also debilitates splicing to A1 (Qiu, Ye and Pintel, unpublished). In contrast, for AAV2, splicing using A1 is independent of A2 (Qiu, Ye and Pintel, unpublished). Also in contrast to AAV2, neither the Rep protein nor additional adenovirus gene products are required to achieve efficient AAV5 promoter activity and pre-mRNA splicing following transfection of a RepCap plasmid clone lacking ITRs into 293 cells (Qiu *et al.*, 2002). The independence of AAV5 splicing is not due merely to its smaller size, but rather to the nature of *cis*-sequences within the context of the intron (Qiu *et al.*, 2004).

AAV5 RNAs generated from P7 and P19, which encode the large and small Rep proteins, respectively, from the large ORF in the 5' half of the genome, use (pA)p at high efficiency (Qiu *et al.*, 2002, 2004). RNAs generated from P41 promoter, however, which encode the virus capsid proteins from the large ORF in the 3' half of the genome, use this nearby site with significantly reduced efficiency; they primarily read through to the distal site (pA)d (Qiu *et al.*, 2004). The intron region of AAV5 contains two relatively consensus AAUAAA signals at nucleotides 2177 and 2191, which are immediately upstream of the first intron acceptor A1 at nucleotide 2204 (Qiu *et al.*, 2004). RNA cleavage and polyadenylation occurs 11–14 nucleotides downstream of the first AAUAAA motif (Qiu *et al.*, 2002). AAV2 also contains a consensus AAUAAA polyadenylation signal in this region; however, its use can only be detected by sensitive PCR techniques (Tullis and Pintel, unpublished).

Sequences within the AAV5 intron are both sufficient and necessary to direct cleavage and polyadenylation of P7-generated transcripts. Efficient polyadenylation of both P7- or P19-driven transcripts and P41-driven transcripts at (pA)p requires a U-rich downstream element (DSE) that overlaps with the A2 3' splice acceptor site (Qiu *et al.*, 2004). In addition, efficient polyadenylation of P7-driven transcripts at (pA)p involves an upstream element (USE) that lies prior to the initiation of the P41 transcripts (between nucleotides 1266 and 1637; Qiu *et al.*, 2004). Because the USE lies upstream of the start of the P41-generated RNAs, these RNAs are not subject to the additional level of control that this element imposes on P7-driven RNAs. The USE is not required if the DSE is intact; however, it can compensate for the loss of DSE function (Qiu *et al.*, 2004). Although the relationship between these two elements is not yet clear, the USE, which is lacking in P41-generated RNAs, probably functions to ensure that the AAV5 P7- and P19-generated RNAs are polyadenylated at (pA)p at high efficiency.

Polyadenylation of P41-generated RNAs at (pA)p is significantly increased when it is produced from a replication-competent molecule such as viral infection or an infectious clone (Qiu *et al.*, 2004). This is not merely due to the presence of the ITRs in *cis*, because ITR-containing templates

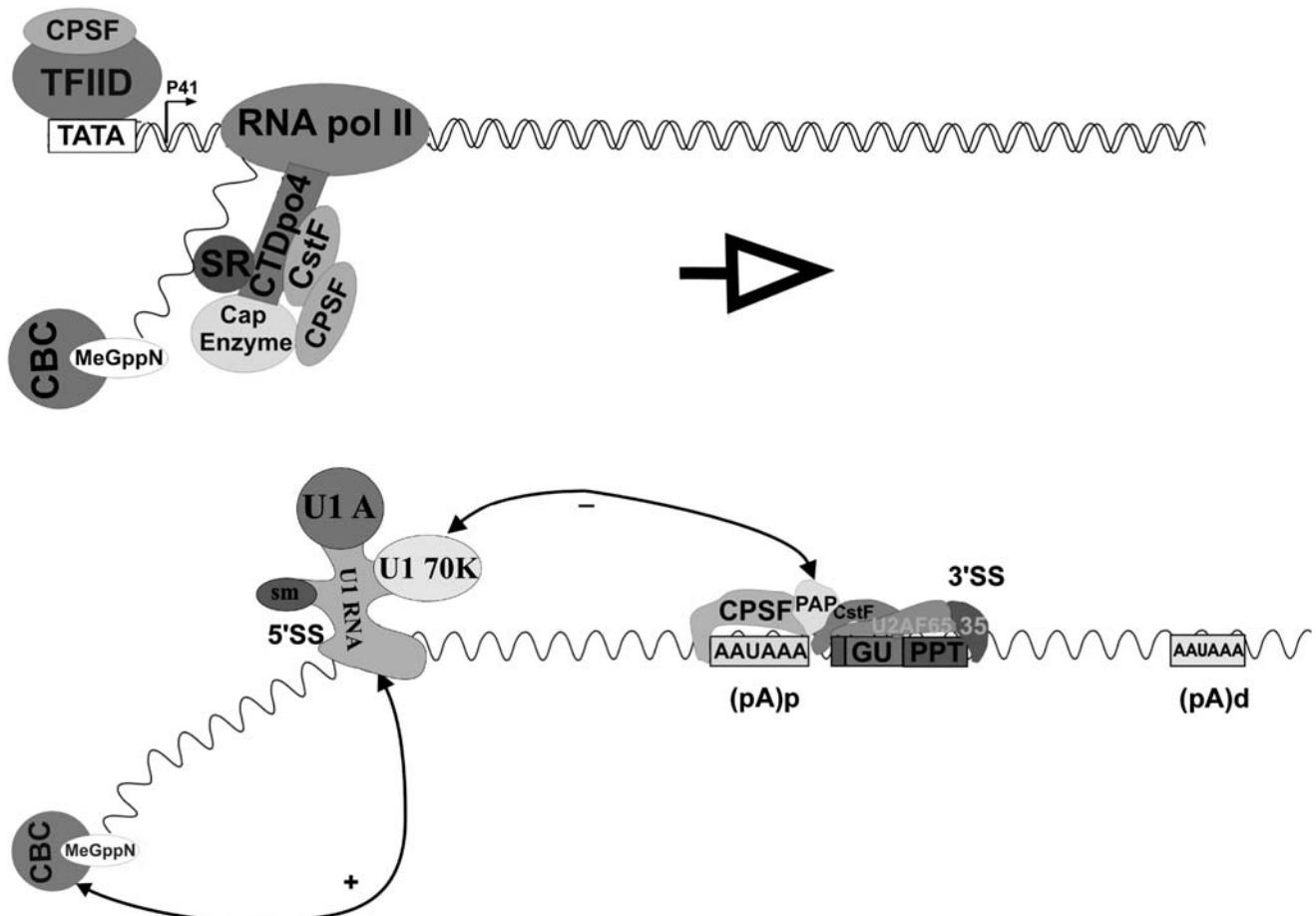
that cannot replicate due to Rep mutation do not show this increase. Although most P41-generated RNAs read-through (pA)p, 25–28 percent of P41-generated RNA during AAV5 co-infection with adenovirus are polyadenylated at (pA)p. Whether these RNAs have any role during AAV5 infection is not known.

As mentioned above, the AAV5 DSE-containing A2 3' splice site plays a dual role in both polyadenylation and splicing, and there is clearly a complex regulation between these two processes. The DSE overlaps, but is not congruent with, the 3' acceptor of the AAV5 intron. The relative strengths of these two signals must allow both for polyadenylation of the upstream promoter-generated RNAs that generate the essential Rep proteins and for efficient splicing to generate the correct ratio of spliced P41-generated RNAs to produce the individual capsid proteins at the appropriate stoichiometry. The relative efficiency of these processes is controlled to a significant degree by the strength of the *cis*-acting signals governing them, and they probably have evolved to program the optimal amounts of viral gene products required for the AAV5 life cycle (Qiu *et al.*, 2004).

Interestingly, when the (pA)p region of AAV5 is replaced with the internal AAUAAA signal from AAV2, which is normally cryptic in AAV2, or when the internal region of the AAV2 intron is inserted into AAV5 and linked to the AAV5 DSE-containing A2 3' splice site, polyadenylation at the AAV2 AAUAAA site becomes highly efficient (Qiu and Pintel, unpublished). This suggests that at least one element that differentiates the use of this site between the two viruses is the presence of the DSE in AAV5.

Polyadenylation at (pA)p increases as the distance between the RNA initiation site and the intron and (pA)p site is increased (Qiu and Pintel, 2004). The steady-state level of RNAs polyadenylated at (pA)p is independent of the promoter used or of the intervening sequence but is dependent upon competition with splicing, inhibition by U1 snRNP binding to the intron donor, and the intrinsic efficiency of the cleavage/polyadenylation reaction (Qiu and Pintel, 2004). Each of these determinants shows a marked dependence on the distance between the RNA initiation site and the intron and (pA)p.

The simplest model to explain the interdependence of splicing and polyadenylation in the AAV5 system is that strong binding of U1 to the donor facilitates both splicing and inhibition of (pA)p and that, perhaps as a consequence of 5' exon definition, U1 binding is stronger when the promoter is close (e.g. P41-generated RNAs, which are inhibited for polyadenylation at [pA]p and splice) and is weaker when the distance is large (e.g. P7-generated transcripts, which splice poorly, escape inhibition by U1 snRNP, and are efficiently polyadenylated at [pA]p) (Figure 18.8). 5'-Terminal exon definition typically requires that factors binding at or near the *cap* site interact with and stabilize the binding of factors (usually U1 snRNP) to the proximal 5' splice site, and this interaction may be more critical when the proximal 5' donor is non-consensus (Lewis *et al.*,



**Figure 18.8** A model proposing a possible explanation for the distance dependence of AAV5 polyadenylation at (pA)p. The top part of the diagram depicts elongating RNA polymerase, with the capping enzymes, polyadenylation factors CstF and CPSF, and SR proteins bound to the RNA pol II CTD. The polyadenylation factor CPSF is shown associated with TATA-bound TFIID, and the cap-binding complex (CBC) is shown associated with the cap structure (MeGppN) on the 5' end of nascent RNA. The bottom part of the diagram suggests how the U1 snRNP (and perhaps the U1 70 K or U1 A protein) may exert its negative effects on polyadenylation at (pA)p, by interacting with the polyadenylation factors associated with the AAUAAA. Also shown is the binding of the splicing factors U2AF(65 + 35) with the adjacent 3' splice site. Stabilization of U1 snRNP to the intron 5' splice site, which facilitates splicing and simultaneously inhibition of (pA)p may occur through interaction with the cap binding complex associated with the 5' cap site as shown, and may be more effective in this regard when the RNA is initiated closer to the donor site (i.e. RNA generated from the P41 promoter), than when the RNA is initiated at a distance (i.e. RNA generated from the P7 promoter). See text for further explanation. See also Color Plate 18.8.

1996). The *cap* structure, via binding to the cap-binding complex (CBC; Izaurralde *et al.*, 1994, 1995) has been suggested to affect both splicing and polyadenylation. A nuclear CBC (Inoue *et al.*, 1989; Colot *et al.*, 1996) facilitates association of U1 snRNP with the cap-proximal 5' splice site, and this interaction is not dependent on a strict spacing between the *cap* and the affected 5' splice site (Lewis *et al.*, 1996). It seems feasible that for AAV5, CBC binding to the *cap* site may stabilize factors binding to the non-consensus AAV5 donor, but only when the *cap* site is very close (78 nucleotides for P41), but not at a distance (1668 nucleotides for P7, 1088 nucleotides for P19). If interactions between components of the CBC and U1 snRNP mediate the distance-related

donor inhibition seen for AAV5, it is due to the strengthening of the association of U1 snRNP to the non-consensus donor site, which would lead to the enhancement of U1's inhibitory activity.

Such a model is also consistent with the distance-related splicing of AAV2 RNA. In that case, the AAV2 Rep protein enhances splicing of proximal P40-generated transcripts to a much greater degree than it enhances splicing of RNAs generated far upstream from P5. It may be that for AAV2, the Rep protein, in the presence of adenovirus, facilitates the stabilization of U1 snRNP to the AAV2 donor site when the RNA initiation site is close to the intron. A potential mechanism that might explain the distance-related processing of

AAV RNA, using the AAV5 system as a model, is depicted in Figure 18.8.

### GOOSE PARVOVIRUS (GPV) AND MUSCOVY DUCK PARVOVIRUS (MDPV)

GPV and MDPV are autonomously replicating viruses that were originally classified into the genus *Parvovirus*, but they are genetically more related to AAVs and accordingly have now been reclassified into the *Dependovirus* genus (Brown *et al.*, 1995b; Zadori *et al.*, 1995). The genome of GPV shares greater than 65 percent identity at the nucleotide level with the AAVs (Brown *et al.*, 1995b; Zadori *et al.*, 1995) and the Rep protein of GPV shares functional homology with the Rep protein of AAV2 (Smith *et al.*, 1999). The gene expression profile of GPV shares some similarity to that of AAV5; however, it also retains some characteristics of the *Parvovirus* genus (Qiu and Pintel, unpublished). GPV contains an efficiently spliced small intron (247 nucleotides) that is similar in size to AAV5. The 5' splice site is located at nucleotide 2208, and the 3' splice sites are located at nucleotide 2436 (A1) and 2454 (A2; nucleotide numbers refer to GenBank accession number NC\_001701).

Also similar to AAV5, the Rep-encoding RNAs generated by the GPV P9 promoter efficiently uses an internal polyadenylation site that has a DSE including a U-rich sequence located within its A2 polypyrimidine tract (Qiu and Pintel, unpublished). However, mutational analysis has shown that an additional U-rich stretch downstream of the intron forms part of the GPV DSE signal (Qiu and Pintel, unpublished). GPV has only one AAUAAA signal, which is located at nucleotide 2416, upstream of the A1 acceptor site. Interestingly, the sequence around that signal is identical to the corresponding region of AAV2, although it is not efficiently used in AAV2 for polyadenylation of AAV2 pre-mRNA (Figure 18.6). The transcription profile of MDPV is similar to that of GPV (Qiu and Pintel, unpublished).

### AVIAN-ADENO-ASSOCIATED VIRUS (A-AAV)

An avian AAV (A-AAV) was first isolated from the Olson strain of quail bronchitis adenovirus (Yates *et al.*, 1973). The genome of A-AAV is 4694 nucleotides in length and has organization similar to that of other AAVs (Bossis and Chiorini, 2003). The entire genome of A-AAV displays 56–65 percent identity at the nucleotide level with the other known AAVs (Bossis and Chiorini, 2003).

The intron of A-AAV is larger than that of AAV2. It is 453 nucleotides in length, with the 5' splice site at nucleotide 1824, and 3' splice acceptor sites are at nucleotide 2247 (A1) and 2277 (A2; nucleotide numbers refer to GenBank accession number AY186198). Like AAV5, splicing of A-AAV RNAs shows less dependence in 293 cells on Ad5. However, in contrast to AAV5, approximately half of the A-AAV RNAs generated from the upstream P5 and P19 promoters are polyadenylated at a (pA)p site (nucleotide 2228) that lies within the intron (Qiu and Pintel, unpublished).

## Erythroviruses

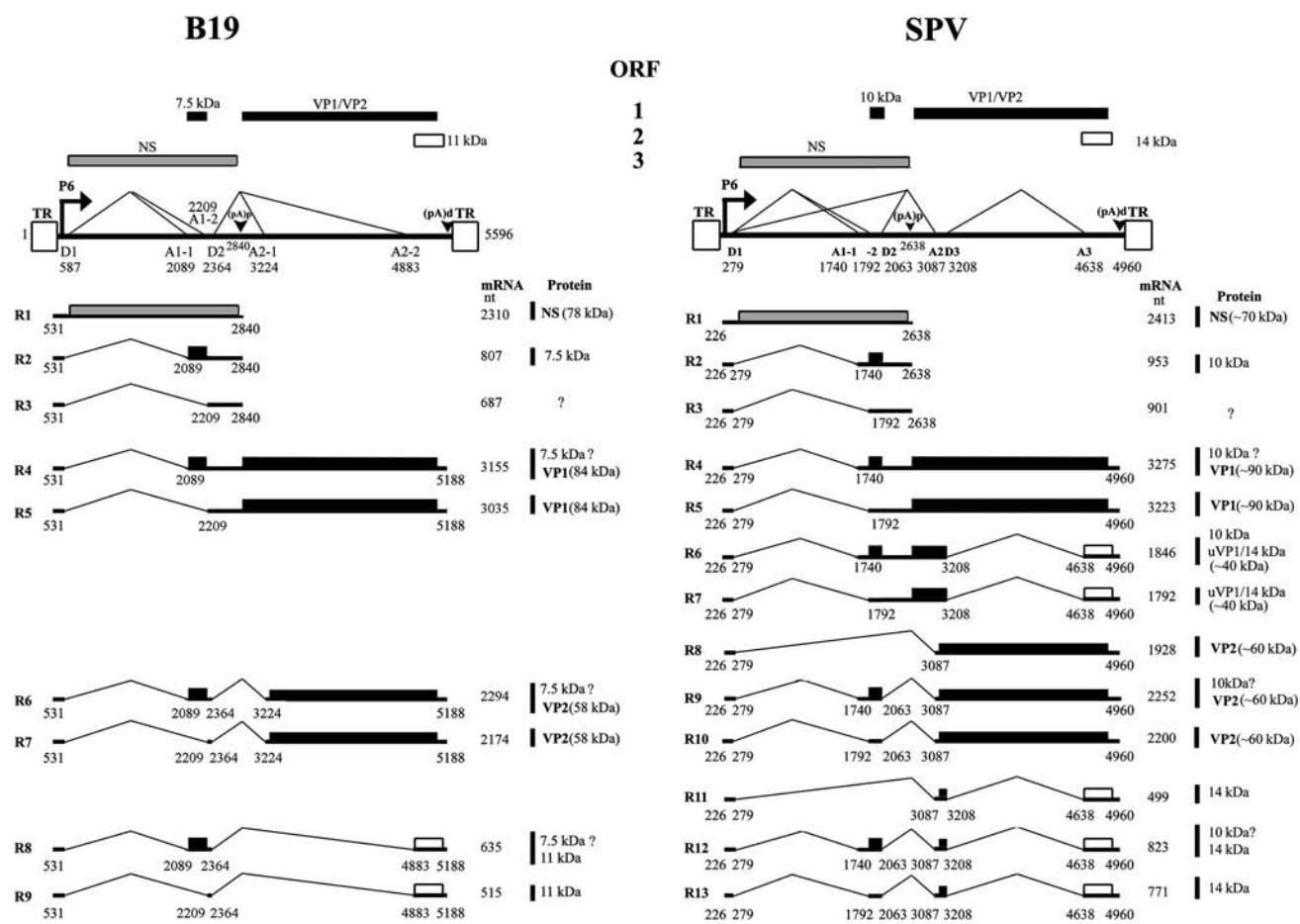
### HUMAN PARVOVIRUS B19

#### GENERAL TRANSCRIPTION ORGANIZATION

A total of nine transcripts have been identified from the B19 genome both in viral infection of permissive cells (Ozawa *et al.*, 1987) and following transfection of non-permissive cells (St Amand *et al.*, 1991; Figure 18.9, *left panel*). Only one promoter for B19 has been identified, at map unit 6 (the TATA box is at nucleotide 499; Blundell *et al.*, 1987; Doerig *et al.*, 1987; Ozawa *et al.*, 1987, 1988a; Liu *et al.*, 1991) and so the processing of P6-generated pre-mRNAs is critical in determining the relative levels of B19 gene products. All transcripts driven by P6 have a short leader sequence of 56 nucleotides (nucleotides 531 to 586; nucleotide numbers refer to GenBank accession number AY386330) at their 5' end. Three transcripts (designated R1, R2, and R3 in Figure 18.9, *left panel*) terminate at a proximal polyadenylation site [(pA)p] at nucleotide 2819 in the middle of the genome. RNA R1 (Figure 18.9, *left panel*) is unspliced and encodes the large non-structural protein NS1, which initiates at an AUG codon at nucleotide 616. RNA R2 (Figure 18.9, *left panel*) is spliced between a donor site at nucleotide 587 (D1) and an acceptor at nucleotide 2089 (A1–1) and encodes a 7.5 kDa protein, the function of which is currently unknown. RNA R3 (Figure 18.9, *left panel*) is spliced between D1 and an acceptor at nucleotide 2209 (A1–2). No protein product of the (pA)p-terminated R3 RNA has yet been identified.

Six additional transcripts that use a polyadenylation site at nucleotide 5170 at the far right side of the genome (pA)d are also generated. All of these RNAs splice the first intron, using D1 and either A1–1 or A1–2. Two of these, R4 and R5, are not spliced further and encode the VP1 protein. For R5, which is spliced between D1 and A1–2, the VP1-initiating AUG at nucleotide 2624 is the eighth AUG present in the mRNA, and it shows reduced translation of VP1 protein *in vitro* (Ozawa *et al.*, 1988b). For R4, and all molecules splicing between D1 and A1–1 (including R6 and R8), the full 7.5 kDa ORF is retained. Whether multiple ORFs are translated from any of these potentially polycistronic mRNAs is not known. RNAs that splice the first intron and also splice a second intron, between a donor site at nucleotide 2363 (D2) and an acceptor at nucleotide 3224 (A2–1; R6 and R7), encode the capsid protein VP2, which, in R7: initiates at the first available AUG at nucleotide 3305. VP2 is translated in the same ORF as VP1. Finally, a set of RNAs that splice the first intron and are also spliced between D2 and a second acceptor, much further downstream at nucleotide 4883 (at A2–2; the R8 and R9 mRNAs), encode the 11 kDa proteins. Although it is produced at high abundance, the function of this protein has not yet been characterized.

Because B19 has a single promoter, temporal regulation of B19 gene expression is also governed to a great extent by processing of the primary P6-generated transcripts (Blundell



**Figure 18.9** Transcription maps of B19 and SPV. The map of B19 is shown on the left, and the map of SPV is shown on the right. In each case, the viral genome is depicted, and includes the location of the viral promoter (P6), the donors and acceptors, the polyadenylation sites (pAp and pAd), and the terminal repeats (TR). The major transcript classes, their size in nucleotides, and the proteins that they encode are shown below. Open reading frames (ORFs) used to encode the viral proteins are shown above the genome.

et al., 1987; Doerig et al., 1987; Ozawa et al., 1987, 1988a; Liu et al., 1991). Accumulation of the NS1 mRNAs that are polyadenylated at (pAp) and not spliced, precedes accumulation of the mRNAs for the structural proteins in non-permissive cells (Yoshimoto et al., 1991).

Small RNAs (<1 kb; R2, R3, R8, R9) are the most abundant RNAs generated during B19 infection (Ozawa et al., 1987; St Amand et al., 1991). Among them, the 0.8 kb small RNA, R2, which encodes a 7.5 kDa protein, is the most abundant species in non-permissive cells as the ratio of A1–1 usage is greater than for A1–2. (Yoto and Pintel, unpublished). R2 is more abundant than R3 in permissive bone marrow cells from a patient with sickle cell anemia (Ozawa et al., 1987). Likewise, R4 is more abundant than R5 in non-permissive cells. The combined VP1 transcripts (R4 + R5) are more abundant than the NS1 transcript (R1) in viral infection (Ozawa et al., 1987; Liu et al., 1992); however, the VP2 transcripts R6 + R7 are even more abundant. Splicing to the downstream A2 acceptor A2–2 is greater than to the upstream A2 acceptor (A2–1) resulting

in more transcripts encoding the 11 kDa protein (R8 + R9), than transcripts encoding VP2 in non-permissive cells (St Amand et al., 1991). As discussed below, there are differences in the relative ratios of the various RNAs generated during viral infection of permissive cells compared with plasmid transfection in non-permissive COS cells, especially in the relative abundance of the NS1-encoding R1 transcripts, which is governed both by a choice between splicing at D1 and polyadenylation at the (pAp) site (Liu et al., 1992).

It has recently been reported that following infection of semipermissive MB02 cells, B19 RNA, rather than splicing between D1 and A1–1 and A1–2, is spliced predominately between A1–2 and a non-consensus (CT rather than GT) donor site 35 nucleotides downstream of D1 (Brunstein et al., 2000). Such splicing would be predicted to generate a protein that initiates at an AUG codon in the NS1 reading frame, two amino acids prior to the non-consensus donor, and would then be fused, after the splice, into the 7.5 kDa ORF. All B19 spliced mRNAs spliced in this way would be predicted to encode a short NS1/7.5 kDa fusion ORF at their

5' ends. As mentioned below, a similar donor site has also been detected at low levels in RNA generated by SPV infection of monkey liver (Vashisht *et al.*, 2004). The generality of this splicing event and how these alternative splice sites are related to the tropism of erythroviruses is not yet clear.

#### DIFFERENCES BETWEEN PERMISSIVE AND NON-PERMISSIVE CELLS, AND REGULATION OF POLYADENYLATION IN THE MIDDLE OF THE GENOME

Replication of B19 is restricted to the erythroid lineage from BFU-E to erythroblasts, with susceptibility to virus increasing with differentiation (Shimomura *et al.*, 1992; Morita *et al.*, 2001). Human bone marrow is permissive and cell lines UT-7/Epo-S1, derived from a megakaryoblastic leukemia cell line (Shimomura *et al.*, 1992; Gallinella *et al.*, 2000; Morita *et al.*, 2001; Morita *et al.*, 2003) and KU812Ep6 (Miyagawa *et al.*, 1999), derived from a chronic myeloid leukemia cell line, are at least semipermissive for viral replication.

In non-permissive cells, the majority of B19 transcripts are spliced at the first donor; however, unspliced transcripts, which polyadenylate at (pA)p and encode NS1, are relatively more abundant than those for VP1 (Liu *et al.*, 1992). In permissive cells, the abundance of RNAs spliced at D1 is increased even further relative to unspliced R1 mRNAs. Furthermore, viral infection in permissive cells is accompanied by increased read-through of the (pA)p site and so the ratio of RNAs polyadenylated distally at the (pA)d site (R4-R9), relative to RNAs, which polyadenylate proximally at (pA)p (R1 + R2 + R3), is also greater in permissive infections (Liu *et al.*, 1992). These two factors significantly increase the capsid-encoding transcripts (R4-R7), as well as RNAs R8 and R9: relative to the level of the NS1-encoding (R1) transcripts in permissive cells. Whether this difference in the RNA profile is directly related to the inability of virus to replicate in non-permissive cells, or whether a cell-type difference in RNA processing prevents replication has not been firmly established. It has been shown that a B19 plasmid, driven to replicate in normally non-permissive COS cells by the SV40 origin of replication, generates a greater relative abundance of RNAs that read-through the (pA)p site, suggesting a model in which replication facilitates the generation of the read-through capsid-encoding transcripts (Beard *et al.*, 1989; St Amand *et al.*, 1991; Liu *et al.*, 1992; Luo and Astell, 1993; St Amand and Astell, 1993). There are additional differences seen in B19 expression in permissive compared with non-permissive cells that have not been fully characterized. In addition, in non-permissive COS cells, most B19 RNAs that are polyadenylated at (pA)p are the spliced R2 RNAs, which encode the 7.5 kDa protein. Therefore, regulation of splicing at the first donor site can be considered to be a critical determinant that governs the relative level of NS1, which is encoded by R1. D1 usage appears to be greater in permissive compared with non-permissive cells.

The B19 (pA)p signal in the center of the genome, ATTAAA, is unusual. Mutational studies have shown that this non-consensus 'ATTAAA' motif is used as a polyadenylation

signal, while the similar motif, 'AATAAC', which is immediately adjacent downstream, is not (Liu *et al.*, 1992; Yoto and Pintel, unpublished). Mutation of the second motif, however, results in enhanced polyadenylation at (pA)p suggesting that the second motif can compete with the first 'ATTAAA' for efficient polyadenylation at this site (Yoto and Pintel). There are a number of examples in which non-consensus polyadenylation signals are used to regulate polyadenylation (St Amand *et al.*, 1991; Tabaska and Zhang, 1999). Non-consensus sequences are typically weaker binding sites for CPSF than consensus sites and this may allow the pre-mRNAs that eventually encode B19 VP1, VP2, and the 11 kDa proteins to read-through the internal polyadenylation site more efficiently and for this signal to remain unused within the VP1-encoding mRNA.

Alternative polyadenylation of B19 RNA is governed by many factors, including competition with splicing of the pre-mRNAs, as has been demonstrated for AAV5 (Qiu and Pintel, 2004). Characterization of the relationship between B19 RNA processing and permissivity to infection will yield important insights into B19 pathogenesis.

#### SIMIAN PARVOVIRUS (SPV)

Although there have been a number of non-human primate erythroviruses identified, including simian parvovirus (O'Sullivan *et al.*, 1994), pig-tailed macaque parvovirus and Rhesus parvovirus (Green *et al.*, 2000), only the transcription profile of SPV has been determined (see Figure 18.9, right panel).

The NS and Cap regions of SPV and B19 are more than 50 percent of homologous (Brown *et al.*, 1995a). Like B19, SPV has a single promoter (P6) and two polyadenylation sites ([pA]p and [pA]d). However, the (pA)p signal in the middle of genome is consensus (AAUAAA) and has a putative GU-rich DSE (Liu *et al.*, 2004).

At least 13 SPV transcripts are generated by alternative splicing and polyadenylation following transfection of non-permissive COS cells (Liu *et al.*, 2004) and viral infection of monkey liver (Vashisht *et al.*, 2004). Although the transcription maps of SPV and B19 are generally similar, they differ in a number of important ways. SPV pre-mRNAs contains three introns, instead of the two introns found for B19. The third intron is within the capsid gene region and RNAs in which this intron is spliced are abundant (there is approximately three times more spliced RNA than unspliced RNA across this region). These RNAs encode the SPV 14 kDa protein (analogous to the B19 11 kDa protein), which initiates at an AUG in the exon preceding the third intron. Also, SPV RNAs were found that were spliced between the donor of the first intron and the acceptor of the second intron (D1 to A2, in Figure 18.9, right panel). A portion of these molecules were not additionally spliced and encode VP2; those from which the third intron was also removed encode the 14 kDa protein. Similar to B19, SPV has a polyadenylation signal ([pA]p) in the middle of genome that directs

efficient polyadenylation; however, unlike B19, the SPV signal is a consensus AAUAAA motif. Efficient polyadenylation at (pA)p of both spliced and unspliced mRNAs (encoding a putative 10 kDa protein, analogous to the B19 7.5 kDa protein and SPV NS1, respectively) was detected. The 14 kDa protein, which is approximately 3-fold more abundant than the VP2 protein, has an unknown function and was localized to both in the nucleus and cytoplasm (Liu *et al.*, 2004).

The first intron donor site D1 is at nucleotide 279 and it is joined to either the A1–1 (nucleotide 1740) or A1–2 (nucleotide 1792) acceptor site (nucleotide numbers refer to GenBank accession number U26342). All SPV RNAs that are spliced between D1 and A1–1 retain an open reading frame for a protein analogous to the B19 7.5 kDa protein. In SPV this protein, which has an apparent molecular weight of 10 kDa, is translated from the R2 molecule that polyadenylates at (pA)p. As is the case for B19, whether this ORF, which is retained in all RNAs splicing between D1 and A1–1, is translated bicistrionically from these RNAs is not known. The second intron donor site D2 (nucleotide 2063) is joined to A2 at nucleotide 3087. Splicing between D1-A2 and D2-A2 is found at approximately equal levels. The novel third intron in SPV RNA uses a donor D3 (nucleotide 3208) and an acceptor A3 (nucleotide 4638) within the capsid gene. Since acceptor A2 is spliced to D1 as well as D2, three SPV transcripts (R8, R9, and R10) could encode VP2. Three transcripts (R11, R12, and R13), which splice the third intron, would be capable of encoding the 14 kDa proteins. Overall splicing at A2 site is very efficient, which guarantees abundant transcripts for VP2. As mentioned above, splicing of the third intron contributes the initiating AUG for the 14 kDa protein, which is the same initiating AUG used for VP2. Because the third intron is spliced at a high level in COS cells, the 14 kDa protein accumulates to levels approximately three times more than VP2 (Liu *et al.*, 2004). Intron 3 is also excised in a minor portion of RNAs from which only the first intron is also spliced. These pre-mRNAs (R6 and R7) would be predicted to encode a fusion between the VP1 unique region and the 14 kDa ORF. Whether this protein is generated during SPV infection is not known.

The basic splice junctions generated during SPV infection of monkey liver tissue (Vashisht *et al.*, 2004) are similar to that from COS cells (Liu *et al.*, 2004). However, a number of additional donor sites were detected in these tissues by RT-PCR analysis (Vashisht *et al.*, 2004). These include a donor site (nucleotide 333) downstream of D1: and two donor sites (nucleotide 3226 and nucleotide 3262) downstream of D3. The relative usage of the donor at nucleotide 333 is predicted to be low, based on its abundance in this assay; however, the relative usage of the donor at nucleotide 3223 and nucleotide 3236 would be predicted to be equivalent to D3 (Vashisht *et al.*, 2004). Interestingly, use of the donors at nucleotide 3223 and nucleotide 3236 would be predicted to prevent expression of the 14 kDa

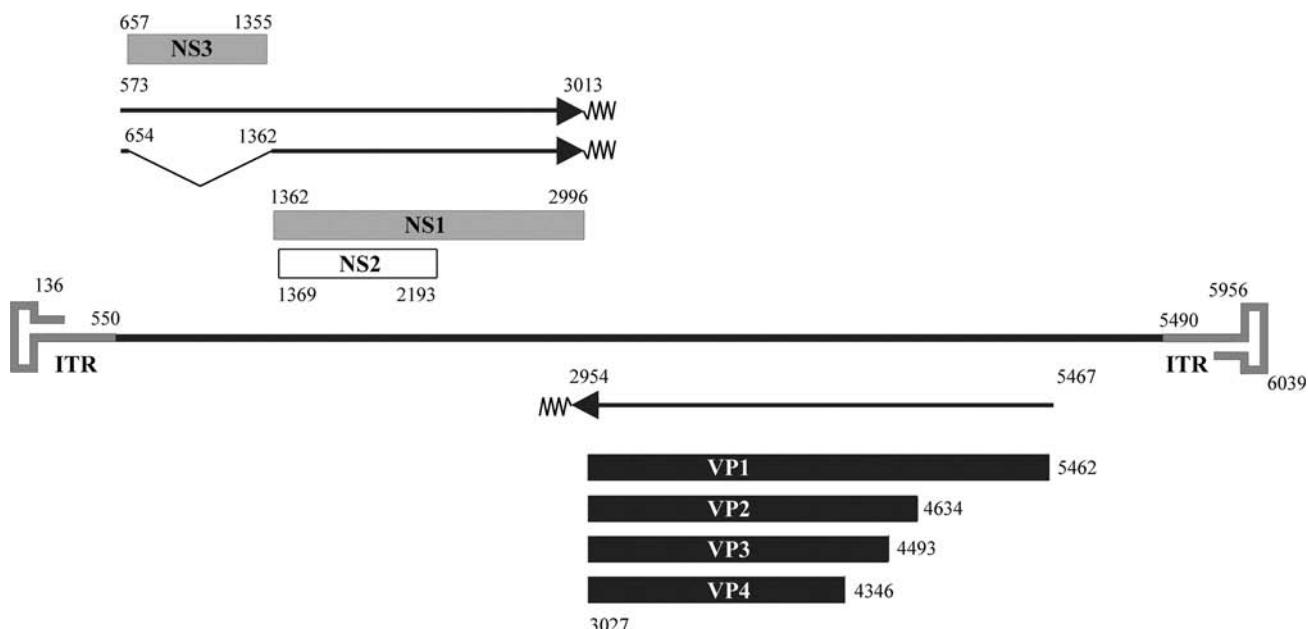
proteins and so it may be useful to confirm the usage of these sites during infection. The RNA profile generated during SPV viral infection in a semipermissive human cell line, UT-7/Epo/S1, was similar to the profile identified in COS cells; however, usage of the rare sites at nucleotide 3223 and 3236 was not detected (Liu *et al.*, 2004).

The small RNAs generated by SPV (the 0.9 kb R2 and R3 RNAs, the 0.8 kb R12: and 0.5 kb R12 + R13 RNAs) are abundant both in viral infection of monkey liver tissue (Vashisht *et al.*, 2004) and following transfection of COS cells (Liu *et al.*, 2004), similar to B19 (Ozawa *et al.*, 1987).

## PARVOVIRUSES OF INVERTEBRATES (DENSOVIRINAE)

The denoviruses (DNVs) are a large group of viruses that infect a number of invertebrate species, including insects and shrimp. Although their capsids and genomes are structurally similar to parvoviruses infecting vertebrates, they share little sequence homology with them, and so they have been classified as a subfamily – Denovirinae – within the Parvovirinae family. They replicate autonomously and have both (+) and (–) sense single-strand genomes that are packaged into separate viral particles. Numerous DNVs have been isolated, but only a subset of these have been characterized at the molecular level. Three groups have so far been identified, and most remain unclassified. One genus, the denoviruses, which contain the denoviruses from *Junonia coenia* (JcDNV), *Galleria mellonella* (GmDNV), and *Mythimna loreyi* (MlDNV), have a genome of approximately 6 kb in length, relatively long ITR structures, and an ambisense genome. A second genus, the brevidenoviruses, which include denoviruses of *Aedes* (AeDNV and AaDNV), and of shrimp, have smaller 4 kb mono- (as opposed to ambi-) sense genomes flanked by different termini. A third genus, the iteraviruses, which include denoviruses of *Caspalia extranea* (CeDNV) and *Bombyx mori* (BmDNV), also are not ambisense, and have genomes of approximately 5 kb in length, which are flanked by short ITRs.

Both the brevidenoviruses and iteraviruses have significant ORFs in each half of the molecule (Boublik *et al.*, 1994; Li *et al.*, 2001; Fediere *et al.*, 2002). The ORFs in the left half of the genome encode the non-structural proteins NS1 and NS2 from unspliced RNAs generated from a promoter at the left-hand end. These proteins are initiated by different AUG codons in two different nucleotide ORFs. It is unclear where the 3' terminus is for the RNAs that encode the NS proteins. The capsid coding ORF in the right-hand end of the molecule is translated from an unspliced mRNA generated from a promoter in the center of the genome. The different capsid proteins are apparently generated from this mRNA by a leaky scanning mechanism.



**Figure 18.10** Transcription map of GmDNV a member of the Densovirus genus of the Densovirinae. The ambisense genome of GmDNV is shown flanked by terminal repeats (ITRs). RNAs encoding the viral non-structural proteins NS1, NS2, and NS3, as well as RNAs in a complementary sense encoding the overlapping capsid proteins VP1–4, are shown. The RNAs overlap in the center of the genome. Polyadenylation is shown by a squiggly line. The different open reading frames (ORFs) that are used are shown in different shading patterns.

The transcription pattern of the *Densovirus* genus has been more fully characterized (Tijssen *et al.*, 2003; Fediere *et al.*, 2004). Maps of GmDNV and MIDNV have been determined in detail and are quite similar (see Figure 18.10; the GenBank accession number for GmDNV is L32896). These viruses have ambisense genomes. The non-structural proteins are expressed from an ORF in the left half of the genome. Pre-mRNA is generated from a single promoter near the left-hand end and is polyadenylated in the center of the genome. An unspliced version of this RNA encodes the NS3 protein from the first available ORF (ORF 1) at the 5' end of the molecule. This pre-mRNA is also spliced to generate a second mRNA, from which an intron that encompasses the NS3 coding region is removed. The resulting spliced RNA encodes the NS1 and NS2 proteins. NS1 initiates at an AUG immediately after the splice donor and continues in ORF 2 to the 3' end of the mRNA. The smaller NS2 is initiated a few nucleotides downstream of NS1, and continues in ORF3, until its termination within the NS1 gene.

The capsid proteins are translated from an ORF on the right-hand side of the genome; however, from a RNA generated in the opposite orientation and from a template of the opposite sense as those encoding the non-structural proteins. A single major transcript is made that is driven inboard from a promoter near the right-hand hairpin and also polyadenylates in the center of the genome. This transcript encodes the multiple major capsid proteins, presumably by a leaky-scanning type mechanism.

## SUMMARY

Because the transcription profiles of numerous parvoviruses have now been determined, some generalities can be drawn. All the parvoviruses use a wide variety of alternative RNA processing strategies to maximize the coding capacity from their small genomes. Different processing strategies result in the generation of multiple RNAs that typically encode one large and 2–5 smaller non-structural proteins that share at least some amino acid sequence, and 2–3 overlapping capsid proteins. Alternative splicing is a key to determining the relative accumulated levels of parvovirus proteins, which is a critical feature of infection.

Most parvovirus genomes are transcribed in overlapping transcription units from multiple promoters and all contain small introns in the center of the genome. All transcripts generated by the multiple promoters of the autonomous parvoviruses MVM, H1, CPV, and FPV and PPV and the adeno-associated viruses type 1, 2, 3, 4, and 6 polyadenylate at the far right-hand end of the genome.

For MVM the small central intron appears to be the focal point for entry of the spliceosome onto MVM RNA. Splicing of the overlapping small intron is very efficient, and the small intron is excised at similar levels from all classes of MVM RNA. Subsequent to small intron recognition and splicing, the upstream NS2-specific exon is defined and excision of the upstream large intron occurs. This second step must be slowed (likely by the large intron non-consensus donors

and acceptors), so that singly spliced RNA can exit to the cytoplasm to encode NS1.

AAV1, 2, 3, 4, and 6 have a single intron that is excised to greater levels from RNAs generated by the intron proximal P40 promoter than from RNAs generated by P19 or P5. Therefore, these viruses generate relatively more Rep78 and Rep52 from unspliced RNAs generated by upstream promoters and more spliced RNAs from P40, which encode the capsid proteins. The level of spliced AAV2-generated RNA increases as infection progresses. Expression of Rep78 and Rep52 requires that unspliced AAV RNAs be transported efficiently to the cytoplasm. Therefore, translation of Rep78 and Rep52 from unspliced RNAs may compensate for the inefficient splicing of P5- and P19-generated RNAs.

Splicing of AAV2 RNA requires both the Rep protein targeted to the transcription template and helper virus co-infection. Rep's role in RNA processing has not been separated from its role as a transactivator of transcription initiation. The distance-dependence of AAV2 RNA splicing is most likely related to the strength of interaction of the U1snRNP with the intron donor, and this interaction is governed by the mechanism that controls 5' exon definition. Therefore, as the size of the 5' terminal exon increases, excision of the intron becomes less efficient. Whether Rep facilitates splicing of P40 RNA by directly influencing RNA processing factors associated with the elongating RNA polymerase, or whether Rep facilitates splicing by localizing the transcription template to helper virus-induced nuclear sites that are rich in RNA processing factors has not been clearly determined. However, most AAV serotypes are much less dependent on helper virus for the transcription and processing of their RNA. Perhaps this is related to a longer co-evolution of AAV2 and adenovirus in a tissue culture system.

Transcripts generated by the left-end promoter of AAV5, GPV, AMDV, B19, and SPV polyadenylate at a site in the center of the genome (pA)p. For AAV5, GPV, and AMDV, transcripts generated from promoters near the center of the genome read through the internal polyadenylation site to the distal site. Polyadenylation of AAV5 P7- and P19-generated RNA at the internal (pA)p site results in reduced accumulation of the Rep proteins encoded by spliced RNA, and thus AAV5 makes little or no Rep68 and Rep40. From the RNA processing standpoint, there is an intriguingly complex set of determinants and interactions that control the choice of splicing and polyadenylation for AAV5 RNA. This choice is clearly related to the distance between the AAV5 promoter and the intron and the (pA)p site it contains and like AAV2, seems most likely to be related to the ability to define the 5' exon of these RNAs. Unraveling the mechanism that controls the distance-related dependence of AAV RNA processing remains of great interest.

Interestingly, all the AAV serotypes that do not use the internal (pA)p site retain a cryptic copy of this motif at the same place within their genomes. The use of this site seems dependent upon a strong DSE that is present in AAV5 (and

GPV) but not the other AAV serotypes. It will be of interest to unravel the evolutionary significance behind the use of (pA)p. However, the protein products produced using the two processing strategies are quite similar.

The various AAV serotypes show a wide spectrum of dependence on helper virus for the expression and processing of their RNA. This ranges from AAV2, which is dependent on both helper virus and Rep for efficient transcription and processing of the P40-generated RNAs, to AAV5, which is less responsive to helper virus and the role of the Rep protein. GPV, which shares significant homology with the AAVs, is helper independent. Further characterization of their expression profiles may shed light on the evolutionary relationship between these viruses.

The erythroviruses B19 and SPV have a single promoter and so alternative processing of a single pre-mRNA molecule generates the full spectrum of RNAs that are expressed by these viruses. Polyadenylation at an internal site is also a critical event for erythroviruses. Use of (pA)p prevents the accumulation of RNAs required to generate the capsid proteins and the regulation of this internal polyadenylation event has been suggested to be a critical determinant of B19 tropism and pathogenesis. In contrast to AAV5, for both B19 and SPV, the internal polyadenylation site remains unused in the capsid encoding VP1 mRNA. It is interesting that B19, SPV, AMDV, GPD, DPV, and AAV5, all of which use an internal polyadenylation site, are clinically pathogenic in their hosts.

In addition to the complex alternative RNA processing strategies that are used to generate multiple mRNA molecules from compact genomes, the expression of the parvovirus genome is further governed, in certain cases, by translational control of these RNAs. For example, the AAV2 capsid proteins VP2 and VP3 are encoded by the same mRNA, and the relative levels at which these two proteins accumulate is controlled by differences in translation initiation. Perhaps the most striking example is found in the densoviruses. This group uses a novel ambisense strategy to express its genome, and multiple capsid proteins are generated from a single mRNA molecule, presumably by a leaky-scanning type mechanism.

The small parvovirus genomes use complex patterns of initiation, processing, export, and translation of their RNA to express their genomes. Characterization of these features will further our understanding of the biology of these viruses, and help unravel their evolutionary relationships. In addition these viruses provide a rich system, amenable to study, in which to examine basic cellular mechanisms of gene expression.

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# Regulation of non-structural protein functions by differential synthesis, modification and trafficking

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In contrast to the large DNA viruses such as poxviruses, the limited complexity of the parvovirus genome means that these viruses generally adapt host cell ‘factories’ for the synthesis of viral components, rather than encoding their own protein/DNA synthesis machinery. This strategy is implemented and orchestrated by a few multifunctional viral regulatory proteins, the non-structural (NS) or Rep proteins, which interfere with host cell physiology at several distinct levels, thereby adapting the existing DNA replication, protein synthesis and transport systems of the host cell. This reprogramming must ensure efficient production of all the viral components necessary for the synthesis of progeny genomes and their protein shells, for dispersing these particles, and eventually for evading the antiviral responses of their host. During parvovirus infections this overwhelming adaptation of the host cell environment is mediated by as few as four proteins, whose individual properties and activities are tightly regulated. To achieve this, they are modulated during the course of infection by post-translational modification, by regulation of their compartmental distribution within the cell, e.g. their nuclear/cytoplasmic distribution, and by interactions with specific cellular partner proteins, forming novel complexes with distinct specificities and activities. The biochemistry, post-translational modification, regulation, and trafficking of the large non-structural NS1 protein of the autonomous parvovirus minute virus of mice (MVM) has been investigated in some detail, and will be the primary focus of this review. In addition, to draw more general conclusions about the regulation of non-structural protein function, observations obtained for the MVM small non-structural NS2 proteins and the adeno-associated virus

(AAV) Rep proteins will be included. Finally, this review concludes with a discussion of the possible interconnections between the activities of different viral NS proteins.

## PRODUCTION AND MODIFICATION OF PARVOVIRUS NON-STRUCTURAL PROTEINS

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Unlike other mammalian DNA viruses, protein production during parvovirus infection is initially constrained by the need to generate a double-stranded transcription template. This S-phase dependent process, mediated by cyclin A (Cotmore and Tattersall, 1987; Bashir *et al.*, 2000), limits parvovirus propagation to actively dividing cells. However, in quiescent cell populations parvoviruses can persist within their target cell until it progresses into S-phase. This cell-cycle transition cannot be induced by the virus, but can be evoked by outside stimuli including transforming agents such as tumor viruses (Cotmore and Tattersall, 1987). Following conversion of the single-stranded virion DNA to a duplex-intermediate, both NS1 and NS2 proteins are expressed from the viral P4 promoter, prior to the onset of capsid protein production. This delay in capsid protein expression is due to its dependence on the NS1 protein, which regulates its synthesis at the transcriptional level by *trans*-activating the viral P38 promoter (Rhode and Richard, 1987). The eventual accumulation of individual parvoviral regulatory proteins is also determined by splicing events and by the differential stability of these polypeptides. The large non-structural NS1 protein is required in stoichiometric amounts

to mediate a variety of viral processes, most notably DNA replication, and this protein progressively accumulates throughout the course of infection, in part due to its relatively long half-life (>6 hours). In contrast, NS2 accumulates early in infection, at a time of strong P4-promoter activity, but its concentration drops at later stages (Schoborg and Pintel, 1991; Corbau *et al.*, 1999) because it has a relatively short half-life (~90 min), being actively degraded by proteasomes through a ubiquitin-independent pathway (Miller and Pintel, 2001). These differences in viral protein accumulation during infection suggest that, at least late in infection, when the steady-state levels of NS2 are significantly reduced (Corbau *et al.*, 1999), the limited availability of these proteins may seriously impinge on viral metabolism. However, the functional implications of such regulation and the significance of the three individual NS2 isoforms, generated through alternative splicing in varying amounts that reflect their mRNA levels (Cotmore and Tattersall, 1990; Schoborg and Pintel, 1991), have yet to be determined.

Production of the adeno-associated virus non-structural (Rep) proteins is regulated at the level of gene expression in a more complex manner. While the autonomous parvovirus NS proteins are expressed from a single promoter, production of AAV Rep-proteins is regulated through two different promoters. Accumulation of the large Rep78/68 proteins is driven by the P5-promoter, while small Rep52/40 proteins are produced from transcripts expressed from the P19 promoter. The Rep78/68 proteins themselves directly modulate expression from these two promoters, suppressing transcription from P5, while activating the P19 promoter and thus assure the desired steady-state levels of each viral protein (Pereira *et al.*, 1997). In addition, like the autonomous parvovirus NS2 proteins, mRNA splicing produces alternate forms of the non-structural proteins (Qiu and Pintel, 2002), so that both large and small Rep proteins exist in two isoforms with disparate carboxy-termini.

Functional differences between the AAV small Reps and the autonomous parvovirus NS2-proteins parallel differences in their expression profiles. Interestingly, both NS2 and Rep52/40 proteins have been implicated in the subcellular trafficking of viral capsid proteins, in assembly of capsid shells and in the packaging of progeny genomes (Cotmore *et al.*, 1997; Wistuba *et al.*, 1995, 1997; Dubielzig *et al.*, 1999; King *et al.*, 2001). However, the autonomous parvovirus NS2 proteins seem to impinge on these processes without exerting any known enzyme activity, while Rep52/40 proteins are actively involved, providing DNA/DNA and DNA/RNA helicase activity (Smith and Kotin, 1998), which is essential, for example, for the efficient packaging of single-stranded virion DNA (King *et al.*, 2001). Functional differences between these proteins inevitably mean that they are required at different concentrations during the course of infection, which is reflected in their alternative modes of regulation. In summary, regulation of the steady-state levels of the individual viral proteins is, at least in part, controlled by the proteins themselves. Differential accumulation of

individual proteins at different phases in the infectious cycle probably reflects progressive changes in viral metabolism.

Although there are differences in the accumulation of individual regulatory proteins at different phases in the life cycle, such modulation cannot necessarily be seen as implicating specific proteins in particular *in vivo* processes. This is partly because each individual protein species serves multiple roles in the viral life cycle and partly because, after the initial onset of viral gene expression, all proteins remain expressed at significant levels throughout the course of infection. This suggests that specific activities of each viral polypeptide may also be regulated by post-translational modification. Protein functions can be altered post-translationally by tagging specific amino acids with small alkyl, fatty acid, carbohydrate or phosphate groups and by proteolytic cleavage of the polypeptide (Cotmore and Tattersall, 1990; Nüesch and Tattersall, 1993). For example, caspase cleavage of the Aleutian mink disease virus NS1 molecule is known to be essential for productive infection (Best *et al.*, 2003). This modification is apparently necessary for the nuclear import of both cleaved and uncleaved forms of NS1, but whether the cleavage influences viral amplification directly, by generating a protein with altered enzymatic activity, or indirectly, by regulating the cellular compartmentalization and hence the activity of the 'uncleaved' NS1 protein (see below), remains to be determined.

Although the spectrum of possible post-translational changes is broad, differential phosphorylation of specific amino acids is perhaps the most widely recognized mode of regulating cellular and viral protein function. It has long been recognized that parvoviral regulatory proteins become phosphorylated at serine and threonine residues during infection and thus could be subject to functional regulation through cellular protein kinases (Molitor *et al.*, 1985; Cotmore and Tattersall, 1986). NS1 and NS2 resolve into several isoforms following 2D chromatography (Santaren *et al.*, 1993) and tryptic phosphopeptide analysis suggests that these isoforms are, at least in part, due to differential phosphorylation. Thus, NS1 yields at least 10 different tryptic phosphopeptides, while NS2 yields at least four (Corbau *et al.*, 1999; Nüesch, unpublished observations). In addition, changes in the phosphorylation pattern of NS1 during the course of infection strongly suggest that these modifications are temporally regulated, as might be expected if proteins carrying different phosphorylation patterns mediated different NS functions. These variations, together with the multitude of potential regulatory sites present in NS1 and NS2, prompted us to investigate interactions between the viral proteins and cellular signalling pathways in more detail.

## FUNCTIONS OF PARVOVIRAL NON-STRUCTURAL PROTEINS

Parvoviral regulatory proteins have been implicated in a wide range of different functions during the viral life cycle. NS1

and the large AAV Rep proteins have been dissected in particular detail and have been shown to contain multiple distinct domains, which act in concert or alone to mediate activities essential for viral DNA amplification, transcriptional regulation, and interactions with the host-cell environment at many different levels. These structural domains are illustrated in Figure 19.1. There are extensive similarities between the large Rep78/68 proteins of AAV and the autonomous parvovirus NS1 proteins, reflecting the similar roles these proteins play in many aspects of viral DNA replication and transcription, so that details discussed below for MVM NS1 may also be relevant for their AAV counterparts.

## Functional interplay between different NS1 domains

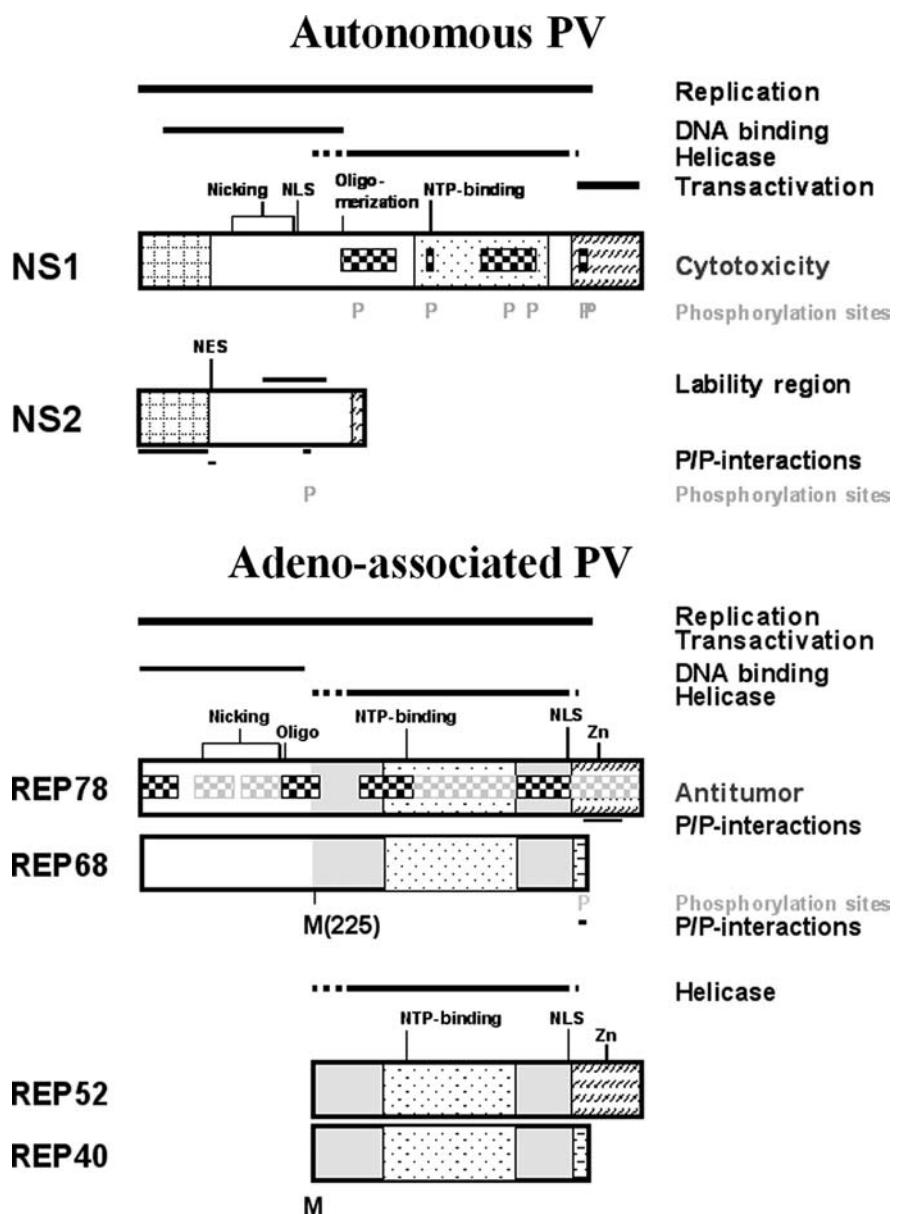
As seen in Figure 19.1, the large non-structural protein of MVM contains an ATP-binding domain (Wilson *et al.*, 1991; Christensen *et al.*, 1995b). Nucleotide triphosphate (NTP)-binding appears to promote NS1 self-assembly into (higher order) oligomers (Nüesch and Tattersall, 1993) and this, in turn, is essential for its ability to bind site-specifically to a consensus duplex DNA-recognition motif, (ACCA)<sub>2–3</sub>, (Cotmore *et al.*, 1995) and contributes to its cytotoxic potential (Li and Rhode, 1990; Legendre and Rommelaere, 1992; Corbau *et al.*, 2000). Hydrolysis of bound NTP allows NS1 (Wilson *et al.*, 1991; Christensen *et al.*, 1995b) and the related Rep proteins (Im and Muzychka, 1990) to transduce energy. A variety of alternative nucleotides can be substituted for ATP, although the efficiency of the resulting energy transfer appears to vary significantly (Zhou *et al.*, 1999). This ATP-driven motor is essential for NS1/Rep duplex DNA unwinding activity (Im and Muzychka, 1990; Wilson *et al.*, 1991; Christensen *et al.*, 1995b; Nüesch *et al.*, 1995). During viral DNA replication, ATPase activity is initially required to separate the strands of the duplex replication origin, generating a partially single-stranded DNA-bubble at the consensus nick site, which then allows NS1 to introduce a site- and strand-specific nick via an energy-neutral *trans*-esterification reaction (Snyder *et al.*, 1993; Brister and Muzychka, 1999; Nüesch *et al.*, 2001). NS1 also serves as a processive 3' to 5' helicase in the ensuing replication complex, hydrolysing ATP to provide the energy needed to unwind the double-stranded DNA template in front of the fork (Nüesch *et al.*, 1998a; Christensen and Tattersall, 2002).

The helicase activity of NS1 is also dependent on its ability to self-assemble into oligomers (Pujol *et al.*, 1997). This self-assembly may, at least in part, be mediated through a novel bipartite motif 261-VETTVT(X<sub>9</sub>)IQT-278 located between the DNA binding and helicase domains of NS1. By analogy with similar helicases, such as the large T antigen of simian virus 40 (SV40 LT; Mastrangelo *et al.*, 1989) and Rep40 (James *et al.*, 2003), NS1 probably assembles into a hexameric-ring structure to carry out this function. In view of the many different activities carried out by multimeric

forms of NS1 during origin recognition (site-specific binding of the double-stranded DNA), origin unwinding and nicking (site-specific interaction with a single-stranded recognition sequence), and helicase activity (non-specific [ss] DNA binding), it is likely that its oligomeric structure fluctuates dynamically *in vivo*, thus increasing the functional potential of the protein by allowing it to adopt different conformations that support different biochemical activities (Hickman *et al.*, 2004).

NS1 carries additional motifs and domains that enable it to support other essential processes in the infectious cycle. For example, it serves as a transcription factor, *trans*-activating capsid gene expression from the viral P38-promoter (Legendre and Rommelaere, 1992) via an acidic carboxy-terminal domain (Legendre and Rommelaere, 1994). NS1 appears to deliver this peptide to the transcription complex via a number of different routes. First, it binds to a copy of its cognate DNA-binding motif (ACCA)<sub>2–3</sub> (Christensen *et al.*, 1995a) positioned in the so-called trans-activation region, or *tar*, element that lies upstream of the P38-promoter (Gavin and Ward, 1990). This interaction is mediated by its amino-terminal DNA-binding domain (amino acids 16–275; Mouw and Pintel, 1998). However, as discussed below, NS1 also interacts with a cellular transcription factor, SP1, which binds to the P38 promoter (Kraday and Ward, 1995) and with members of the general transcription machinery TBP and TFIIB (Lorson *et al.*, 1998), so that its trans-activation domain appears to be positioned in the transcription complex via multiple interactions.

Multiple NS1-domains must also work in concert during various aspects of viral DNA amplification. As discussed previously, NS1 both controls the initiation of viral DNA replication and serves as part of the replication fork that amplifies viral templates and generates progeny single-stranded genomes. NS1 interacts directly with the viral replication origins via its amino-terminal DNA-binding domain. As seen in Figure 19.1, this region also contains a hydrophobic metal coordination site and an active site tyrosine motif that are similar to those found in the replicator proteins of bacteriophages, single-stranded plasmids and Gemini viruses (Koonin and Ilyina, 1993). Besides being directly involved in recognition of the (ACCA)<sub>2–3</sub>-site (Nüesch *et al.*, 1995), this part of the protein is essential for the *trans*-esterification reaction that introduces a site- and strand-specific nick into the replication origin (Nüesch *et al.*, 1995). It has been shown that both Mg<sup>2+</sup> and Mn<sup>2+</sup> ions can function as cofactors for this nicking reaction in AAV, while Ca<sup>2+</sup> or Zn<sup>2+</sup> ions stimulate the process only marginal or not at all (Yoon-Robarts and Linden, 2003). The nicking reaction leaves NS1 covalently attached to the 5' end of the DNA at the nick site (Nüesch *et al.*, 1995; Cotmore and Tattersall, 1988; Snyder *et al.*, 1990), while generating a new 3' nucleotide that serves as an essential primer for subsequent strand-displacement synthesis, driven by DNA polymerase δ. This synthesis amplifies viral DNA via a unidirectional rolling-circle mechanism (see Chapter 14) in which the NS1



**Figure 19.1** Domain structure of parvovirus non-structural proteins. Functional domains of the parvovirus regulatory proteins have been extensively mapped using site-directed mutagenesis and analysis of wild-type and mutant polypeptides in biochemical assays performed in vitro and in vivo (for references see text). **Structural features:** owing to alternative splicing autonomous parvovirus, NS1 and NS2 proteins share a common amino-terminus of 84 amino acids (cross-hatched box), while the main body and the (unique) carboxy-termini of the three NS2 isoforms, NS2<sub>P</sub>, NS2<sub>Y</sub> and NS2<sub>L</sub> (hatched box) differ because of differential splicing and the use of alternative reading frames (Cotmore and Tattersall, 1990). In contrast to the NS proteins of autonomous parvoviruses, AAV REP proteins all share sequence but are different lengths due to the use of alternative promoters at map positions 5 and 19. The small REP proteins, REP52/40, initiate at methionine M225 in the large REPs (light gray box, REP78/68 amino acids 225 to 531 REP52/40 amino acids 1 to 306). This common domain present in all four REP proteins harbors the nuclear localization signal (NLS; amino acids 493 to 509), an NTP-binding motif, and a helicase domain. As in NS1, the helicase domain (dotted box; NS1 amino acids 394–486; REP78/68 amino acids 329–421; REP52/40 amino acids 105–196) shares a high degree of homology with the helicase domain of SV40 LT (Astell et al., 1987; Jindal et al., 1994). The amino-terminus of the large REP proteins contains the oligomerization, DNA-binding, and nicking motifs. Recently, this domain was crystallized and its structure determined (Hickman et al., 2002). The last 80 carboxy-terminal amino acids of REP78 and REP52 (hatched box) contain a zinc-finger motif (amino acids 534–594), while the carboxy-terminal 14 amino acids of REP68 and REP40, generated through splicing events, lack this feature. **Functional motifs and domains present in the individual non-structural proteins:** NS1 (MVMp; 672 amino acids). Individual motifs and domains involved in viral DNA amplification are scattered throughout the polypeptide. Only the extreme carboxy-terminal 67 amino acid are dispensable for this NS1 function. The site-specific DNA binding domain is located between amino acids 16 and 275 (Mouw and Pintel, 1998). Within this

polypeptide that is covalently attached to the 5' end of the nicked strand is presumed to be translocated with the fork, functioning as part of the replicative helicase. The NS1 helicase domain is composed of five parallel beta-sheets and contains an ATP-binding pocket and a Mg-binding site (Astell *et al.*, 1987; Jindal *et al.*, 1994). During viral DNA amplification, NS1 interacts directly with cellular proteins from the basic DNA replication machinery and with cellular transcription factors. As discussed below, interactions with such cellular partners can directly influence the properties of the polypeptide, activating it to carry out specific reactions, e.g. nicking origin DNA (Christensen *et al.*, 2001). The ability of NS1 to interact site-specifically with its cognate DNA-recognition motif, (ACCA)<sub>2-3</sub>, plays a pivotal role during virus propagation. This site is not only found in the viral origins of replication and the *tar* element, but is also distributed at multiple sites throughout the genome (Cotmore *et al.*, 1995), so that additional, as yet undocumented, NS1 functions requiring site-specific DNA-binding to these elements may exist.

The complex domain structure of NS1, and the need to coordinate combinations of these domains for specific functions, suggests that the conformation of this polypeptide

may be carefully regulated. As seen for many cellular proteins, conformational changes in a protein can be induced through a variety of different mechanisms, including interactions with small compounds or metal ions, interactions with partner proteins, differential self-assembly and post-translational modification. All these strategies appear to be used to modify both NS1 and the large Rep proteins. As discussed previously, ATP binding induces oligomerization of NS1, which is required before it can bind site-specifically to its DNA recognition motif. The resulting NS1/DNA complex is most stable in the presence of a non-hydrolyzable ATP-analogue, such as ATP- $\gamma$ -S, while induction of ATPase activity (e.g. by supplying the cofactor Mg<sup>2+</sup>), reduces NS1's DNA-binding affinity (Christensen *et al.*, 1995a; Cotmore *et al.*, 1995). These findings tend to suggest that the presence of a non-hydrolyzable analogue in its NTP-binding pocket locks the NS1 multimer in a conformation that promotes self-assembly and/or interaction with the DNA, while ATP hydrolysis reduces both the affinity of NS1 for the nucleotide and in turn changes its conformation so that it releases the DNA. Whether the presence of alternative nucleotides such as GTP, which promote DNA-binding but support NS1's helicase activity very inefficiently (Christensen *et al.*,

*domain, a hydrophobic metal coordination site, 126-wHcHvligg-134, similar to the copper-binding site of cytochrome B (Koonin and Ilyina, 1993) and an active site tyrosine, 210-YfltK-214, are located. These two essential motifs are involved in site-and strand-specific nicking, while the two tyrosines, Y188 and Y197, play an important role for site-specific DNA binding (Nüesch *et al.*, 1995). The sequence and structure of the helicase domain, which spans amino acids 394–486, show remarkable similarities to that of SV40 LT. This domain harbors the NTP-binding pocket, 399-GpaSTGKiiqal-411, where K405 interacts with the alpha-phosphate group, the nucleoside interaction site around D467, and the two negative charges, EE443/444, which are thought to bind its Mg<sup>2+</sup> cofactor that is required to hydrolyse the nucleotide (Li and Rhode, 1990; Nüesch *et al.*, 1992; Jindal *et al.*, 1994). The carboxy-terminal 67 amino acids serve as a transactivator domain for P38 promoter activation (Legendre and Rommelaere, 1994). In addition, an interaction site, 261-VETTVT(X<sub>9</sub>)IQT-278, which controls self-assembly into (higher order) oligomers (Pujol *et al.*, 1997), and a bipartite nuclear localization signal, 194-KK(X<sub>31</sub>)KKK-218 (Nüesch and Tattersall, 1993), have been mapped. Regions within the polypeptide that are implicated in the induction of CPE are indicated with chequered boxes (Corbau *et al.*, 2000). The six target phosphorylation sites identified for NS1, S283 (Nüesch, unpublished observations); T403, T435 (Corbau *et al.*, 2000), S473 (Dettwiler *et al.*, 1999), T585, and S588 (Daeffler *et al.*, 2003), are indicated. T435 and S473 are known targets for PKC $\alpha$  (Dettwiler *et al.*, 1999; Nüesch *et al.*, 2001). NS2 (MVMp 188 amino acids): A nuclear export signal, NES 96-MtkkFgtLtl-105 (Eichwald *et al.*, 2002), and a variety of sites that interact with cellular partner proteins have been identified in the small non-structural proteins. SMN-proteins interact within the common amino-terminus of all four MVMp NS proteins (amino acids 1–84), CRM1 with the region harboring the NES (Eichwald *et al.*, 2002; Miller and Pintel, 2002), members of the 14-3-3 family (Brockhaus *et al.*, 1996) with the region around T149, which needs to be phosphorylated (the mapped phosphorylation site is indicated; Bodendorf *et al.*, 1999; Salome, unpublished). A lability region involved in proteosome-mediated, ubiquitin-independent, degradation of the polypeptides is located between amino acids 131–146 (Miller and Pintel, 2001). REP78/68 (AAV2; 606, 545 amino acids): Domains involved in viral DNA amplification are spread throughout most of the polypeptide (amino acids 1–519), except the unique carboxy-termini. The site-specific DNA binding domain (amino acids 1–208; Im and Muzyczka, 1992) harbors the active site tyrosine, Y156 (Davis *et al.*, 2000), a hydrophobic metal coordination site, 89-fHmHvlv-95 with a critical glutamic acid E83 (Yoon-Robarts and Linden, 2003), and one part of the oligomerization signal (oligo; amino acids 162–182; 332–346). The helicase domain has been crystallized (James *et al.*, 2003). This domain, which is common to all four REP proteins (amino acids 225–519), harbors the NTP-binding pocket, where K340 coordinates with the alpha-phosphate group of the nucleotide (Chejanovsky and Carter, 1990). This region includes an NLS, 493-KRP(X)<sub>9</sub>PKRVR-509 (Yang *et al.*, 1992; Kleinschmidt *et al.*, 1995), which allows all four REP proteins to become translocated efficiently to the site of replication. Regions within the large REP proteins that are thought to be involved in ras/E1A-induced transformation-inhibition (Yang *et al.*, 1992) are indicated by chequered boxes (black for strong, grey for partial inhibitory effects). Regions that are important for interactions with cellular proteins, i.e. PKA and PRKX with REP78(52) (Di Pasquale and Stacey, 1998), and 14-3-3 with REP68(40), and the target phosphorylation site, S535, which is involved in regulation of the 14-3-3 interaction (Han *et al.*, 2004), are indicated. REP52/40 (AAV2; 382, 321 amino acids): The helicase domain located in the common region of all four REP proteins is essential during packaging of single-stranded virion DNA into preformed capsids (Smith and Kotin, 1998; Dubielzig *et al.*, 1999; King *et al.*, 2001).*

1995a,b), plays a role in regulating NS1 activity *in vivo*, however, remains to be determined. In addition to the Mg<sup>2+</sup>-binding site at amino acids 443/444 that supports its ATPase activity (Jindal *et al.*, 1994; Christensen *et al.*, 1995b), NS1 also has a metal ion-binding site at residue 126, which is essential for its nucleolytic activity. It seems possible that co-factor binding to this (uHuHuuu) site, which closely resembles the copper-binding site of cytochrome B (Koonin and Ilyina, 1993), could also induce local conformational changes in the polypeptide.

Post-translational modification, particularly phosphorylation, is commonly used in eukaryotic cells to induce local conformational changes in polypeptides that prime them for particular functions. Such modifications can often be detected by changes in the electrophoretic mobility of the protein under denaturing conditions, as observed for phosphorylated forms of parvoviral non-structural proteins (Corbau *et al.*, 1999). These local conformational changes may, in theory, open ATP-binding and/or metal coordination sites, allowing co-factor-binding and the consequent activation or modulation of specific enzyme activities. Alternatively, post-translational modification may render specific peptide domains available for interaction with target DNA or partner proteins, forming ternary complexes that support new activities. Similarly, directly modified peptide domains within a single viral polypeptide may interact with each other in different ways, promoting or impairing specific biochemical activities.

## Adaptation of cellular processes

The formation of multiprotein complexes is a key-feature in ensuring efficient production of viral progeny. Viral non-structural proteins may serve as scaffold proteins for these complexes, coordinating the activities of cellular enzymes. Alternatively, particular functional domains of NS may be activated in these complexes via interactions with specific cellular protein partners. This could be achieved either by working in concert with the interaction partner(s), or through changes in NS or cellular protein configurations. Several cellular interaction partners for both NS1 and NS2 have been identified to date, which illustrate a variety of these types of interaction. For example, during viral DNA replication NS1 and the large Rep proteins of AAV interact with the cellular single-strand DNA-binding protein, replication protein A (RPA; Christensen and Tattersall, 2002; Stracker *et al.*, 2004). By recruiting this central element from the cellular replication machinery, the virus is able to subvert and adapt cellular replication complexes for its own preferentially amplification. NS1 also interacts with components of the basic transcription machinery (Lorson *et al.*, 1998), and selected transcription factors (Krady and Ward, 1995) and, although the mechanism of these interactions has not been explored in depth, it seems possible that the viral protein once again usurps the cellular machinery by preferentially recruiting essential elements to the viral template.

In a very different type of interaction, the virus also usurps a cellular transcription factor, called glucocorticoid-modifying element-binding protein (GMEB), to mediate site- and strand-specific nicking at the left-end replication origin of MVM. In the parvovirus literature this heterodimeric factor is called parvovirus initiation factor (PIF), and it binds site-specifically to a position in the active form of the left-end origin that allows it to interact directly with NS1 bound at its adjacent cognate recognition site, forming a ternary complex that stabilizes the NS1-DNA interaction (Christensen *et al.*, 2001). This enhanced binding allows NS1 to remain on the DNA while it unwinds the duplex DNA at the adjacent nick site, which is an essential prelude to nicking (Christensen *et al.*, 2001; Nüesch *et al.*, 2001). Thus, although NS1 itself possesses all the required enzyme activities, it is unable to nick the origin without PIF. In AAV the conformation of the viral replicator protein, and its ability to nick the origin, is altered through distinct site-specific interactions with the hairpin-structure of the substrate DNA, rather than by interactions with a cellular partner. In addition, structural data obtained for the DNA-binding domain of the AAV5 Rep78/68 protein suggest that conformational switches occur during the nicking reaction itself (Hickman *et al.*, 2004).

Adaptation of cellular responses that result in NS1-induced cytotoxicity are much less well understood. In part this may be because different death pathways are triggered in different virus/host cell systems. Thus, while some autonomous parvovirus infections clearly induce apoptosis in the host cell (Rayet *et al.*, 1998), some cells, like the murine A9 cell line that is a standard host for the prototype form of MVM (MVMp), resist various arms of this cell death pathway, but eventually die from necrosis. In addition, cell death induced by NS1, as measured, for example, by colony formation inhibition assays (Li and Rhode, 1990; Legendre and Rommelaere, 1992), is most likely the end-product of multiple as yet undefined activities. These may include virally-induced cytostatic effects, such as cell cycle arrest in late S/G2-phase (Op De Beeck and Caillet-Fauquet, 1997; Op De Beeck *et al.*, 2001), reprogramming of cellular signaling pathways (Anouja *et al.*, 1997; Lachmann *et al.*, 2003; Nüesch *et al.*, 2003), and the induction of cytopathic changes (Caillet-Fauquet *et al.*, 1990).

NS1 expression induces a series of morphological alterations in the cell, termed cytopathic effects (CPE), which cause the cell to round-up and detach from its substrate (Caillet-Fauquet *et al.*, 1990; Corbau *et al.*, 2000). Processes which precede the establishment of this phenotype include the formation of subnuclear structures, termed autonomous parvovirus replication (APAR) bodies, which are the sites of early viral DNA replication (Cziepluch *et al.*, 2000; Bashir *et al.*, 2001), and which accumulate a variety of viral and cellular proteins (Cziepluch *et al.*, 2000; Bashir *et al.*, 2001; Young *et al.*, 2002a,b). Within the cytoplasm, MVM is also able to modulate the dynamics of distinct cytoskeletal filaments, which may explain why cells appear to collapse late

in infection (Nüesch *et al.*, 2004). To correlate the known activities of NS1 with its ability to induce cell death, we generated a number of mutant NS1 genes, and characterized their biochemical properties *in vitro* and their ability to induce CPE after transfection (Corbau *et al.*, 2000; Daeffler *et al.*, 2003). During the course of infection many different NS1 activities may contribute to the induction of cell death, but our investigations revealed that relatively few NS1 functions are required to induce efficient rounding-up and detachment of the host cell from its culture substrate. Neither replication activities nor activities involved in *trans* activation of the P38 promoter were required or even contributed to this phenotype at early times after transfection (Corbau *et al.*, 2000). In fact, the only NS1 activities required for CPE induction appeared to be the presence of an intact ATP-binding pocket and NS1's ability to self-assemble into higher order oligomers. Interestingly, mutants that affected consensus phosphorylation sites in the protein impaired its ability to induce CPE, and these tended to be clustered within distinct peptide domains (Corbau *et al.*, 2000). In general, these studies suggested that morphological alterations leading to cell collapse are not induced by any intrinsic biochemical property of the viral proteins, but rather result from the assembly of NS1-containing multiprotein complexes. In view of the observed cellular phenotype resulting from expression of NS1 variants, it seemed likely that MVM-induced CPE is mediated via the host cell cytoskeleton (Nüesch *et al.*, 2005). We recently showed that NS1 was able to interact directly with CKII $\alpha$ , the catalytic subunit of casein kinase (Nüesch and Rommelaere, 2004). Formation of this novel complex suggests that the viral protein is able to recruit additional enzyme activities. In combination with other interaction partners, in particular the cytoskeleton filament protein tropomyosin (Nüesch and Rommelaere, 2004), this novel interaction may allow NS1 to interfere actively with cellular signaling and hence change the host cell physiology. It may also act as an adaptor protein, bringing together cellular proteins that are normally unable to interconnect. Whether this, for instance, allows the formation of the nuclear (APAR) bodies remains to be elucidated.

We still have only a relatively superficial understanding of how the viral regulatory proteins coordinate the progress of viral infections. However, it seems likely that most of the enzyme activities discussed above could be subject to higher order regulation, through modulated cellular signaling events. For example, post-translational modification could influence co-factor affinity and/or the rate of ATP-hydrolysis, induce conformational changes in the proteins and/or modify interaction domains involved in oligomer formation, protein/DNA interactions, or affinity for cellular proteins partners. Since parvoviral non-structural proteins are subject to a complex and varying pattern of phosphorylation during the course of infection (Molitor *et al.*, 1985; Cotmore and Tattersall, 1986, 1990; Collaco *et al.*, 1997), signaling by cellular protein kinases could provide a potent

mechanism for regulating the coordinated progress of individual NS functions.

## Functions and properties of the small non-structural NS2 proteins

At least three forms of the small MVM non-structural NS2 proteins, with slightly different carboxyterminal peptides, are generated during infection. These small, 25 kDa, polypeptides are dispensable for virus amplification in some cell types, notably transformed non-murine cells (Naeger *et al.*, 1990). However, they are essential for productive replication in cells from its natural rodent host, where they exert multiple regulatory properties. These polypeptides lack known enzyme activities, but interact with multiple cellular partner proteins. In particular, they bind the nuclear export factor chromosome region maintenance 1 (CRM1) and play a central role in the control of nuclear export, including control of progeny virion egress from the nucleus (Eichwald *et al.*, 2002; Miller and Pintel, 2002). Other known interaction partners, like 14-3-3 family members, tend to suggest that NS2 proteins are involved in the modulation of cellular signaling, since 14-3-3 proteins are known influence the regulation of cellular protein kinases and phosphatases. However, direct evidence for such intervention remains elusive. Like NS1, NS2 becomes phosphorylated at multiple residues during infection and is a target for several different cellular kinases *in vitro* (Nüesch, unpublished results). Moreover, phosphorylation and dephosphorylation of these proteins influences their intracellular distribution (Cotmore and Tattersall, 1990) and ability to interact with cellular partner proteins (Bodendorf *et al.*, 1999; Salome, unpublished observations). Thus, as previously discussed for NS1, the small parvoviral non-structural proteins seem to interconnect with cellular signaling pathways in order to become primed for distinct functions during infection.

## Unique features of adeno-associated virus Rep proteins

There are many functional, structural and sequence similarities between the large Rep proteins of AAV and autonomous parvovirus NS1 polypeptides, but there are also notable differences. For example, both the large and small AAV Rep proteins are produced in two forms, which differ at their carboxy termini. Although most of the biochemical activities associated with these forms appear indistinguishable, only the larger product from each promoter (Rep78 and Rep52 from the P5 and P19 promoters, respectively) have distinctive carboxy-terminal zinc-finger motifs. Recent investigations have shown that these domains mediate interactions with the cellular protein kinases PKA and PRKX (Di Pasquale and Stacey, 1998; Di Pasquale and Chiorini, 2003). On the other hand, the carboxy-terminal peptides of Rep68 and Rep40 have been shown to associate with members of the

14-3-3 family, suggesting interconnections between these AAV regulatory proteins and cellular signaling pathways that are not seen for their larger counterparts (Han *et al.*, 2004).

In contrast to the close homology seen between the large Rep and NS1 proteins, the structure and function of the small Rep52/40 and NS2-proteins are strikingly different. Rep52 and Rep40 are abbreviated forms of Rep78 and Rep68 respectively, which initiate at amino acid 225 in the large Rep sequence and thus lack the DNA-binding and nuclease activities of the large Reps, while retaining their 3' to 5' helicase function (Smith and Kotin, 1998). Rep52 and 40 proteins interact physically with AAV capsids (Dubielzig *et al.*, 1999), and their helicase activity is essential for the efficient packaging of single-stranded virion DNA, although this function can also be mediated less efficiently by the larger Rep78/68 polypeptides (King *et al.*, 2001).

## REGULATION OF NS PROTEIN ACTIVITY BY PHOSPHORYLATION

### Non-structural protein activities depend on their phosphorylation pattern

Evidence for the regulation of NS1 function by post-translational modification has emerged from a variety of *in vitro* and *in vivo* studies. Specific inhibitors and site-directed mutagenesis have been used to investigate individual regulatory events, and NS1 functions and properties have repeatedly been shown to be dependent on the phosphorylation state of the protein (Nüesch *et al.*, 1998a). For example, phosphorylated forms of NS1 produced in mammalian cells were shown to be fully replication competent in a kinase-free *in vitro* rolling-circle replication system, while under- or dephosphorylated forms of NS1 were impaired (Nüesch *et al.*, 1998b). This differential activity was traced to the loss of NS1's intrinsic ATPase and helicase functions upon dephosphorylation (Nüesch *et al.*, 1998a). Interestingly, concomitant with this loss of function, dephosphorylated NS1 polypeptides showed an increased affinity for their cognate DNA-recognition motif, an observation that was supported by data from site-directed mutagenesis (see below), and perhaps suggests that these modifications regulate NS1's role in the viral life cycle, balancing its activity as a DNA-bound transcription factor with its involvement in viral DNA replication. Differential phosphorylation may also play an important role in regulating the interplay between different domains in the viral polypeptide, thus helping to prime NS1 for its multiple *in vivo* functions.

In contrast, AAV Rep-driven DNA amplification is negatively regulated by phosphorylation. Hyperphosphorylated Rep proteins, generated in the presence of okadaic acid, were significantly impaired for site-specific DNA binding, nicking, and helicase functions, but these activities were restored when the proteins were treated with a variety of phosphatases. These observations led the authors to speculate

that a cellular phosphatase, PP2A, may play an important role in regulating AAV DNA replication (Narasimhan *et al.*, 2002). In accord with these findings, bacterially produced Rep proteins, which are not phosphorylated, are fully competent for AAV replicative functions (Chiorini *et al.*, 1994), whereas their NS1 counterparts are not (Nüesch *et al.*, 1998a).

These contrasting findings are of particular interest since they suggest that there are important differences in the way these viruses interact with signaling cascades in their host cell. Release from latency and the establishment of a productive AAV infection relies upon the intercession of helper adeno-, herpes or papilloma viruses, which are themselves able to interfere with cellular signaling pathways. Thus, it is possible that AAV may have adapted to the presence of these helper viruses by using cellular phosphatases, rather than protein kinases, to regulate their DNA replication. In summary, there are several examples where cellular signaling pathways have been shown to regulate the activity of parvovirus non-structural proteins, but the details of these interactions appear to differ substantially between viral genera, perhaps adapting them for specific life styles and host cell tropisms.

### Properties of non-structural proteins are regulated through interactions with cellular kinases

Although the MVM NS1 protein is a target for a number of different kinase families *in vitro*, including casein kinase II, protein kinase A (PKA), and cdc2, only members of the protein kinase C (PKC) family, PKC $\alpha$  and PKC $\eta$ , have been shown to modulate distinct biochemical activities *in vitro* (Nüesch *et al.*, 1998a; Dettwiler *et al.*, 1999; Lachmann *et al.*, 2003) and *in vivo* (Lachmann *et al.*, 2003; Nüesch *et al.*, 2003). As described below, regulation of NS1 by PKC $\alpha$  has been analyzed in great detail, but although PKC $\eta$  phosphorylation is known to be essential for NS1-dependent viral DNA amplification, its exact role and target phosphorylation site(s) remain elusive. Since PKC $\eta$  phosphorylation is not needed for the nicking and DNA-helicase functions of NS1, but is essential to coordinate rolling-circle replication mediated by the cellular DNA replication machinery, it seems likely that PKC $\eta$  phosphorylation modulates interactions with components of these replication complexes. Together these two members of the PKC family have an overwhelming impact on MVM NS1 replicative functions *in vivo* and *in vitro* and thus the cellular PDK/PKC signaling pathway appears to play a major role in regulating NS1 activity during infection.

Because of the pre-eminent role played by this kinase family, the majority of analyses performed to date have focused on the influence of consensus PKC phosphorylation sites and their corresponding signaling pathways. This approach has identified several consensus PKC phosphorylation sites in NS1 that are targeted by cellular kinases *in vivo* (i.e. S283, T403, T435, S473, T585, and S588), and site-directed mutagenesis of these amino acids to alanine,

which mimics dephosphorylated serine or threonine residues, was shown to alter its activity profile. With the caveat that even this very conservative amino acid substitution might influence NS1 activity in ways that do not relate directly to its dephosphorylated state, mutational analysis of all of the consensus PKC phosphorylation sites in NS1 has provided interesting insights into how NS1 functions can be modulated *in vivo* (Corbau *et al.*, 2000; Nüesch *et al.*, 2001; Daeffler *et al.*, 2003).

A number of lines of experimental evidence suggest that T435 and S473, PKC $\lambda$  phosphorylation sites located within the NS1 helicase domain, are essential for the molecule's DNA unwinding activity and hence that phosphorylation of these residues is essential for viral DNA replication. NS1 proteins with alanine substitutions at these positions, T435A and S473A, were shown to be inactive *in vivo* and *in vitro* (Dettwiler *et al.*, 1999; Nüesch *et al.*, 2001), while defective wild-type NS1 polypeptides, generated in cells expressing dominant-negative PKC $\lambda$  mutants, were not naturally phosphorylated at these sites but regained both their *in vitro* helicase activity and their ability to support DNA replication following rephosphorylation with recombinant PKC $\lambda$  *in vitro* (Nüesch *et al.*, 2003). The S473A mutant was analyzed in particular detail. Although it lacked helicase activity, it was able to form homo-oligomers (Corbau *et al.*, 2000) and interact with its cognate DNA-recognition motif (Nüesch *et al.*, 2001), and it was able to nick the viral origin *in vitro* in a strand- and site-specific fashion when the nick site was presented in a single-stranded form. In fact, like experimentally dephosphorylated NS1 polypeptides, mutant S473 showed reduced ATPase activity (Nüesch *et al.*, 1998a, 2001) and, perhaps in consequence, showed enhanced affinity for its (ACCA)<sub>2-3</sub>-recognition motif. Since S473A was still able to *trans*-activate the P38-promoter, PKC $\lambda$  phosphorylation can be viewed as modulating NS1 function, freeing it to act as a transcription factor while impairing its ability to support DNA amplification. Thus the precise phosphorylation status of NS1 appears to prime it for performing specific roles in the viral life cycle.

Mutational analysis of other putative PKC-phosphorylation sites suggests that they may also play a role in modulating NS1 activities, perhaps by allowing conformational changes in the polypeptide. Such conformation-dependent regulation is quite common in cellular proteins, such as protein kinase C (Newton, 2003; Newton and Johnson, 1998), and mutation of several putative NS1 PKC phosphorylation sites suggest that they function in a similar way. For example, mutant T363A targets a PKC consensus site located within the hinge region that separates the DNA-binding domain of NS1 (amino acids 16–275) and its helicase-domain (amino acids 391–486). This oligomerization-competent NS1-mutant is a strong site-specific DNA-binding protein, but is impaired for many activities essential for DNA replication, *trans*-activation of the P38 promoter, and cytotoxicity (Corbau *et al.*, 2000; Nüesch *et al.*, 2001). Since no motifs that are known to be involved in DNA replication or *trans*-activation are modified in this mutant, effects mediated by

the conformation of the polypeptide may be indicated. However, whether or not this site is regulated by phosphorylation remains uncertain, since to date T363 phosphorylation has not been detected *in vivo*. An additional example of the impact of phosphorylation on NS1 conformation was obtained by mutagenesis of T403, a consensus PKC phosphorylation site located within the ATP-binding pocket. This mutant NS1 polypeptide consistently resolved into two distinct species on SDS-PAGE (Corbau *et al.*, 2000), suggesting that changes in this domain may induce NS1 to switch between different conformations. Since this site seems to be a target for phosphorylation *in vivo* (Corbau *et al.*, 2000), the T403A phenotype could indicate that post-translational modification within the adenosine triphosphate (ATP)-binding site might indeed modulate the affinity of NS1 for ATP or its rate of ATP hydrolysis by altering the conformation of this domain.

Another consensus PKC (and casein kinase II [CKII]) phosphorylation site, T278, has yet to be shown to be phosphorylated *in vivo*, but its position in the NS1 molecule is so tantalizing that it merits consideration. This site is located within an essential bipartite self-association motif (amino acids 261–278) present in the hinge region of NS1, between its DNA-binding and helicase domains, so that phosphorylation at this site could be expected to influence NS1 oligomerization. This prediction was supported by mutagenesis of T278 to alanine, which completely abolished NS1's ability to co-transport cytoplasmic mutants of NS1 into the nucleus. Interestingly, this mutant also showed an impaired ability to induce morphological alterations in the host cell associated with CPE (Corbau *et al.*, 2000). This result suggests that many NS activities, including its cytotoxic potential, could be regulated by phosphorylation, in this case by modulating its ability to form oligomers.

Phosphorylation of critical interaction sites is commonly used to regulate protein/protein interactions among cellular proteins. A clear example of such modification in parvovirus non-structural proteins involves their ability to interact with 14-3-3 proteins, since members of this family only bind to phosphorylated forms of their-binding site. Thus, NS2 binds specific 14-3-3 proteins (Brockhaus *et al.*, 1996) but mutation of NS2 residue T149 to alanine both abolishes phosphorylation of this molecule and renders it unable to interact with 14-3-3 proteins (Bodendorf *et al.*, 1999; Salome, unpublished observations). Similarly, 14-3-3 proteins bind to the carboxy-terminal peptide of AAV Rep68 (and Rep40), where the interaction is dependent on the phosphorylation of S535 (Han *et al.*, 2004). An example of phosphate-modulated interactions with other cellular proteins emerged from recent differential affinity chromatography studies in which MVM NS1 was shown to interact with the catalytic subunit of casein kinase II (CKII $\alpha$ ). This reaction was thought to be dependent upon phosphorylation of a consensus PKC $\lambda$  phosphorylation site, S473, since mutagenesis of this residue impaired the interaction (Nüesch and Rommelaere, 2004). In summary, examples are beginning to emerge that suggest parvoviral protein function can be modulated by cellular

kinases. In addition to the direct modification of specific binding sites, it is also possible that protein/protein interactions are regulated through induced conformational changes in the viral protein, leading to *de novo* exposure of potential binding sites.

## Temporal regulation of NS1 function

Changes in the phosphorylation pattern of NS1 occur during the course of infection, suggesting that the activity profile of this protein could be temporally regulated by such modifications (Corbau *et al.*, 1999). In particular, we were interested to see if such changes correlated with the induction of cytolysis, since it seemed possible that this activity might be suppressed early in infection, to allow efficient progeny production, but induced at later times to facilitate release of progeny virions. To explore this hypothesis site-directed mutants targeted at potential regulatory elements in NS1 were generated. Mutation of residues T585 and S588, located within consensus PKC phosphorylation sites in the carboxy terminus of NS1, appeared to give the postulated changes in cytotoxicity when measured in a single-round infection (Daeffler *et al.*, 2003). Since these two NS1 residues are known to be phosphorylated *in vivo*, they represent credible potential regulatory elements. In general these results suggested that at least some cytotoxic effects of NS1 constitute specific viral functions, rather than occurring as mere side-effects of virus amplification.

Interestingly, this study identified two potential regulatory elements within the same NS1 domain that had opposite effects. Thus, while mutating T585 to alanine strongly reduced its ability to induce cytolysis, a similar mutation at position S588 enhanced its cytotoxic potential as measured by the formation of larger plaques (Daeffler *et al.*, 2003). Both these mutations yielded replication competent MVM variants, but additional (putative) regulatory sites were also identified that altered the cytotoxic potential of NS1, but gave non-viable mutants. Thus, NS1 mutant S283A showed enhanced CPE, while mutagenesis of the PKC $\lambda$  phosphorylation sites T435 and S473 gave proteins with impaired cytotoxicity (Corbau *et al.*, 2000). Taken together these findings suggest that the cytotoxic potential of NS1 may be subject to post-translational regulation and that several different phosphorylation sites may be involved in triggering this activity. In addition, as suggested previously, differential phosphorylation could influence the protein's affinity for its cellular partner proteins, which seem to be essential for the induction of CPE (Nüesch, unpublished observations).

## REGULATION BY TRAFFICKING AND CELLULAR COMPARTMENTALIZATION

The functional impact of viral proteins may also be controlled *in vivo* by their subcellular distribution. Controlled

targeting of an effector protein to its site of action has frequently been described for cellular proteins, e.g. protein kinases involved in cell signaling (Newton, 1997), and emerging evidence supports the idea that the activity of parvoviral non-structural proteins may also be modulated by targeting to specific subcellular microdomains. Such regulation would require that the proteins are (actively) transported to their site of action, contain signals allowing them to bypass compartmental barriers (e.g. the nuclear envelope), and are specifically targeted to particular microdomains.

To date, only nuclear/cytoplasmic trafficking has been investigated in detail, while transport mechanisms and/or targeting of polypeptides to other site(s) remains largely speculative. Since the non-structural proteins mediate critical processes including transcriptional regulation, DNA amplification (Weitzman *et al.*, 1996; Bashir *et al.*, 2001) and the packaging of progeny single-stranded (ss)DNA genomes (Wistuba *et al.*, 1997; Lombardo *et al.*, 2002) at sites within the cell nucleus, it is important that these proteins are efficiently transported through the nuclear envelope. The large viral non-structural proteins have bipartite nuclear localization signals, located in the MVM NS1 polypeptide at residue 194 (194-KK[X<sub>31</sub>]KKK-218) and in the AAV2 Rep proteins at residue 491 (491-KKR[X<sub>11</sub>]KRRR-509), arguing for a nucleoplasmin-like nuclear import mechanism (Yang *et al.*, 1992; Nüesch and Tattersall, 1993; Kleinschmidt *et al.*, 1995). In contrast, the small size of the NS2 proteins should allow them to pass through the nuclear pores without specific targeting signals (Cotmore and Tattersall, 1987), but since these proteins are predominantly (>70 percent) cytoplasmic, this mode of nuclear entry must either be rather inefficient and/or NS2 must be actively exported from this compartment.

Parvoviral non-structural proteins are also thought to interfere directly with a variety of cytoplasmic processes. For example, MVM infection affects cell physiology by modulating cellular signaling (Anouja *et al.*, 1997; Lachmann *et al.*, 2003; Nüesch *et al.*, 2003) and, in particular, NS1 is known to influence cytoskeleton dynamics, leading to degradation of actin, vimentin, and tropomyosin filaments (Nüesch *et al.*, 2004; Nüesch and Rommelaere, 2004). Since these effects are observed in the cytoplasm, forms of NS1 that are retained in this compartment may carry specific modifications that adapt them for cytoplasmic functions. Alternatively, they may continually shuttle between the nucleus and cytoplasm, and/or trigger the observed effects while in the nucleus, using cellular or viral mediator proteins.

In addition to its bipartite nuclear localization signal, NS1 contains signals/motifs within its carboxy-terminal domain that appear to influence its intracellular distribution. Although approximately 30 percent of wild-type NS1 is found in the cytoplasm, deletion of its acidic carboxy-terminal domain reduced this cytoplasmic component, allowing more than 95 percent of the polypeptide to become nuclear (Nüesch and Tattersall, 1993). This suggests that the carboxy-terminal 67 amino acids of wild-type NS1 contain some sort

of nuclear export or cytoplasmic retention signal, although candidate sequences have yet to be identified. Interestingly, variants carrying this deletion were found to be significantly less toxic than wild-type polypeptides (Legendre and Rommelaere, 1992), in line with the idea that NS1 cytotoxicity may be predominantly triggered in the cytoplasm rather than the nucleus (as discussed further below). If correct, this would indicate that the cytotoxic activity of NS1 is regulated in its host cell, in part, by controlling its subcellular distribution.

In contrast, the cytoplasmic accumulation of NS2 suggests that the main activities of this protein may occur in the cytoplasm. NS2 has a nuclear export signal (NES; 96-MtkkFgtLti-105; Eichwald *et al.*, 2002), and its nuclear/cytoplasmic distribution is phosphorylation dependent (Cotmore and Tattersall, 1990). Thus, while unphosphorylated forms of NS2 are found in both the nucleus and cytoplasm of infected cells, phosphorylated NS2 polypeptides remain exclusively cytoplasmic. This suggests that phosphorylated NS2 is either specifically retained in the cytoplasm, or is rapidly exported from the nucleus upon phosphorylation. In fact, only dephosphorylated forms of NS2 are able to interact physically with CRM1 and hence become associated with the nuclear export machinery (Bodendorf *et al.*, 1999). Moreover, since NS2 mutants that are impaired in their ability to interact with CRM1 accumulate within the nucleus, this supports the idea that at least some NS2 activities are modulated by its regulated transport through the nuclear pore (Eichwald *et al.*, 2002; Miller and Pintel, 2002).

In addition to modulating transport between pre-existing cellular compartments, parvovirus infection creates new cellular environments (Nüesch *et al.*, 2004), such as the novel subnuclear APAR bodies described by Cziepluch and colleagues (Cziepluch *et al.*, 2000), which are the site of early viral DNA amplification (Weitzman *et al.*, 1996; Cziepluch *et al.*, 2000; Bashir *et al.*, 2001; Young *et al.*, 2002a,b). Many cellular proteins accumulate in these structures, together with the viral NS1 (Cotmore and Tattersall, 1987; Cziepluch *et al.*, 2000; Bashir *et al.*, 2001), NS2 (Young *et al.*, 2002a) and AAV Rep proteins (Weitzman *et al.*, 1996). Although signals and mechanisms mediating this accumulation of viral proteins remain elusive, it is intriguing to speculate that targeting of viral proteins to such microdomains could act as an important regulatory mechanism during infection.

## DRIVING PARVOVIRUSES THROUGH THE REPLICATION CYCLE

As summarized above, specific functions of the non-structural proteins appear to be regulated during infection by diverse processes including modification of their properties and enzyme activities, changes in their ability to interact physically with cellular partner proteins, and changes in their targeting to distinct compartments and microdomains in

the host cell. Protein function may be regulated in a temporal fashion during the course of infection, as the relative importance of the different activities performed by each polypeptide changes, and this appears to be reflected in changes in the phosphorylation pattern of NS1 (Corbau *et al.*, 1999). In turn, these differences in post-translational modification suggest that the viral proteins influence cellular signaling cascades, perhaps by altering their subcellular distribution or by modifying their activity. In this section, experimental evidence is reviewed that suggests that both of these events occur during the viral replication cycle.

Investigations with MVM suggest that the control of the cellular PDK/PKC signaling pathway and NS1 regulation by that pathway, are interconnected. Thus, on one hand, PKC $\lambda$  and PKC $\eta$  modulate NS1 activities *in vitro* and *in vivo* but, on the other hand, the subcellular distribution of these kinases and their activator kinase, PDK-1, is altered following viral infection (Dettwiler *et al.*, 1999; Lachmann *et al.*, 2003; Nüesch *et al.*, 2003). Within the PDK/PKC signaling pathway such changes in cellular distribution generally result in kinase activation (Frodin *et al.*, 2000, 2002; Newton, 2003), suggesting that the NS1 modulating kinases, PKC $\lambda$  and PKC $\eta$ , are specifically activated during MVM infection and could, in association with selective adaptor proteins present at their new locations, exhibit modified substrate specificity (Jaken and Parker, 2000; Nüesch unpublished, observations). Thus, targeting the non-structural proteins to specific (micro)domains could cause them to be phosphorylated at novel sites. This possibility is supported by findings that late in infection NS1 phosphorylation events are observed at new PKC consensus sequences, for example, at T585 and S588 (Daeffler *et al.*, 2003).

However, recent developments do provide insight into mechanisms that allow the viral non-structural proteins to influence some intracellular signaling pathways. Notably, the non-structural proteins of both AAV and MVM have been shown to interact directly with specific cellular kinases, and thereby modulate the activity of these proteins (Di Pasquale and Stacey, 1998; Di Pasquale and Chiorini, 2003; Nüesch and Rommelaere, 2004). As discussed previously, hyperphosphorylation of AAV Rep78 inhibits its ability to support DNA replication, but Rep interacts with cellular protein kinases PKA and PRKX, effectively inhibiting their activity and allowing the AAV replication cycle to proceed. Interestingly, Rep78 interactions with cellular kinases were also shown to modulate both cellular transcription mediated through the cAMP-responsive element binding site (CREB) family of transcription factors and the later stages of adenovirus replication (Di Pasquale and Stacey, 1998; Di Pasquale and Chiorini, 2003). Similarly, interaction of autonomous parvovirus NS1 with CKII $\alpha$  was shown to alter the substrate specificity of this cellular kinase, inducing *de novo* phosphorylation of both viral and cellular proteins (Nüesch and Rommelaere, 2004; Nüesch, unpublished observations).

In addition to these direct interactions with the catalytic subunits of cellular kinases, modulator proteins involved in

cellular signaling are also targeted. For example, NS2 (Brockhaus *et al.*, 1996) and Rep68 (Han *et al.*, 2004) interact with members of the 14-3-3 family of proteins, which leads to hyperphosphorylation of the viral proteins (Bodendorf *et al.*, 1999; Salome, unpublished observations) correlating, in the case of AAV Rep, with inhibition viral DNA amplification (Han *et al.*, 2004). By interfering with the activity, substrate specificity and subcellular distribution of protein kinases and/or their associated modulator proteins could potentially trigger changes in entire signaling cascades, leading to the observed changes in the phosphorylation patterns of both viral (Han *et al.*, 2004; Nüesch, unpublished observations; Salome, unpublished observations) and cellular target proteins (Anouja *et al.*, 1997; Nüesch and Rommelaere, 2004) during the course of infection.

Since the nuclear envelope remains intact until late in infection (Nüesch *et al.*, 2005), the activity spectra of cytoplasmic forms of NS1 may be determined by their effective sequestration both from potential substrates and from potential mediators of post-translational modification. The subcellular location of NS1 is known to influence its pattern of post-translational modification, since cytoplasmic mutants lack specific PKC $\lambda$  phosphorylations that are found in the wild-type protein (Nüesch *et al.*, 2003). This suggests that newly synthesized NS1 must enter the nucleus to become activated by PKC $\lambda$ , at least early in infection. In fact, PKC $\lambda$  phosphorylation of NS1 at residues T435 and S473 seems to be essential for both activating the helicase function of this protein and for allowing it to induce CPE in the host cell (Corbau *et al.*, 2000). Since the latter activity appears to involve targeting the host cell cytoskeleton and most likely occurs in the cytoplasm (Nüesch *et al.*, 2004), this tends to suggest that active export of modified forms of NS1 from the nucleus may be required. Thus, complex transport and targeting mechanisms, in particular between the nucleus and cytoplasm of infected cells, may be needed to fine-tune the required cascade of non-structural protein functions.

As mentioned previously, NS2 interferes with the nuclear export machinery by physically interacting with and thus coopting or sequestering, the nuclear export factor CRM1 (Bodendorf *et al.*, 1999). Mutations in NS2 which impair its ability to bind CRM1 lead to the accumulation of NS2 in the nucleus, but also lead to subsequent nuclear retention of newly produced virions (Eichwald *et al.*, 2002; Miller and Pintel, 2002). Thus phosphorylation of NS2, which both impairs its ability interact with CRM1 (Bodendorf *et al.*, 1999) and leads to its sequestration in the cytoplasm (Cotmore and Tattersall, 1990), potentially prevents it from subverting normal nuclear export processes. These findings illustrate how post-translational modification of specific viral peptides can impact on intracellular trafficking patterns.

Although a direct link has yet to be established, it is possible that NS2 is involved in regulating the nuclear/cytoplasmic transport of NS1 and/or its post-translational modification, particularly in view of the observation that signals or motifs in the carboxy-terminal peptide of NS1 affect the extent of

its nuclear retention (Nüesch and Tattersall, 1993). Since NS2 is itself tolerated by the host cell, NS2-dependent modulation of NS1 function was suggested by the observation that NS2 enhances NS-induced cytotoxicity (Brandenburger *et al.*, 1990; Legrand *et al.*, 1993). This observation can be interpreted in two ways. First, NS2 may influence cellular signaling pathways by interacting with and sequestering or subverting, the cellular 14-3-3 regulatory proteins (Brockhaus *et al.*, 1996). Alternatively, NS2's ability to interfere with the nuclear export machinery (Bodendorf *et al.*, 1999; Eichwald *et al.*, 2002; Miller and Pintel, 2002) could lead to changes in the subcellular distribution of NS1 and/or an NS-modifying kinase, especially kinases such as PKC $\lambda$  that themselves contain a nuclear export signal (Perander *et al.*, 2001). Thus, without NS2, the cytoplasmic accumulation of NS1 molecules carrying the necessary T435/S473 phosphorylations might be restricted, and their ability to influence cytoplasmic events leading to CPE consequently diminished. In summary, it seems likely that the NS2 proteins indirectly impinge on the ability of NS1 to fulfil specific roles during infection. If NS2 is viewed predominantly as a modulator of NS1 function, rather than an effector protein in its own right, this could explain why certain cells, such as transformed human fibroblasts, can support the productive replication of NS2-null mutant viruses (Cotmore *et al.*, 1997; Naeger *et al.*, 1990). From this perspective, in such cells deregulation of certain subcellular trafficking patterns and/or changes in the distribution of activated kinases could promote productive infection without the intercession of NS2.

## SUMMARY

Clearly, there are many ways in which the functions of parvoviral non-structural proteins could be regulated during infection. Since this is a relatively new area of interest, few pathways have been explored in detail, and the discussion presented here is thus necessarily speculative. However, the available data does suggest that most of the major modifications observed in the MVM NS1 polypeptide are mediated by a relatively limited group of kinases. Thus, adaptation of a limited number of cellular signaling cascades may be sufficient to coordinate the viral replication cycle, especially if the intracellular trafficking and substrate specificity of the regulating kinases are themselves controlled by (viral) adaptor proteins. These types of regulation have both been described for members of the PKC family in uninfected cells (Newton, 1997; Jaken and Parker, 2000; Newton, 2003). However, much work still needs to be done to characterize this regulation during the parvovirus life cycle, to identify which viral events trigger activation, which cellular proteins/complexes are involved, and which factors control the intracellular trafficking and organization of specialized microdomains.

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# Synthesis, post-translational modification and trafficking of the parvovirus structural polypeptides

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The nuclear invasion, assembly, and maturation of nuclear viruses requires efficient transport across the nuclear membrane of their structural proteins at several steps of the infection process. In the *Parvoviridae*, the karyophilic capsid proteins must traverse across the nuclear membrane at three steps of the viral life cycle:

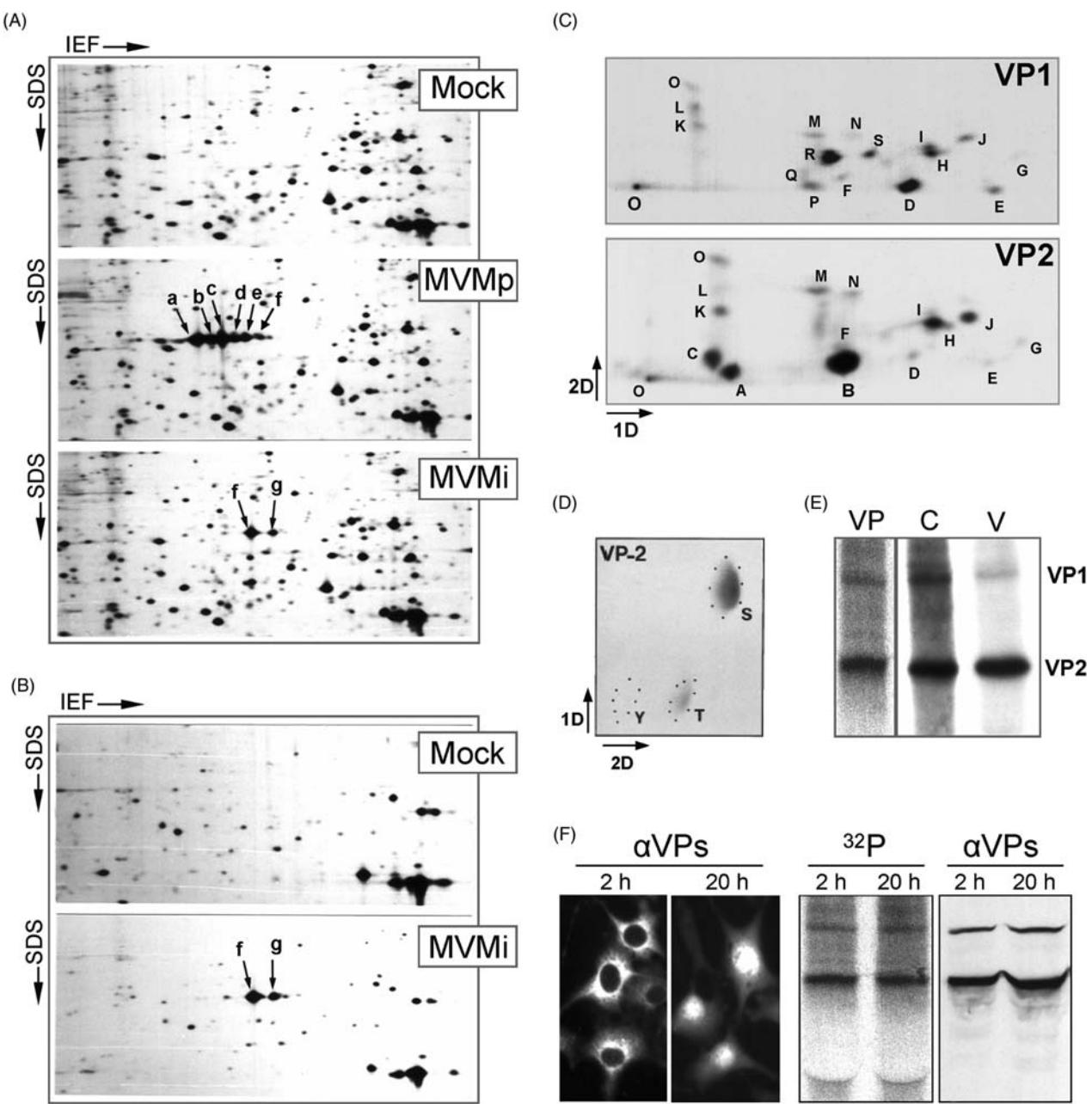
- entry of the infectious particle;
- transport from the cytoplasm to the nucleus of *de novo* synthesized proteins;
- exit of newly formed virions.

These processes imply a temporal and physical regulation of the movement of the viral structural components inward and outward from the nucleus, that must be exerted by determinants within amino acid sequences and post-translational modifications of the proteins. Additional key factors of the intracellular traffic of viral components is the accessibility of the protein signals to the transport machinery of the cell along the assembly and maturation. Understanding these processes is essential for gaining an integrated view of viral morphogenesis. We investigated these questions in the nuclear transport of the capsid proteins of the autonomous parvovirus minute virus of mice (MVM) during the import at the initiation of the infection, in the capsid assembly, and along the nuclear export of the mature virion.

## **POST-TRANSLATIONAL MODIFICATION OF PARVOVIRUS STRUCTURAL PROTEINS**

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The entire pattern of viral protein species has been determined for the prototype strain of the parvovirus minute virus of mice (MVM<sub>P</sub>) in permissive A9 mouse fibroblast cells by high resolution 2D gel electrophoresis (Santaren *et al.*, 1993). The <sup>35</sup>S-Met pulse-labeled minor VP1 and major VP2 structural polypeptides from infected cells were resolved with a high degree of microheterogeneity using isoelectric focusing, whereas most cellular proteins were resolved as single species. Post-translational modification of MVM capsid proteins was previously noticed in a similar study for MVM and H-1 (Peterson *et al.*, 1978), as well as for the canine parvovirus (CPV; Parrish, personal communication) suggesting that post-translational modification may be a common feature of parvovirus structural proteins. In the best resolution obtained in the neutral central region of the gels, VP2, the major capsid protein of MVM<sub>P</sub> could be resolved as a consistent pattern of three abundant (named a, b, c), two intermediate (d, e) and one meager (f) neutral isoelectric focusing species or subtypes (Santaren *et al.*, 1993; see Figure 20.1A, *central panel*). The VP2 modification occurred soon after or during the translation and preceding the transport of the protein, as all the newly



**Figure 20.1** Post-translational modifications of parvovirus MVM structural proteins. Autoradiogram of 2D analysis of the VP2 isoelectric species of the MVMP and MVMi strains synthesized in (A) NB324K human transformed fibroblasts, and in (B) mouse primary bone marrow cells. Cells were labeled 12–16 hpi with 0.5 mCi/ml of  $^{35}$ S-methionine and samples resolved by isoelectric focusing (IEF) and PAGE (SDS) in the second dimension. Only the central neutral region of the gels is shown. The six VP2 subtypes of MVMP were named in order of increasing acidic pl, and the two MVMi subtypes were designated by matching the spots of the films. (C) Phosphorylation pattern of parvovirus MVMP capsid proteins. 2D maps by thin-layer chromatography (TLC) of tryptic phosphopeptides of VP-1 and VP-2 proteins isolated from purified  $^{32}$ P-labeled capsids. Plates were exposed to autoradiography with intensifying screen at  $-70^{\circ}\text{C}$ . Peptides designated with the same letter in both proteins share identical 2D migration. 1D: first dimension, 2D: second dimension. (D) Phosphoamino acids composition of VP-2. Thin-layer 2D analysis of phosphoamino acids of VP-2 isolated from purified  $^{32}$ P-labeled MVMP capsid. Markers: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine. (E) Distinct VP phosphorylation in viral particles. Immunoprecipitated structural proteins (VP) and purified empty (C) and DNA-full (V) MVMP particles labeled with  $^{32}$ P-orthophosphate were resolved in 10 percent SDS-PAGE, blotted to nitrocellulose filters and exposed for autoradiography in phosphoimager. (F) Cytoplasmic phosphorylation of MVMP structural proteins. Left: Immunofluorescence analysis of the subcellular distribution of expressed VP proteins in permanently transfected A9 mouse fibroblasts synchronized by a double block (Cotmore and Tattersall, 1987), and stained at 2 and 20 hours post-aphidicolin release. Right: VP proteins immunoprecipitated from transfected A9 cells labeled with  $^{32}$ P-orthophosphate were blotted to filters and exposed to autoradiography ( $^{32}$ P) or probed with an anti-MVM capsid serum ( $\alpha$ VPs).

synthesized VP2 subtypes were equally represented in  $^{35}\text{S}$ -Met pulse-labeling at 5 minutes.

Defining the nature of the modification in viral structural proteins is a challenging issue in parvoviruses and in other systems. For example, the major capsid protein of polyomavirus (VP1) is also differentially modified after translation by several types of substitutions like phosphorylation and acetylation, which contribute to at least six isoelectric forms of the protein with a controversial function in capsid assembly (Bolen *et al.*, 1981; Garcea *et al.*, 1985). Similarly, although the MVM capsid proteins are modified by conventional phosphorylation in amino acid residues (see below), the higher number of VP phosphorylation sites (Maroto *et al.*, 2000), the possibility of radiolabeling with  $^3\text{H}$ -adenosine (Peterson *et al.*, 1978), and the fact that the VP2 phospholabel did not increase toward the more acidic region of the 2D gels (Santaren *et al.*, 1993), indicate that phosphorylation does not account for the six resolved isoelectric subtypes. In this sense, it may be worth mentioning that MVM empty capsids gradient-purified from  $^{32}\text{P}$ -orthophosphate-labeled cells harbor a high quantity of radiolabel migrating in a heterogeneous fashion in denaturing sodium dodecyl sulphate (SDS)-acrylamide gels, which is resistant to nucleases and distinct from conventional phosphoamino acids (our unpublished observations). These findings denote the presence of an abundant phosphorylated component of the parvovirus capsid, the nature of which deserves further research.

The role of the 2D-subtypes of VP proteins in parvovirus biology and the sites of non-conventional modifications in the polypeptide chains are also unresolved questions. In MVMp, the 2D pattern of VP2 modification was uncoupled from the nuclear translocation and the assembly of this protein, since all the six VP2 subtypes were packaged at the induced stoichiometry into purified empty capsids (Santaren *et al.*, 1993). Unlike conventional phosphorylation in amino acids (see below), the 2D-pattern of VP2 modification is conserved in the MVMp infection of the human transformed permissive NB324K cell type (Figure 20.1A), suggesting that the origin and the physiological stage of the permissive host cell may be not important for this type of modification. A further clear discrimination between both types of modifications can be deduced from the different domains of the protein where they occur. While the most prominent phosphorylated amino acids mapped to the N-terminal sequence of VP2 (see below), the pattern of 2D modification was identical for the VP2 and VP3 proteins isolated from purified DNA-full virions (Santaren *et al.*, 1993), indicating that the six isoelectric species arise from the carboxy-terminal part common to both proteins.

Interestingly, when compared with the highly homologous hematotropic MVMi strain, the pattern of VP2 modification resulted in a viral strain-specific feature. In the infection of the NB324K human transformed fibroblast cells, which are permissive to both viruses; the VP2 major capsid protein of MVMi was resolved in high resolution 2D

gels as only two subtypes: one migrating at the position of the subtype f of MVMp, and another subtype (g) focusing to a more acidic point of the gel (Figure 20.1A, *bottom panel*). This simple 2D-pattern of VP2 modification was strictly conserved in samples harvested from MVMi-infected primary mouse bone marrow cultures (Figure 20.1B), which are permissive to MVMi cytotoxic and productive infection (Segovia *et al.*, 1991). All together these experiments indicate that the post-translational modification of MVM capsid proteins resolved in 2D gels is mainly independent of the host cell physiology, but it is determined by the virus genotype.

## PARVOVIRUS CAPSID PHOSPHORYLATION

While the nature and biological significance of the 2D-post-translational modifications remain unclear,  $^{32}\text{P}$ -labeled VP proteins assembled in MVMp capsid could be purified from nitrocellulose filters free of other phosphorylated components (Maroto *et al.*, 2000) and subjected to 2D tryptic phosphopeptide analysis by standard procedures (Boyle *et al.*, 1991). Both VP2 and VP1 proteins harbored a characteristic complex pattern of phosphorylation (Figure 20.1C), in which the amino acid serine was the main phosphate acceptor, followed by threonine at a level several-fold lower (Figure 20.1D). Thus, although they share a common 3D structure in the capsid (Agbandje-McKenna *et al.*, 1998), the VP subunits forming the MVM particle show distinct patterns of phosphorylation, an important feature of the parvovirus T = 1 icosahedral capsid that cannot be resolved by crystallography.

This pattern of VP phosphorylation was essentially maintained in established cell lines of different origins, though the overall level of capsid protein phosphorylation may differ depending on the transformation stage of the host cell (Maroto *et al.*, 2004). Digestion with secondary proteases revealed a minimum number of 18 Ser or Thr residues phosphorylated to different levels in both polypeptides (Figure 20.1C). Significantly, while most weakly phosphorylated phosphopeptides were shared by both polypeptides, the major ones were specific for either type of capsid subunit (Figure 20.1C; Maroto *et al.*, 2000). Since the entire VP2 sequence is contained within VP1, these data suggest that the 143 amino acid-long VP1-specific region plays a major role in the final distribution of MVM capsid phosphorylation sites. This sequence may act as an acceptor of phosphates in its numerous contained Ser and Thr residues and it could affect the folding of the protein and thus its accessibility to cellular Ser/Thr kinases.

In the assembled capsid, the main phosphopeptide of VP2 (named B) was mapped to its N-terminal domain using a combination of techniques and data, such as the heavy loss of phosphorylation accompanying VP2 to VP3 cleavage and the site-directed mutagenesis of terminal serine residues

(Maroto *et al.*, 2000). Since the MVM capsid is formed by approximately 50 VP2 subunits, this sequence is the major phosphorylated domain of the extracellular viral particle. The rest of the approximately 12 phosphorylation sites remain unassigned. Finding the biological roles of parvovirus capsid phosphorylation awaits the difficult task of precisely mapping the phosphorylated residues, and thus it remains largely undefined. One major exception is the VP2 N-terminal phosphorylated domain, in which phosphoserines play an important role in the traffic of the MVM virion from the nucleus to the cytoplasm (see below). The other phosphorylation sites, occupied at low stoichiometry in the viral particle, could, however, also modulate important biological functions of the entire coat. We have attempted to map other phosphorylation sites in the MVM capsid taking the advantage of the fact that most of the 14 amino acid differences between the i and p strains localize to Ser or Thr residues (Astell *et al.*, 1986). However, the VP2 from both viruses infecting the permissive NB324K cells show a similar 2D phosphopeptide map (our unpublished observations), illustrating the difficulty of assigning phosphorylation sites in other parvovirus coats on the single basis of homology between primary amino acid sequences.

A matter of current investigation in this laboratory is the relationship between VP protein phosphorylation of parvovirus capsid and the physiology of the host cell. It is noteworthy that the nuclear translocation of VP proteins is cell cycle regulated (Hernando and Almendral, unpublished data), which allows the cytoplasmic accumulation of newly synthesised capsid proteins in highly synchronized transfected cells (Figure 20.1F, *left*). Under these experimental conditions, the level of VP phosphorylation is similar between the protein occurring in the cytoplasm and that translocated into the nucleus later in the cell cycle (Figure 20.1F, *right*), suggesting that the main VP phosphorylations occur in the cytoplasmic compartment prior to nuclear transport. However, the VP phosphorylation may be altered in the subsequent steps of the virus cycle. Indeed, the level of VP1 phosphorylation is several-fold lower in purified DNA-full virions as compared with empty capsids (Figure 20.1D), suggesting that the removal of phosphates from most VP1 capsid subunits may be a requisite for genome encapsidation and virus maturation (Hernando *et al.*, 2005).

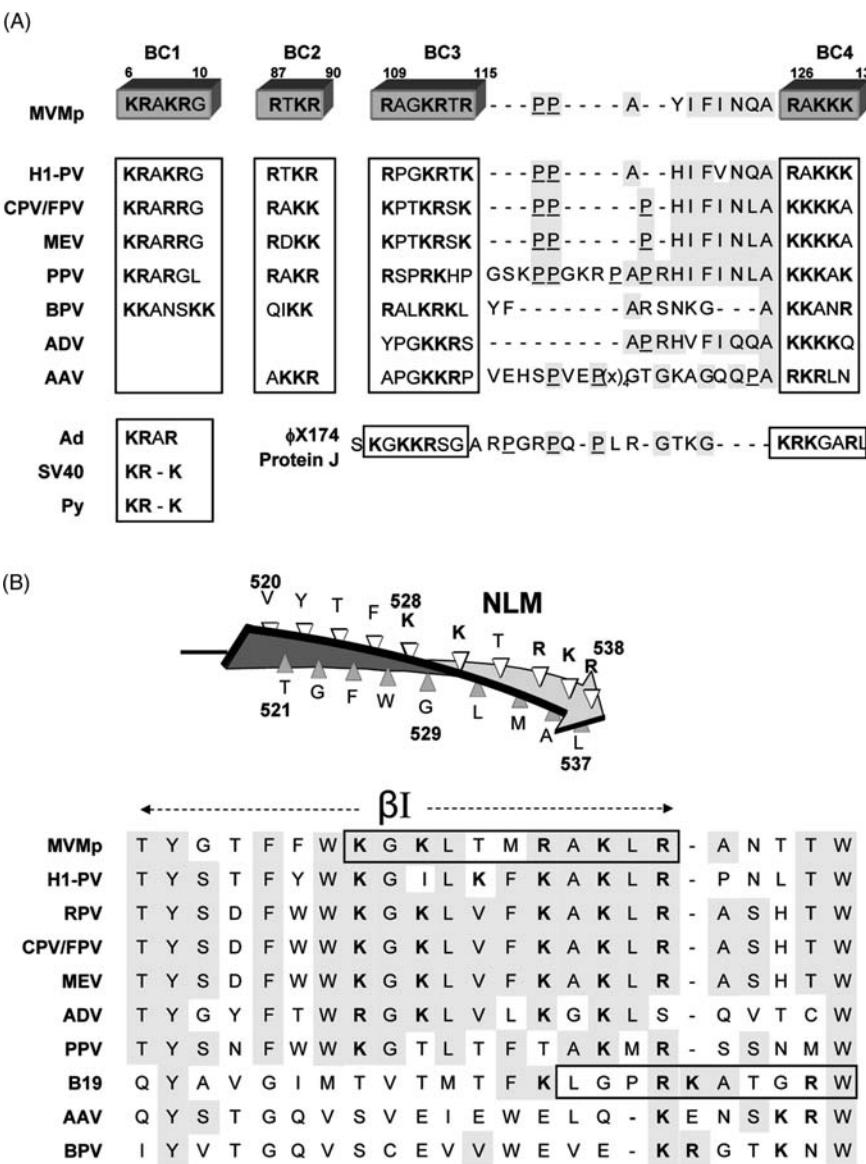
## THE INCOMING VIRION TARGETING THE NUCLEUS

The nuclear membrane offers a second barrier to those viruses, such as the *Parvoviridae*, that need components of eukaryotic replication and transcription machinery for their multiplication. In the absence of nuclear membrane disintegration, when the viral genome is first delivered by the incoming particle, the traffic of viral macromolecules must proceed across the central aqueous channel of the

nuclear pore complex (NPC), a large structure with an 8-fold rotational symmetry built of proteins named nucleoporins (Ryan *et al.*, 2000). It is intriguing that the functional diameter of the NPC central channel for non-deformable cargo (Dworetzky and Feldherr, 1988; Panté and Kann, 2002) is precisely in the range of the 25 nm diameter of the parvovirus capsid (Tattersall and Cotmore, 1988). This coincidence introduced a certain controversy to our understanding of the exact configuration of virion entering through the NPC upon the multiple processes and interactions occurring along the cytoplasm (Vihinen-Ranta *et al.*, 2004). Physically the virion could traverse the NPC in the intact configuration, but a partial or a complete capsid disassembly, as reported for larger viruses (Ojala *et al.*, 2000; Trotman *et al.*, 2001; reviewed in Whittaker *et al.*, 2000), could alternatively be required for an efficient delivery of the genome into the nucleus.

A number of studies indicate that the capsid of the incoming parvovirus virion must undergo important structural changes to deliver the genome into the nucleus and initiate the infection. In MVM and CPV, the capsid is formed by approximately 10 subunits of the larger VP1 protein (84 kDa) and 50 subunits of VP2 (62 kDa), the entire VP2 coding sequence being contained in VP1 which has an additional specific 142 amino acids sequence at its N-terminus (Tattersall *et al.*, 1977) not ordered in the crystal structure (Tsao *et al.*, 1991; Agbandje-Mckenna *et al.*, 1998). Only VP2 can assemble into empty and DNA-full capsids, but VP1 is required for the infectivity of the virions (Tullis *et al.*, 1993). The VP1 sequence is located in the interior of the MVM and CPV capsid and is not accessible to proteases or antibodies, but can be exposed in purified virions heated under controlled temperature or treated with urea (Cotmore *et al.*, 1999; Vihinen-Ranta *et al.*, 2002). These processes may mimic a conformational change occurring during the natural entry pathway, since micro-injection of antibodies against the VP1 arm of CPV in the cytoplasm blocks infection prior to complete particle disassembly (Vihinen-Ranta *et al.*, 2002).

Indeed, the VP1-specific sequence harbors several important functions to initiate the viral infection. As outlined in Figure 20.2A, in addition to the domain containing phospholipase activity (Zadoni *et al.*, 2001), the VP1-specific region contains four basic clusters of amino acids (named BC-1 to BC-4) which are highly conserved within parvoviruses and in some other DNA viruses. BC1 and BC2 fit conventional nuclear localization sequences (NLS) formed by a short stretches of basic amino acids either in a single or as two domains present in most karyophilic polypeptides (Kalderon *et al.*, 1984; Robbins *et al.*, 1991). These sequences are recognized in the import direction by shuttling transport receptors of the importin/karyopherin family (reviewed in Mattaj and Englmeier, 1998; Weis, 2002). Nuclear transport activity was demonstrated mostly for BC1 as peptide coupled to an heterologous protein (Vihinen-Ranta *et al.*, 1997) as well as in the context of an entire



**Figure 20.2** Nuclear import signals in the parvovirus capsid proteins. (A) Alignment of clusters of basic amino acids (BC) conserved in the VP1 specific n-terminal region of parvoviruses. Amino acid residues are indicated in the single letter code. The conservation of BC1 with other karyophilic dsDNA viruses (left) and BC3 and BC4 with the protein J of the ssDNA bacteriophage  $\phi$ X174 (right) are highlighted with basic residues of the BC boxes in bold. Proline residues scattered along the space region between BC3 and BC4 are underlined and possible homologous positions are shadowed. (B) Representation of the NLM of MVM as disposed on the  $\beta$ -strand I of a capsid subunit, with the basic amino acids at the face exposed to the solvent in bold. Below: Disposition of the NLM in parvoviruses. Residues in the region of the  $\beta$ -strand I of MVM conserved in more than five other parvoviruses are shadowed, and basic residues are in bold. Major sequences with probed nuclear transport activity have been boxed. Viral acronyms are: AAV, adeno-associated virus; Ad, adenovirus; ADV, aleutian disease virus; B19, parvovirus B19; BPV, bovine parvovirus; CPV, canine parvovirus; FPV, feline parvovirus; H1-PV, parvovirus H1; MEV, mink enteritis virus; MVM, minute virus of mice; PPV, porcine parvovirus; Py, polyomavirus; RPV, rat parvovirus; SV40, simian virus 40.

singly expressed VP1 protein (Lombardo *et al.*, 2002), and it was essential for CPV infectivity (Vihinen-Ranta *et al.*, 2002) and for the MVM viral particle to initiate infection (Lombardo *et al.*, 2002). These data and the high level of homology of BC1 with N-terminal domains of capsid proteins of double-stranded (ds) DNA karyophilic viruses (see Figure 20.2A) that promote nuclear targeting (Nakanishi

*et al.*, 1996; Shii *et al.*, 1996), strongly suggest that many of the members of the Parvoviridae use factors of the karyopherin  $\alpha/\beta$  transport pathway to dock their genomes to the NPC in association with VP1.

In contrast, BC3 and BC4 did not behave like NLS as microinjected peptides (Vihinen-Ranta *et al.*, 1997) and did not show transport activity for VP1 either expressed alone

or in the context of the complete MVM genome (Lombardo *et al.*, 2002). A likely function of the BC3 and BC4 sequences in the parvovirus life cycle may be inferred from their significant homology with the two basic DNA-binding domains of the protein J of the  $\phi$ X174 bacteriophage (see Figure 20.2A, *right*), involved in the tethering of the ssDNA viral genome to the capsid inner surface at the final stage of morphogenesis (Hafenstain and Fane, 2002). The basic domains of the J protein, together with critical proline residues present in the spacing region (Hafenstain *et al.*, 2004), are associated and probably contribute to order between 8 and 10 percent of the genome in the icosahedral capsid as resolved in the X-ray structures of Microviridae (McKenna *et al.*, 1992, 1994). Similarly, the BC3–BC4 basic domains with a high degree of conservation between different members of *Parvoviridae* and the scattered proline residues in between (Figure 20.2A), may bind the negatively charged ssDNA and contribute to the packaging of the 12–18 percent icosahedrally ordered genome found in the CPV and MVM capsids (Chapman and Rossmann, 1993; Xie and Chapman, 1996). Interestingly, the available genetic analyses support this hypothesis, since DNA-full MVM virions lacking BC3 and BC4 could be harvested and purified but failed to reinitiate the infection, and they showed a packaging defect in the form of large viral DNA species migrating in agarose gels at the mRF position (Lombardo *et al.*, 2002), also appearing in pure preparations of VP1-null mutant (Tullis *et al.*, 1993) but not of wild-type (wt) virions. A detailed comparison of the degree of ordered genome between the high resolution structures of the B19 parvovirus (Kaufmann *et al.*, 2004) that lacks BC3 and BC4 and consistently shows the VP1 N-terminal region exposed on the virion surface (Rosenfeld *et al.*, 1992) and the AAV2 (Xie *et al.*, 2002), which shows the higher homology to the  $\phi$ X174 protein J (Figure 20.2A), should be a valuable study to test this hypothesis.

## NUCLEAR TRANSLOCATION OF THE CAPSID PROTEIN SUBUNITS

The parvovirus capsid proteins are karyophilic polypeptides that must traverse the nuclear membrane in the ongoing infection to package the viral genome being replicated in the nucleus. A major issue in the assembly pathway of the parvovirus is therefore the configuration of the VP proteins competent for translocation across the nuclear membrane. For this process, the size of the capsid in respect to the functional diameter of the NPC is again an important issue, since the viral structural protein subunits synthesized *de novo* could be competent themselves for nuclear translocation, or a cytoplasmic capsid assembly preceding nuclear invasion could be required. As nuclear transport is an active process driven by a specific protein NLS, a method to address the parvovirus assembly pathway is synonymous with the

identification of nuclear import sequences in the capsid proteins.

For MVM, both VP1 and VP2 singly expressed proteins efficiently target the nucleus of transfected cells (Tullis *et al.*, 1993; Lombardo *et al.*, 2002), suggesting that each protein must carry its own nuclear transport sequence. VP2, the major capsid forming polypeptide, lacks any consensus NLS in its primary amino acid sequence, but it contains a structured domain with nuclear targeting capacity for the unassembled protein subunits mapped in the  $\beta$ -strand I of the MVM capsid, which has been named the nuclear localization motif (NLM; Lombardo *et al.*, 2000). Unlike conventional transport sequences, the parvoviral NLM is located within an amphipathic beta-strand that in the resolved 3-D crystal structure of MVM and CPV capsids (Tsao *et al.*, 1991; Agbandje-McKenna *et al.*, 1998), shows all the charged basic amino acids placed within the region of the strand facing the solvent in the interior surface of the capsid and the hydrophobic amino acids facing toward the protein core (see Figure 20.2B). If the NLM is aligned among the capsid proteins of representative members of the *Parvoviridae* (Figure 20.2B, *bottom*), the number of basic residues of the exposed face, the predicted overall amphipathic character of the sequence (Chapman and Rossmann, 1993), and key residues of the hydrophobic face, which are essential for its activity in MVM (for example, G529 and L531; Lombardo *et al.*, 2000), are strictly conserved in the *Parvovirus* genus, suggesting that the nuclear transport of the capsid proteins also proceeds by a similarly structured motif in these viruses as well. Although the homology among residues decreases when comparing with BPV and members of the genus *Erythrovirus* and *Dependovirus*, the  $\beta$ -strand configuration of this protein region is conserved in the crystal structure of B19 and AAV2 capsids (Xie *et al.*, 2002; Kaufmann *et al.*, 2004). Furthermore, a powerful nuclear transport activity not involved in capsid assembly was also demonstrated for the c-terminal basic residues of  $\beta$ -strand I in B19 (Pillet *et al.*, 2003). The  $\beta$ -strand I is internal in all the resolved structures of the parvovirus capsid, strongly suggesting some cytoplasmic folding of an unassembled major capsid protein subunit.

The configuration of the VP protein complex, which is competent for nuclear transport is still a matter of discussion, since the VP protein subunits can be isolated in different oligomerization stages in AAV-infected cells (Wistuba *et al.*, 1997) and upon CPV capsid denaturation (Yuan *et al.*, 2001). Experimental studies that shed light on this process involved between the cooperative interaction between the VP2 and VP3 proteins of AAV for nuclear transport (Hoque *et al.*, 1999) and the VP1 and VP2 proteins of MVM (Lombardo *et al.*, 2002). In the latter system, a genetic analysis suggested an *in vivo* interaction between VP subunits, as incompetent VP1 subunits mutated at the NLM and the BC1 and BC2 sequences were efficiently translocated into the nucleus by the co-expression of wt VP2 proteins, and transport-incompetent VP2 subunits mutated at the NLM were

partly co-transported by the VP1-specific NLS sequences. On the basis of these data and since VP1 is synthesized in the course of the infection at a 1:5 ratio in respect to VP2 (Tattersall *et al.*, 1977; Jongeneel *et al.*, 1986; Cotmore and Tattersall, 1987; Schoborg and Pintel, 1991), it was postulated that a trimer of VP proteins is the assembly intermediate competent for nuclear transport (Lombardo *et al.*, 2000, 2002). This hypothesis is structurally supported by the high stability of putative trimers as measured by the buried surface area on oligomer formation (Wu and Rossmann, 1993; Xie and Chapman, 1996) owing to the intertwined nature of the 3-fold symmetry-related subunits in parvovirus capsid (Tsao *et al.*, 1991; Agbandje-McKenna *et al.*, 1998) and the exposure of the basic residues of the NLM to the solvent in the predicted structure of the VP trimer (Figure 20.3). In addition, the phenotype of two sets of mutants constructed in the MVM capsid also supports the configuration of a VP trimer translocating to the nucleus (Figure 20.4). First, the introduction of bulky residues or the truncation of side chains, involved in interactions at the interfaces between trimeric subunits of MVM capsid (e.g.

the K153A mutant; Reguera *et al.*, 2004), produced the accumulation of VP trimers that could be isolated in sedimentation gradients (Figure 20.4, *lower*; Riobos *et al.*, 2005). These VP trimers were competent in nuclear translocation in spite of the fact that they are absolutely unable to form capsid (Figure 20.4, *upper panels*). Second, mutations inactivating the NLM (double mutant K530N and R534T, named  $\Delta$  NLM) that halt nuclear transport and capsid assembly of VP2 subunits (Figure 20.4, *upper*; Lombardo *et al.*, 2000) produced the cytoplasmic accumulation of VP oligomers sedimenting mostly at the trimer position, whereas the VP1/VP2 oligomers translocated into the nucleus by the activity of the NLS of the VP1 specific region assembled into VP entities larger than capsid and sedimenting toward the bottom of the gradients (Figure 20.4, *lower*; Riobos *et al.*, 2004). Thus, biochemical and genetic analyses consistently support the cytoplasmic configuration of a VP trimer as the major intermediate of parvovirus capsid assembly competent for nuclear transport.

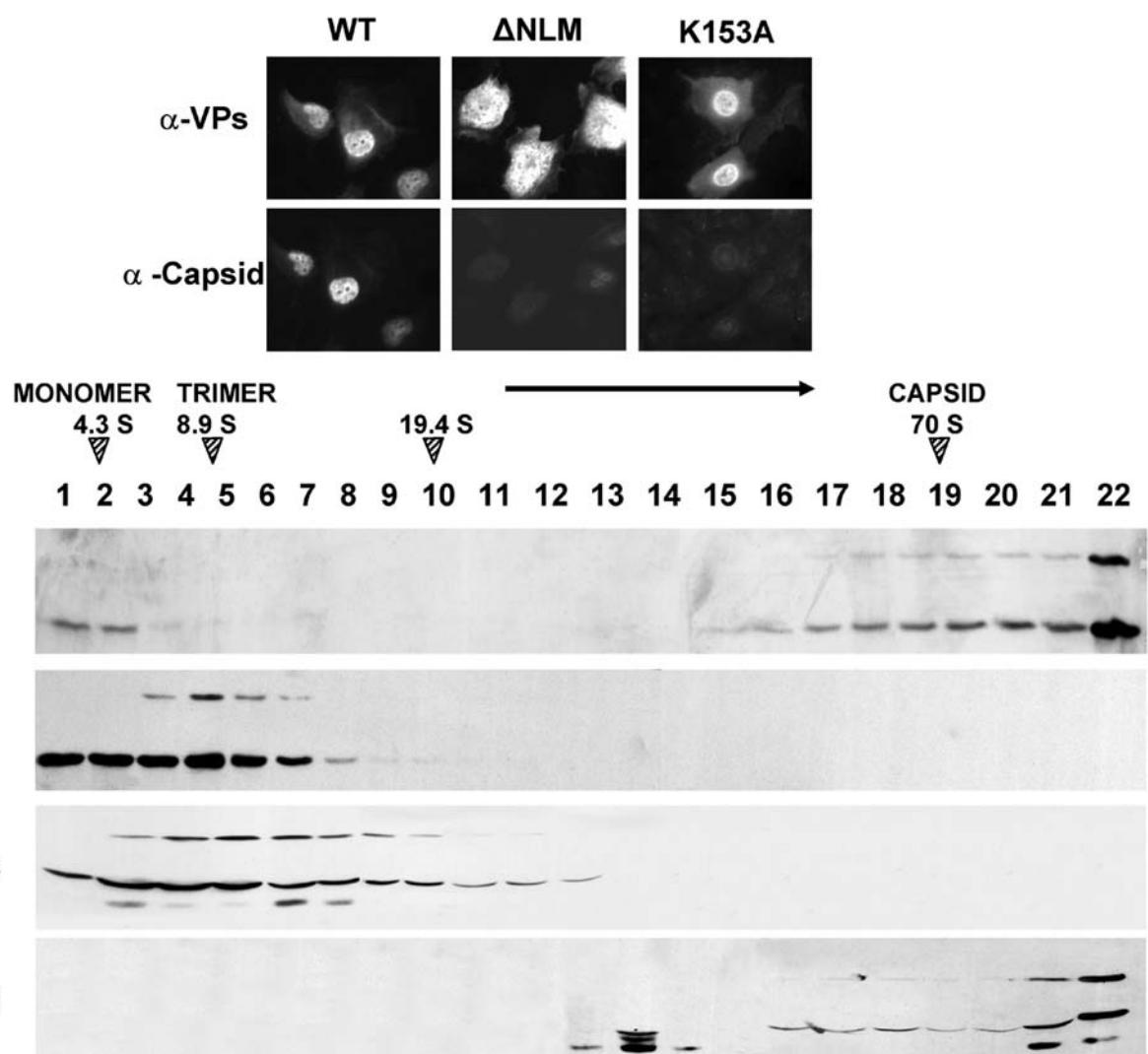
A number of poorly characterized additional factors must contribute to the proper VP oligomerization, transport,



**Figure 20.3** Structure of the NLM of MVM displayed in a VP2 trimer. The figure shows a trimer of VP subunits of the MVM capsid viewed down an icosahedral three-fold axis from the interior of the particle. The  $\beta$ -strand I containing the mapped nuclear localization motif (NLM) is shown as space-filling model colored white, with some of the basic residues important for transport in blue, and the rest of the protein in ribbon showing the  $\beta$ -barrel fold. Each subunit in the trimer has been drawn in a different color. The figure was generated with the program RasMol (Sayle and Milner-White, 1995) from available coordinates (PDB, Accession No. 1MVM). See also Color Plate 20.3.

and capsid assembly. For example, the capacity of VP2 to assemble in MVM capsids, while VP1 cannot under similar high nuclear accumulation (Tullis *et al.*, 1993; Lombardo *et al.*, 2002), and the difficulties to isolate VP1-only oligomers (our unpublished observations), may indicate an inappropriate folding of the VP1 protein subunits. In support of this, deletion mutants of the VP1-specific region triggered an important ubiquitination that could be significantly prevented by the co-expression of VP2 proteins (Lombardo *et al.*, 2002). This suggests that, in keeping with its major structural role, VP2 may act as a scaffolding factor assisting the cytoplasmic VP1 folding to avoid its degradation and the formation of transport competent trimers.

In addition, although CPV and MVM capsid formation can be detected in the cytoplasm of heterologous expression systems (Yuan *et al.*, 2001; Hernando *et al.*, 2005), it is rather well experimentally supported that the nucleus is the subcellular compartment where the parvovirus capsid first assembles in the natural infection of host cells (Wistuba *et al.*, 1997; Hoque *et al.*, 1999; Lombardo *et al.*, 2000). This is in agreement with the complete inability of cytoplasmic accumulated VP trimers to assemble into capsid (Figure 20.4; Riolobos *et al.*, 2005), suggesting that the nuclear milieu may be a source of factors with chaperone activity necessary for the final stages of parvovirus assembly and maturation.

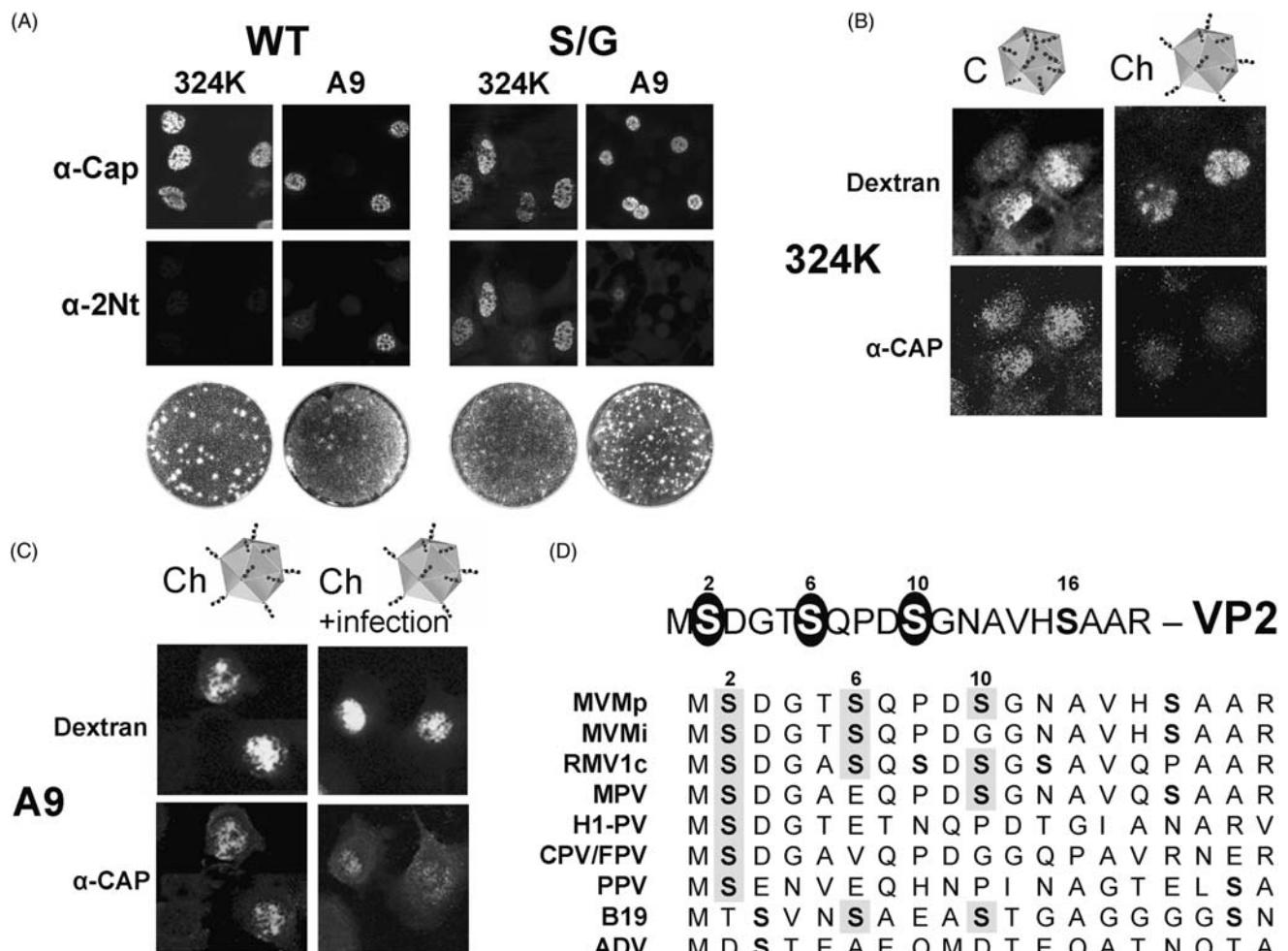


**Figure 20.4** Nuclear translocation of parvovirus capsid assembly intermediates. Phenotype of subcellular distribution and oligomerization of punctual MVM capsid mutants engineered at the exposed basic face of the NLM (mutant  $\Delta$ NLM), or at the face of contact between VP trimers (mutant K153A). Upper: Transfected cells were stained for immunofluorescence with an anti-MVM polyclonal antiserum recognizing protein subunits ( $\alpha$ -VPs) and a capsid-specific monoclonal antibody ( $\alpha$ -capsid). Lower: Sedimentation analysis of the indicated VP mutants of MVM capsid transfected in permissive cells. Homogenates were centrifuged for 6 hours across a 5–30% sucrose gradient (left to right in the figure) and the VP proteins in the fractions were subjected to immunoblot analysis with an  $\alpha$ -MVM capsid antiserum. Markers were: bovine serum albumin, 4.3 S;  $\beta$ -amylase 8.9 S; thyroglobuline, 19.4 S; purified MVM empty capsid, 70 S.

## ACTIVE NUCLEAR EXPORT OF THE MATURE PARVOVIRUS VIRION

It is generally assumed that non-enveloped viruses exit from the nucleus inducing disorganization and lysis of the nuclear membrane. However, there are accumulating evidences in different karyophilic viruses for release of infectious virus into the culture medium before patent signs of cytopathic effects in the host cells. Parvovirus empty capsids and DNA-full virions accumulate in the nucleus late in the infection cycle. The traffic of both types of particles inside the cell at the final stages of the infection was traced for MVM

with specific antibodies; a monoclonal antibody recognizing a capsid-specific epitope configured at the surface of both types of particles (Lopez-Bueno *et al.*, 2003), and a polyclonal antibody raised against the N-terminal sequence of VP2 (2Nt) which is exposed out of the protein shell only in virions with encapsidated DNA (Tattersall *et al.*, 1977; Paradiso *et al.*, 1984). In the MVM infection of highly synchronized transformed (NB324K) and non-transformed (A9) cell lines, the assembly of capsid and the subsequent maturation of virions could be followed along the time with these specific antibodies. Significantly, late in the infection cycle the intranuclear signal of virions is lost in contrast to the extended accumulation of empty capsids (Figure 20.5A, left).



**Figure 20.5** Nuclear export of mature virions. (A) Upper: Nuclear exit of wt and the S/G mutant lacking 2Nt phosphorylation in transformed (NB324K) and non-transformed (A9) cells. Shown are representative fields of cells stained with a monoclonal antibody recognizing assembled particles ( $\alpha$ -Cap) and DNA-filled virions ( $\alpha$ -2Nt) visualized by confocal microscopy. Lower: Plaque-forming capacity of wt and S/G mutant in NB324K and A9 cell monolayers. Note the differences in plaque morphology and 324K/A9 ratio between the viruses. Confocal analysis of the subcellular localization of purified MVM empty capsids either native (C) or with 2Nt exposed by heating at 50°C, 10 min (Ch) microinjected into the 324K (B) or the A9 nucleus (C). Viral particles were injected with dextran-FITC and analyzed 1 hour post-injection after staining with the  $\alpha$ -CAP serum. Shown are representative injected cells with the FITC-dextran marking intact nuclear membrane. (D) The 2Nt sequence in parvoviruses. Upper: The three serines of 2Nt phosphorylated in the MVMp infection of NB324K cells are highlighted. Lower: Comparison of the N-terminal sequence of the major capsid protein of several parvoviruses, showing shadowed the serines residues conserved at the three phosphorylated positions of MVMp, and the rest in bold. Viral acronyms are as in previous figures.

This phenomenon denoted a specific nuclear egress of virions prior to nuclear membrane disruption, that was supported by the distribution of both types of viral particles in the nuclear and cytoplasmic compartments of fractionated cells analyzed by centrifugation to equilibrium (Maroto *et al.*, 2004).

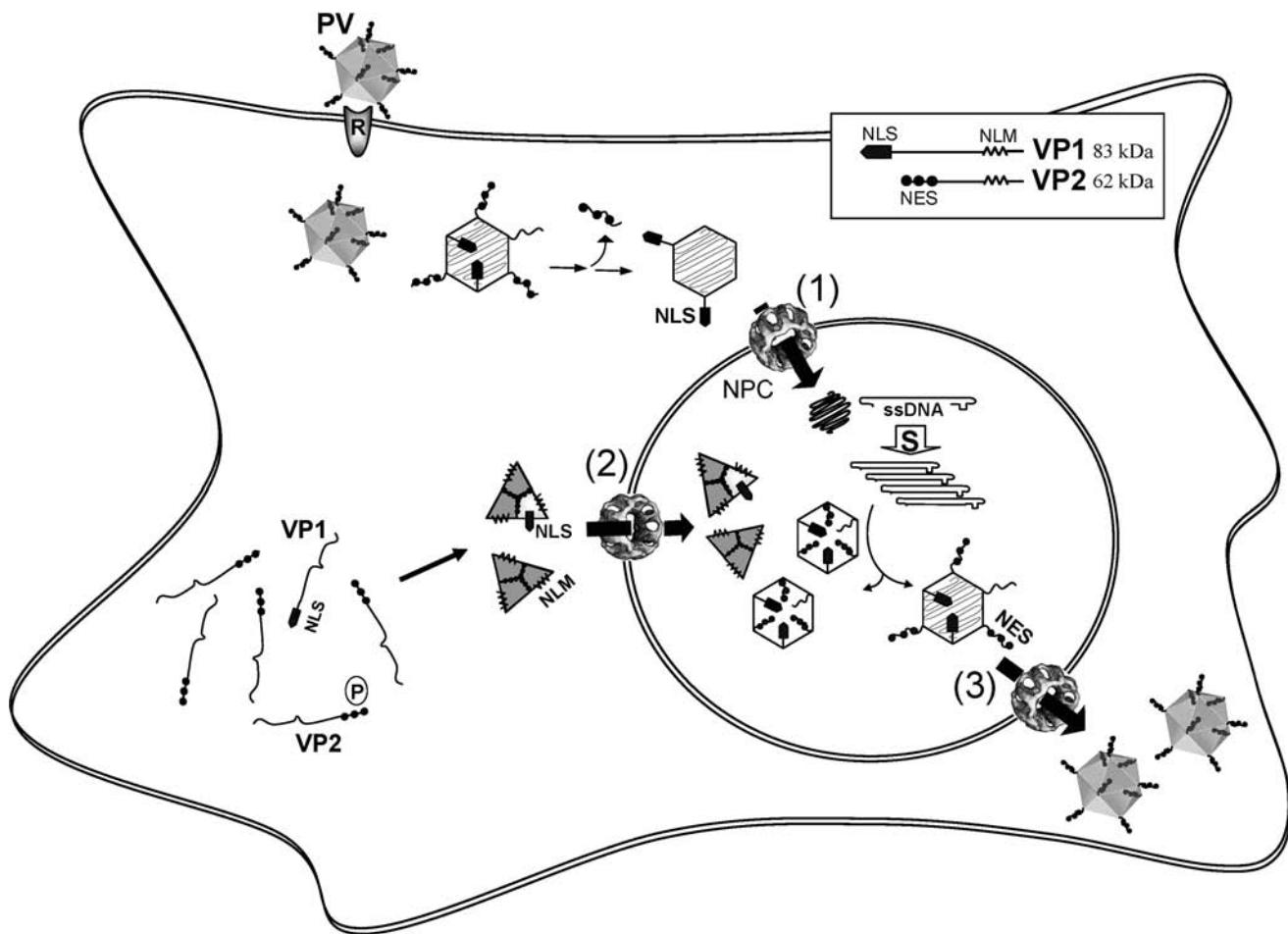
The identification of the signal used by the virions to exit from the nucleus derived from the biological analysis of the pattern of MVM capsid phosphorylation (see Figure 20.1C, p. 292). The most prominent phosphopeptide of MVM capsid, phosphopeptide B of VP2, was mapped to the 2Nt domain and showed the serine residues at positions 2, 6, and 10, phosphorylated in NB324K transformed cells (Maroto *et al.*, 2000). Virions lacking these phosphoserines (named S/G mutant) mature normally but showed a distinct nuclear export phenotype in respect to the wt. The S/G virion remained mostly accumulated within the nucleus of transformed cells, though efficiently exit the non-transformed A9 nucleus, reversing the characteristic plaque-forming capacity of MVMp in these cell types (Figure 20.5A, lower). The role of 2Nt as a signal for nuclear export of the MVM capsid and the capacity of this signal to access different export routes, was evidenced in the analysis of the intracellular traffic of purified microinjected MVM particles (Maroto *et al.*, 2004). As shown in Figure 20.5C, phosphorylated MVM capsid exits the nucleus of transformed NB324K only if they are previously subjected to a heat-induced structural transition detectable by fluorescence that exposes the 2Nt (Hernando *et al.*, 2000; Carreira *et al.*, 2004). However, an exposed 2Nt was not sufficient for the capsid to exit the non-transformed A9 nucleus, as undefined factors of the early stages of the MVM infection were also required (Figure 20.5D). These data and the efficient exit and plaque forming capacity of the S/G mutant suggested an alternative MVM export route independent of capsid phosphorylation operating in non-transformed A9 cells.

Although the routes of export accessed by the non-conventional 2Nt export signal in different cell types are largely unknown, some of their features, shared with the better established export mechanisms of cellular components, could be characterized. In the eukaryotic cells, proteins and RNA are actively exported from the nucleus through the NPC by the recognition of nuclear export sequence (NES) (Nakielny and Dreyfuss, 1999) by specific receptors such as the cellular chromosome region maintenance 1 protein (CRM1 or exportin1; Ossareh-Nazari *et al.*, 1997; Stade *et al.*, 1997). CRM1 binds to leucine-rich and other types of NESs (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997) and can be inhibited by the antibiotic leptomycin B (LMB; Kudo *et al.*, 1998), although other less characterized nuclear export receptors are resistant to LMB and recognize distinct types of signals (Stuven *et al.*, 2003). Indeed, the exit of the MVM virion in transformed NB324K cells was not significantly sensitive to the LMB inhibitor (Maroto *et al.*, 2004), suggesting that the virus could use in this cell some of the atypical phosphorylation-dependent nuclear export routes

(Kaffman *et al.*, 1998; Ohno *et al.*, 2000), which are inactive in the non-transformed cells. In sharp contrast, a patent export inhibition by LMB in A9 cells indicated that the CRM1 factor must be involved in MVM virus nuclear exit, although no consistent indications of a direct interaction between MVM particles and CRM1 could be demonstrated with specific antibodies. Interestingly, the CRM1-binding domain of the non-structural NS2 protein (Bodendorf *et al.*, 1999; Ohshima *et al.*, 1999) that is important for viral spreading in culture (Eichwald *et al.*, 2002; Miller and Pintel, 2002) undergoes a natural selection in immunodeficient mice to NS2 mutant proteins with higher capacity to sequestrate CRM1 in the perinuclear region (Lopez-Bueno *et al.*, 2004). This sequestration triggered a general non-specific increase in the release of viral non-structural proteins and capsids from the nucleus probably due to the loss of integrity of the nuclear membrane (Lopez-Bueno *et al.*, 2004). Such an indirect CRM1 involvement may also facilitate the unrelated mechanism of active nuclear exit of mature MVM virions. Finally, the conservation of the 2Nt serine residues phosphorylated in MVM among different parvoviruses (Figure 20.5D), particularly the phenotypically relevant serine at position 2 (Maroto *et al.*, 2000) preceding an acidic residue, suggests that the nuclear exit of other parvovirus virions may also use alternative phosphorylation and CRM1-dependent pathways in different host cells.

## SUMMARY

The diagram shown in Figure 20.6 summarizes the main messages of this review, which focuses on the regulation exerted on parvovirus life cycle by the trafficking of the structural proteins across the nuclear membrane. The model is exemplified for MVM, but given the high degree of conservation of the VP transport signals among parvoviruses mentioned above (Figures 20.2, p. 295, and 20.5), a similar scheme may apply to other members of the family. Three types of nuclear transport sequences have been identified in the MVM capsid proteins, two displaying import activity, the NLM domain common to VP1 and VP2, and the conventional NLS (BC1 and BC2 domains) of VP1, and one export sequence (NES) in the N-terminal region of VP2. The incoming virions initiating the infection harbor the VP2 N-terminal sequence exposed outside the particle and the two types of import sequences hidden in the shell. During the transport across the cytoplasm, the VP2 end is cleaved-off and a conformational change of the capsid triggers the exposure of the NLS of VP1 that allows access to the cellular nuclear import machinery. As the infection proceeds the synthesized structural proteins, which have been phosphorylated in the cytoplasm, oligomerize into trimers thus exposing the NLM and the NLS of VP1, being subsequently brought into the nucleus for capsid assembly. Empty capsids are first formed, which contain all the



**Figure 20.6** Nuclear transport of capsid proteins and viral particles in the Parvovirus life cycle. Processes outlined in the model: (1) Entry of the incoming virion: NES cleavage and exposure of the NLS of VP-1 along the transport of the incoming virion through the cytoplasm to deliver the genome across the NPC. (2) Translocation across the nuclear membrane: The synthesized VP1 and VP2 structural proteins are phosphorylated in the cytoplasm and translocated as trimers into the nucleus by the NLM activity. Viral DNA is amplified in the S-phase of the cell cycle and encapsidation presumably occurs in pre-assembled empty capsids. (3) Active nuclear exit of the newly formed virions. The mature virion can actively egress from the nucleus prior cell lysis by the NES mapped at VP2 N-terminus exposed on the capsid surface. Upper right: Nuclear transport sequences identified in the VP proteins of MVM: NLS: conventional nuclear transport sequence in linear configuration identified in VP1; NLM:  $\beta$ -stranded nuclear localization motif in VP1 and VP2; NES: phosphorylated (P) nuclear export sequence in VP2. NPC: nuclear pore complex.

nuclear transport sequences hidden inside. In the packaging step, as the viral genome becomes partially ordered in a process probably contributed to by dephosphorylation of the coat and the ssDNA-binding activity of the BC3-BC4 domains of VP1, a structural change is imposed leading to the exposure of the NES of VP2 out of the protein shell, specifically driving the mature virion outside the nucleus.

In conclusion, the signals regulating the nuclear/cytoplasmic shuttling of the parvovirus capsid proteins are exposed to the cellular transport machinery under distinct configurations. The intracellular plasticity of the parvovirus coat allows the exposure of the ends of the VP proteins, most probably across the channel at the 5-fold axis

(Tsao *et al.*, 1991; Agbandje-McKenna *et al.*, 1998), driving the traffic of the viral particle inward and outward into the nucleus. It is disturbing that sequences harboring key signals for parvovirus life cycle cannot be resolved in the crystal structure of the capsid owing to their flexible unordered configuration. The parvoviral coat transitions not involving disassembly may represent a common structural solution evolved by metastable viral capsids to traverse the nuclear membrane. However, the nuclear translocation of the capsid protein subunits driven by structured transport signals (NLM-like), which requires correct protein folding and oligomerization, probably plays a key role in the quality control of viral morphogenesis. In addition, the function

of both types of transport signals can be regulated by phosphorylation. Identifying the cellular factors interacting with the VP proteins that mediate their phosphorylation and nuclear membrane translocation should provide relevant insights into the mechanisms of gene delivery by the parvovirus capsid and its biotechnological applicability.

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# Molecular interactions involved in assembling the viral particle and packaging the genome

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The life cycle of viruses involves both disassembly and reassembly of the virion structure within the same cell. It is intriguing to learn how these reciprocal processes are accomplished and how different viruses have solved the problem. The capsid is a protective vehicle for transporting the viral genome through extreme environments in the extracellular world, and this requires a stable structure as in the case of the parvovirus capsid. However, this protective coat has to be or become metastable to allow its efficient assembly at the appropriate site following cell entry. So there might be subtle differences between newly assembled capsids, which leave the cell and capsids entering the cell by different routes. Alternative routing requires orientation and flexibility, which are emerging issues in the study of virion structures. Here we discuss the available data relating to capsid assembly and genome packaging of parvoviruses, which might also shed some light on the disassembly process that occurs during infection.

## VIRION COMPOSITION AND STRUCTURE

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### **Virion composition**

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The parvovirus virion is composed of two to four capsid proteins and a single-stranded genome of about 5000 nucleotides in length (for reviews see Siegl *et al.*, 1985; Cotmore and Tattersall, 1987; Tijssen, 1990; Tijssen and Bergoin, 1995; Berns, 1996). The proteins have approximate molecular weights between 60 and 96 kDa with the major coat protein representing about 80 percent of the total capsid mass. They are encoded from a single gene by

overlapping in-frame DNA sequences leading to polypeptides with a common core sequence, a common C-terminus yet different N-termini. The capsid is made up of 60 equivalent copies of the core sequence containing polypeptides with a varying amount of minor capsid proteins (between 10 and 20 percent of total capsid mass) differing in the N-terminus. The extent to which minor capsid proteins are incorporated within the capsid is species dependent and might also vary within the same species under different production conditions. The unique N-terminus of VP1 of most parvoviruses contains a motif related to the catalytic domain of phospholipase A<sub>2</sub> (PLA<sub>2</sub>), which plays an important role in viral infection (Zadori *et al.*, 2001; Dorsch *et al.*, 2002; Girod *et al.*, 2002). In some species of autonomous parvoviruses (e.g. minute virus of mice [MVM], H1) VP3 is a proteolytic processing product of VP2. The capsid proteins are not modified by carbohydrates and virions do not contain lipids or low molecular weight histone-like proteins. The presence of polyamines has been shown for members of the densoviruses (Kelly and Elliott, 1977), but not for the other parvoviruses. Phosphorylation of all three capsid proteins has been described for some autonomous parvoviruses (Santaren *et al.*, 1993; Maroto *et al.*, 2000). Mature infectious virions have masses of approximately  $5.4\text{--}6.2 \times 10^6$  Da and contain about 30 percent DNA and 70 percent protein. The virion is unusually resistant to inactivation. It is stable up to about 60°C and tolerates pH values between 3 and 9.

### **Virion structure**

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The 60 capsid protein subunits are assembled in an icosahedral shell with  $T = 1$  structural symmetry in which they

occupy symmetrically equivalent positions and have a diameter of about 18–26 nm (Cotmore and Tattersall, 1987; Berns, 1996). Atomic or near-atomic structures have been resolved for the canine parvovirus (CPV), the feline panleukopenia virus (FPV), MVM, the *Galleria mellonella* densovirus (*Gm*DNV), the porcine parvovirus (PPV), the adeno-associated virus type 2 (AAV2), and to a lower resolution for human parvovirus B19 (Tsao *et al.*, 1991; Agbandje *et al.*, 1993, 1994; Simpson *et al.*, 1998; Simpson *et al.*, 2002; Xie *et al.*, 2002). Images were also reconstructed from electron cryo-microscopy pictures of B19, Aleutian mink disease virus (AMDV), AAV2 and AAV5 (Chipman *et al.*, 1996; McKenna *et al.*, 1999; Kronenberg *et al.*, 2001; Walters *et al.*, 2004). They show a similar structural arrangement at the inner surfaces but variability at the organization of the outer surface. Each subunit is folded as a  $\beta$ -barrel core composed of eight-strands of antiparallel  $\beta$ -sheets and four large protruding loops that form the surface ‘spike’ features unique to each capsid species. In common is a cylindrical channel at the 5-fold symmetry axis surrounded by a canyon-like depression, a dimple-like depression at the 2-fold axis, and small protrusions at each of the 3-fold rotation axes except in the case of B19.

The outside of the *Gm*DNV capsids is much smoother than that of the vertebrate parvoviruses owing to the truncation of common external loop structures (Simpson *et al.*, 1998). The loops from neighboring subunits interact by intertwining at the 3-fold axis, which may additionally stabilize trimers as compared with dimers or pentamers (Figure 21.1). However, adjacent subunits at the 5-fold axis also form sling-like connections to their neighbours in the AAV2 capsid or interdigitate in CPV, FPV, MVM, PPV, and *Gm*DNV capsids. However, these interactions look less elaborate than at the 3-fold axis.

Quantification of protein–protein interactions by determining the available interacting surface area (Jones and Thornton, 1996) at the different symmetry elements supports this interpretation (Table 21.1). An exception is *Gm*DNV, which shows only a slightly increased interaction surface at the level of trimers compared with dimers or pentamers. Also the number of H-bonds between subunits indicates a preferred stability of trimers and pentamers. Again *Gm*DNV differs in this respect from the other parvoviruses. The formation of these linking loops at the 3-fold and 5-fold axes certainly determines steps of capsid assembly and key residues involved in the strongest interactions are presumably critical for assembly.

Of all the different structures that have been resolved, the inner surface of the capsid is more conserved than the outer surface. It is smoother and does not show the pattern of notching seen at the outer surface. The pattern and density of charged amino acids is characteristically rearranged between the inside and outside surfaces especially at the 3-fold related subunits. The most striking feature of the DNA structure in the CPV capsid is the complete lack of interactions between the phosphates and basic amino acids

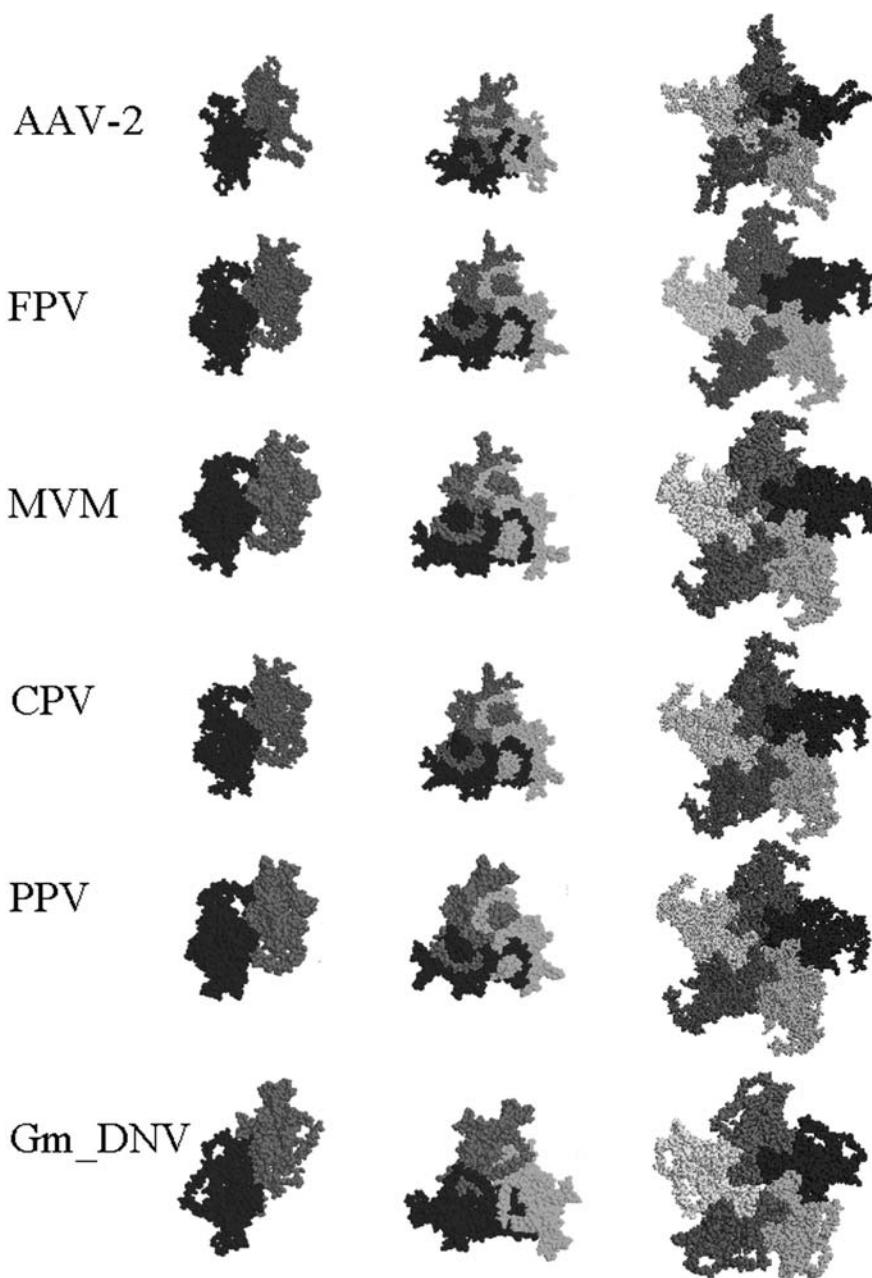
(Chapman and Rossmann, 1995). The single-stranded DNA (ssDNA) structure can be considered to be flipped inside out, with bases pointing outwards towards the protein and phosphates being surrounded by metal ions or other counterions on the inside. The capsid proteins interact with the bases mainly by hydrogen bonding. There is potential for a low level of sequence specificity in the interaction with the 30 or so DNA fragments that interact with the interior of the capsid. Any possible contribution of short basic sequences in the N-terminal portion of the minor capsid proteins of many parvoviruses towards charge neutralization of packaged DNA is rather unlikely since they are not required for genome packaging in the case of MVM or AAV2 (Tullis *et al.*, 1993; Wu *et al.*, 2000) and they are not present in all parvoviruses.

In the case of CPV and MVM, there is structural evidence that the VP2 N-termini of some of the capsid proteins have traversed through channels at the 5-fold symmetry axes from the inside of the capsids to the outside (Tsao *et al.*, 1991; Agbandje-McKenna *et al.*, 1998; Xie *et al.*, 2002). This is probably a result of genome packaging as assayed by VP2-specific antibodies (Cotmore *et al.*, 1999). In empty capsids of MVM and AAV2, the N-termini are not accessible from the outside and, in the case of AAV2, they are probably located at the 2-fold symmetry axes inside the capsids (Cotmore *et al.*, 1999; Kronenberg *et al.*, 2001). Upon a heat or pH stimulus, the VP1 N-termini also become exposed at the capsid surface of several parvoviruses by a conformational transition possibly at the cylindrical channels along the 5-fold axes (Cotmore *et al.*, 1999; Vihinen-Ranta *et al.*, 2002; Canaan *et al.*, 2004). This transition also occurs only with full capsids and is functionally associated with the infection and disassembly processes. Interestingly, the channels around the 5-fold axes are filled in AMDV and B19 (Chipman *et al.*, 1996; McKenna *et al.*, 1999) and the N-termini of VP1 are accessible from the outside in empty and full B19 capsids (Rosenfeld *et al.*, 1992).

## A PROPOSED PATHWAY OF PARVOVIRUS ASSEMBLY

### The AAV2 paradigm

Based on a detailed isotopic pulse chase study of AAV2 virion assembly, Myers and Carter (Myers and Carter, 1980; reviewed in Carter *et al.*, 1990) established the now commonly accepted concept of parvovirus assembly. The central question was, whether the viral genome is introduced into a preformed capsid or the capsid assembled around the genome. The basis for answering this question was the identification of AAV particles which differed with respect to their buoyant density in CsCl gradients and their sedimentation velocity in sucrose gradients.



**Figure 21.1** Visualization of parvovirus capsid protein interactions. Capsid protein subunits (indicated by different colors) of adeno-associated virus type 2 (AAV2), feline parvovirus (FPV), minute virus of mice (MVM), canine parvovirus (CPV), porcine parvovirus (PPV) and *Galleria mellonella* densovirus (GmDNV) are viewed from outside at the 2-fold (first row), 3-fold (second row) and 5-fold (third row) symmetry axes. Note intertwining interactions at the 3-fold and 5-fold axes, which are not present at the 2-fold axes. Quantification of the interacting surface areas is shown in Table 21.1. (The images were generated and kindly provided by W. von der Lieth.) See also Color Plate 21.1.

Cells were labeled with [<sup>3</sup>H]-thymidine or [<sup>35</sup>S]-methionine and accumulation of the radioactive label in different classes of AAV particles was recovered after different chase periods. The main results were:

- After a short [<sup>35</sup>S]-methionine pulse, radioactive label appeared within 5–10 minutes in empty AAV ( $\rho = 1.32 \text{ g/cm}^3$ ) particles, reaching a maximum after about 30 minutes and chased from the empty

particles to the more dense fractions of AAV ( $\rho > 1.32 \text{ g/cm}^3$ ).

- [<sup>3</sup>H]-Thymidine label was not detected in empty particles but accumulated in fully packaged AAV particles ( $\rho = 1.41 \text{ g/cm}^3$  and  $\rho = 1.45 \text{ g/cm}^3$ ) and defective interfering particles (DI) with similar kinetics to the appearance of [<sup>35</sup>S] label in these fractions.
- Accumulation of [<sup>3</sup>H] and [<sup>35</sup>S] label into the DNA-containing particle fractions was slow and lasted for at

**Table 21.1 Quantification of protein–protein interactions**

Virus	ASA	% ASA	H-Bond number
AAV2_DI	1400	4.8	20
FPV_DI	1821	5.8	8
MVM_DI	1736	5.5	16
CPV_DI	1807	5.8	18
GM_DNV_DI	1864	8.2	18
PPV_DI	1853	6.19	16
AAV2_TRI	4942	16.9	49
FPV_TRI	5069	16.6	63
MVM_TRI	5104	16.3	41
CPV_TRI	5258	16.6	64
GM_DNV_TRI	2405	10.6	12
PPV_TRI	5314	17.7	60
AAV2_PENTA	2318	7.9	18
FPV_PENTA	2140	7.0	18
MVM_PENTA	2230	7.1	22
CPV_PENTA	2772	8.5	20
GM_DNV_PENTA	1807	8.0	14
PPV_PENTA	2212	7.0	21

ASA: [ $\text{\AA}^2$ ] accessible surface area between two subunits at the indicated symmetry; % ASA: percentage of interface surface of the total surface of one residue; H-Bond: number of H-bonds between two subunits at the indicated symmetry; DI: 2-fold symmetry; TRI: threefold symmetry; PENTA: 5-fold symmetry; protein–protein interactions were calculated by C.-W. von der Lieth according to Jones and Thornton (1996).

least 4 hours. It continued also in the presence of a DNA synthesis inhibitor.

- An unusual sedimenting fraction (AAV-SP;  $\rho = 1.41 \text{ g/cm}^3$ ; sedimenting at about 60S) was labeled more slowly than empty particles but more rapidly than the other DNA containing particles. The DNA was completely sensitive to DNase I digestion converting the SP sedimentation characteristics to those of empty capsids. This particle class was interpreted as an intermediate between empty and full or as partially packaged capsids.
- Inhibition of DNA replication reduced labeling of mature virions, but increased accumulation of empty capsids.

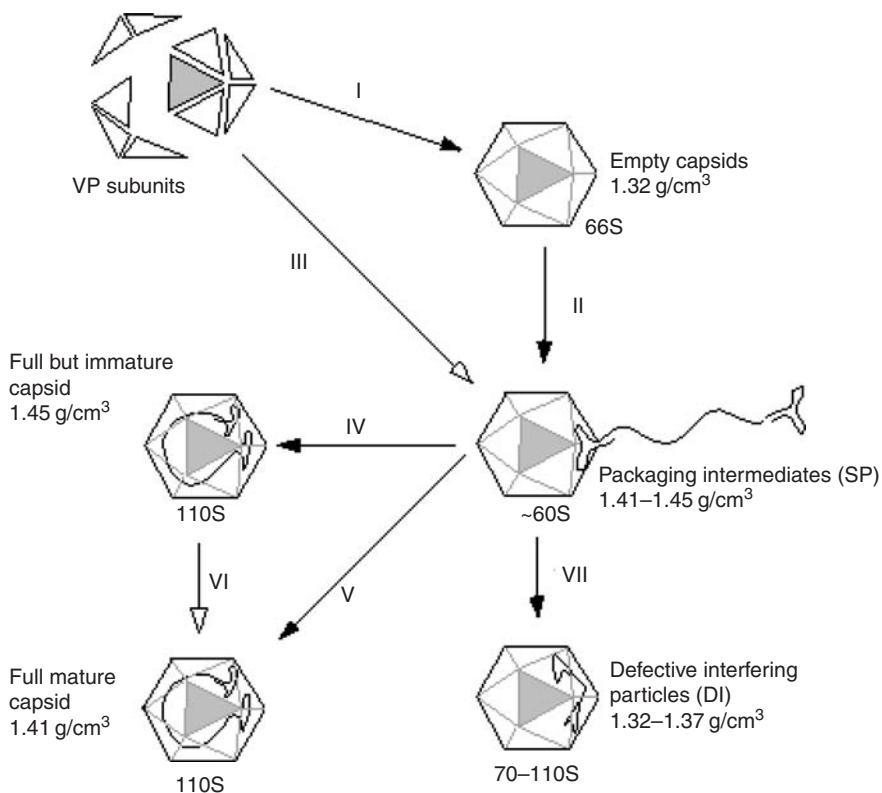
From these data the authors deduced a model in which replicated strand-displaced ssDNA associates with preformed empty capsids to form the intermediate structure AAV-SP in which the DNA is associated with the capsid but not protected (Figure 21.2). This intermediate is then slowly converted into a full capsid with a density of  $\rho = 1.41 \text{ g/cm}^3$  or  $1.45 \text{ g/cm}^3$ . For these two capsid species they could not observe a precursor–product relationship. Although this interpretation explains the observed data, it does not consider the pool of non-assembled capsid proteins or capsomers as a substrate for genome packaging. A consequence of introducing the viral genome into preformed capsids is that packaging is a vectorial process. Based on the finding that 5' termini are more accessible to digestion with DNase I

in partially encapsidated genome populations than 3' termini, it was later concluded that the packaging process occurs in a preferred direction (King *et al.*, 2001) supporting the hypothesis of Myers and Carter. Finally, the involvement of a helicase activity in the packaging process (King *et al.*, 2001) is also most consistent with a translocation process into preformed or partially formed capsids. Full length single-stranded AAV DNA generated by replication of AAV2 genomes containing mutations in the helicase motif of small Rep proteins associated with capsids, but failed to be completely translocated into the capsid.

### Comparing AAV2 to the autonomous parvoviruses

Fractions with similar characteristics as described for AAV2 have also been described for autonomous replicating parvoviruses (for a review see Cotmore and Tattersall, 1987) except for possible packaging intermediates sedimenting at 60S. In a report by Richards *et al.* (1977) of analysis of the kinetics of MVM assembly, the formation of empty capsids appeared just prior to full capsids after infection of synchronized cells. A precursor–product relationship between empty and full particles could not be established in this study. However, they observed that the  $\rho = 1.46 \text{ g/cm}^3$  labeled particles were quantitatively converted to the  $\rho = 1.42 \text{ g/cm}^3$  class in the nucleus suggesting a maturation pathway from a less stable, higher density full particle to the more stable and compact final product. In a more recent study, Yuan and Parrish (2001) followed the pulse-chase kinetics of CPV capsid proteins by analyzing non-assembled VP proteins, empty capsids and full capsids by sedimentation velocity analysis on sucrose gradients. Very similar to the results obtained by Myers and Carter (1980), the [ $^{35}\text{S}$ ]-Meth label disappeared from the empty capsid pool just before the pool of full capsids increased; however, the proposed precursor–product relationship was not quantitative. Interestingly, they also observed a large pool of labeled non-assembled VP proteins, which also decreased before the full capsid pool increased. The contribution of this pool of capsid proteins or capsomers – whether it is derived from non-assembled capsid proteins or from disassembled capsids – to the assembly of full capsids remains to be considered.

Overall, the data available so far are most plausibly explained by the hypothesis that the strand-displaced replicated DNA binds to an empty preformed capsid and that the DNA is then introduced in a slow process to form full particles. Direct evidence for this scenario, for example by reconstitution of the packaging reaction *in vitro* using preformed capsids, is still lacking. Two reports on AAV2 *in vitro* packaging could provide the basis for proving this hypothesis (Ding *et al.*, 1997; Zhou and Muzyczka, 1998). An open question remains as to whether the genome docking reaction occurs with preformed capsids or is embedded in the capsid assembly process (Figure 21.2; reaction III). The latter would



**Figure 21.2** Possible pathways for parvovirus assembly. Dark arrows indicate pathways proposed by Myers and Carter (Myers and Carter, 1980), based on pulse-chase experiments using <sup>35</sup>S-labeled amino acids and <sup>3</sup>H-labeled nucleotides. Reaction I: Assembly of empty capsids; the equilibrium is on the side of capsids in the nucleus. Reaction II: Proposed SP intermediate remains constant after its formation in the first hours of packaging. Reaction III: Not considered by Myers and Carter (Myers and Carter, 1980); possible according to Yuan and Parrish (Yuan and Parrish, 2001). Reaction IV: 1.45 g/cm<sup>3</sup> fraction shows similar pulse-chase kinetics as SP intermediate; delayed compared to reactions II and V. Reaction V: Nearly quantitative precursor-product relationship between empty capsids (1.32 g/cm<sup>3</sup>) and mature virions (1.41 g/cm<sup>3</sup>) implying reactions II and V. Reaction VI: No precursor-product relationship observed in AAV2; but reported for MVM. Reaction VII: Accumulation of 1.35 g/cm<sup>3</sup> and 1.37 g/cm<sup>3</sup> fractions is faster than that of SP and slightly slower than that of empty capsids. The reasons for abortive packaging are not known.

suggest that empty capsids are more of a by-product than a substrate and that encapsidation requires early interaction of partially-assembled capsid proteins with genome termini.

The pathway discussed above involves capsid assembly and genome packaging as two separate steps. Available data describing these steps will be discussed in the following two sections.

## CAPSID ASSEMBLY

Although the capsid assembly process has been studied by genetic, cell biological, and biochemical techniques, only certain facets of the overall process are currently understood.

### Genetic analysis

Genetic analysis showed that expression of the major capsid proteins of autonomous parvoviruses is sufficient

for capsid assembly, e.g. of MVM (Pintel *et al.*, 1984), CPV (Lopez de Turiso *et al.*, 1992; Saliki *et al.*, 1992), PPV (Martinez *et al.*, 1992), ADV (Christensen *et al.*, 1993), mink enteritis virus (MEV; Christensen *et al.*, 1994), duck parvovirus (DPV; Le Gall-Recule *et al.*, 1996) and B19 (Kajigaya *et al.*, 1989; Brown *et al.*, 1991; Kajigaya *et al.*, 1991). For B19 and MVMi, it has been reported that VP1 alone cannot assemble into capsids (Wong *et al.*, 1994; Lombardo *et al.*, 2000). However, VP1 deletion mutants of B19 – expressed in SF9 cells using the baculovirus system, which extended the VP2 N-terminus by about 70 amino acids – formed capsids of normal appearance (Wong *et al.*, 1994). Longer versions of VP1 assembled progressively less efficiently and produced capsids of markedly dysmorphic appearance. This suggests that capsid formation tolerates only limited numbers of molecules with the VP1 unique sequence.

Deletion of the first 25 amino acids of B19 VP2 had no effect on capsid assembly of baculovirus expressed VP proteins (Kawase *et al.*, 1995). Further N-terminal truncations of up to 30 amino acids failed to self-assemble, but

could co-assemble with normal VP2 into capsids. This region corresponds to the beginning of the  $\beta$ A antiparallel strand. Truncations beyond 30 amino acids prevented both self-assembly and co-assembly. Also in a baculovirus expression system, N-terminal deletions of CPV VP2 up to amino acid number 14 were tolerated with regard to capsid assembly; however, deletion of 24 amino acids led to a loss of hemagglutination and an altered morphology of the resulting virus-like particles (VLPs) (Hurtado *et al.*, 1996).

The authors proposed a role of the arginine, asparagine, glutamic acid, arginine (RNER) sequence motif in correct capsid formation. Deletions in loops 1, 3, and 4 prevented capsid formation of CPV, whereas a deletion in loop 2 had little or no effect on capsid morphogenesis. In a recent report 28 amino acids involved in non-covalent interactions between trimeric capsid protein subunits of MVM were individually mutated to alanine (Reguera *et al.*, 2004). Only a small number of these side chains were needed for correct oligomerization and capsid assembly. These subunits showed either hydrophobic contacts between trimeric subunits with high intersubunit association energies or participated in intertrimeric hydrogen bond or salt bridge formation.

Similarly to the autonomous parvoviruses, expression of the major capsid protein VP3 of AAV2 on its own is sufficient to form capsids provided that it accumulates in the cell nucleus (Hoque *et al.*, 1999a), while in the absence of a nuclear localization signal (NLS) no capsid assembly was observed if VP3 was expressed alone (Steinbach *et al.*, 1997; Hoque *et al.*, 1999a). Following transfection of cells with a proviral plasmid, capsids could be found that consisted exclusively of VP3 molecules, also suggesting that VP1 and VP2 are not required for assembly (Rabinowitz *et al.*, 1999; Warrington *et al.*, 2004). Expression of single VP1, VP2, or combined VP1 and VP2 after mutation of all other possible VP translation start codons in the context of the whole AAV2 genome, yielded no particles in capsid preparations (Warrington *et al.*, 2004), whereas coexpression of VP3 led to assembly of capsids composed of VP1 and VP3, or VP2 and VP3 (Warrington *et al.*, 2004; King *et al.*, unpublished observation).

Alanine-scanning and insertional mutagenesis of AAV2 capsid proteins provided evidence that mutations within the  $\beta$ -barrel structure of VP3 tend to prevent assembly (Wu *et al.*, 2000). Also, insertions at the extreme N- or C-termini of VP3 impaired capsid assembly. A number of insertion mutants that are defective for capsid formation cluster between amino acids 224 and 311 (N-terminal region of VP3) and between 607 and 693 (C-terminal region of all three capsid proteins; Wu *et al.*, 2000).

The last result is in agreement with an earlier observation that an intact C-terminus of AAV2 VP proteins is required for capsid assembly (Ruffing *et al.*, 1994). However, insertion of a 6xHis-tag at the C-terminus of the cap open reading frame (ORF) was tolerated when the tag was present solely in VP3, whereas it was defective for particle assembly when the

insertion was present in all three capsid proteins (Zhang *et al.*, 2002). Insertion of up to 238 amino acids at the N-terminus of VP2 were tolerated for capsid assembly, when they were expressed with sufficient VP3 (Yang *et al.*, 1998; Warrington *et al.*, 2004). Several point mutations in the AAV2 *cap* gene prevent capsid assembly indicating further critical amino acids in the assembly process (Warrington *et al.*, 2004).

Although additional parvovirus gene expression is not required for VP proteins to assemble into capsids (Pintel *et al.*, 1984; Kajigaya *et al.*, 1989, 1991; Clemens *et al.*, 1992; Christensen *et al.*, 1994; Cohen *et al.*, 1995; Wistuba *et al.*, 1997; Hernando *et al.*, 2000), several observations suggest that it can influence directly or indirectly the overall process. In a murine cell line, assembly of MVM capsids was largely blocked when mutant viruses that were not able to translate the non-structural protein NS2 were used (Cotmore *et al.*, 1997). This was not observed in a human cell line, suggesting an indirect effect of NS2 on capsid assembly in mouse cells. Similar mutants of CPV NS2 did not prevent capsid formation (Wang *et al.*, 1998), however, it should be noted that NS2 proteins of CPV and MVM are quite divergent. Co-expression of AAV2 Rep proteins together with all three capsid proteins influenced the intranuclear distribution of capsids suggesting a direct or indirect interaction with the assembled capsids (Wistuba *et al.*, 1997). In the absence of adenovirus and AAV2 Rep proteins, the majority of the capsid proteins remained associated with the nucleoli in HeLa cells, while co-expression of Rep proteins with the VP proteins led to a release of the capsids from nucleoli or a fragmentation of the nucleoli together with assembled capsids. When the capsid proteins of CPV were expressed alone in mammalian cells, they assembled an increased proportion of VP1 into empty capsids compared with capsids formed in infected cells (Yuan and Parrish, 2001), also suggesting an influence of other viral gene products on the assembly process.

## Cellular localization of capsid assembly

There seems to be a connection between nuclear localization of capsid proteins and capsid assembly (Wistuba *et al.*, 1997; Hoque *et al.*, 1999a; Lombardo *et al.*, 2000; Yuan and Parrish, 2001). Co-expression of AAV2 VP3 with either VP1 or VP2, which both contain nuclear localization signals, allowed nuclear accumulation of VP3, probably due to interaction of the capsid proteins in the cytoplasm prior to nuclear transport (Ruffing *et al.*, 1992; Hoque *et al.*, 1999a). Nuclear co-transport requires only one of the minor capsid proteins, as assembly of VP1/VP3 capsids and VP2/VP3 capsids occurs with comparable efficiency to wt capsids containing VP1, VP2, and VP3 (Hoque *et al.*, 1999a,b; Warrington *et al.*, 2004). A correlation of capsid assembly with nuclear localization of the capsid proteins was also suggested by immunolocalization of assembled AAV2 capsids in HeLa

cells exclusively to the nucleus (Wistuba *et al.*, 1997). N-terminal VP2 deletion mutants that were unable to accumulate in the nucleus in COS1 cells did not assemble into capsids. N-terminal addition of an NLS conferred assembly competence to VP3 (Hoque *et al.*, 1999a). Likewise, MVM VP2 mutants which affected nuclear transport also affected assembly (Lombardo *et al.*, 2000). Furthermore, a panel of CPV mutants within possible NLS sequences or a mutant with a reported assembly defect (Tresnan *et al.*, 1995) also showed a close correlation between capsid assembly and nuclear localization (Yuan and Parrish, 2001).

On the other hand, in baculovirus infected Sf9 cells, VP2 proteins and empty capsids of CPV accumulated in the cytoplasm around the nucleus (Yuan and Parrish, 2001). A similar observation was reported for MVM (Lombardo *et al.*, 2002). However, the assembly efficiency in insect cells was rather low. Nuclear accumulation may simply facilitate assembly by increasing the local concentration of capsid proteins in accordance with the observation that capsid assembly of AAV2 is a VP protein concentration-dependent process (Wistuba *et al.*, 1997). If the critical concentration is achieved in the cytoplasm (e.g. in insect cells), capsid assembly might also occur in the cytoplasm. Alternatively, or in addition, the nucleus might provide factors that facilitate or chaperone the assembly process. The appearance of assembled AAV2 capsids first in the nucleolus of infected cells (Wistuba *et al.*, 1997) could be an indicator for both interpretations. There are still no reports of a systematic attempt to identify nuclear proteins involved in the capsid assembly process.

The presence of nuclear localization signals on the minor capsid proteins might also control the stoichiometry of major and minor capsid proteins in the capsid (Lombardo *et al.*, 2000). In MVM, not only short basic NLS-like sequences in VP1, but also a conformation-induced nuclear localization motif (NLM) present in VP1 and VP2, contribute to nuclear accumulation of the capsid proteins which cooperate in nuclear transport (Lombardo *et al.*, 2000, 2002). The data suggest that VP1/VP2 trimers form in the cytoplasm in addition to VP2 trimers and translocate to the nucleus thereby determining VP1-VP2 stoichiometry of about 1:5 in the capsid. The region of VP protein interaction maps to residues 60–161 (Lombardo *et al.*, 2000). VP2 seems also to assist cytoplasmic folding of VP1 as it is able to prevent ubiquitination of certain VP1 mutants when it is co-expressed. This lends further support to the concept of cytoplasmic oligomerization of the VP subunits (Lombardo *et al.*, 2002).

## Biochemical analysis of capsid assembly

Sedimentation velocity analysis of soluble cytoplasmic pools of newly synthesized capsid proteins in a productive AAV2 infection clearly showed capsid protein oligomerization involving all three capsid proteins but no capsid formation in the cytoplasm, in agreement with the

immunolocalization data (Wistuba *et al.*, 1995, 1997). More detailed analysis of the capsid proteins sedimenting at low S-values showed a pool containing all three capsid proteins sedimenting in a broad peak between 6S and 10S in the cytoplasm, whereas in the nucleus a more defined peak at 7S – probably VP monomers – was separated from a 10S–15S peak containing mainly VP2 and VP3. This fraction might represent VP trimers or pentamers. Most strikingly, there was a dramatic accumulation of capsid proteins at about 60S, the sedimentation position of empty capsids (and the postulated SP intermediate), in the nucleus, indicating a significant shift from VP oligomerization towards capsid formation in this compartment (Wistuba *et al.*, 1995). Over the course of infection the nuclear pool further increased, whereas the cytoplasmic pool decreased. There also exists an insoluble pool of capsid proteins and capsids, which is difficult to analyze, but which might contribute to the assembly reaction (Wistuba *et al.*, 1995).

Attempts to assemble AAV2 capsids *in vitro* using renatured baculovirus expressed VP proteins showed oligomerization of capsid proteins without a defined assembly intermediate or end product, even at high capsid protein concentrations (Steinbach *et al.*, 1997). In the presence of a HeLa extract, however, rare capsid assembly events were noted. This again suggests that (nuclear) assembly factor(s) may be required for capsid formation. The only cellular protein that has been found to be associated with AAV2 virions is nucleolin, a protein with many functions, which shuttles between the cytoplasm and nucleus and is enriched in interphase nucleoli (Qiu and Brown, 1999). This interaction was shown by co-immunoprecipitation of capsids, using antibodies against nucleolin, by co-purification of nucleolin with AAV2 virions in CsCl gradients and by fluorescent immuno-co-localization of AAV2 capsids and nucleolin in AAV2/adenovirus infected cells.

*In vitro* overlay assays showed that nucleolin bound to capsids but not to denatured or dissociated capsid proteins. Interestingly, nucleolin only seems to be associated with AAV2 capsids that have been prepared by solubilization of an insoluble capsid fraction using 2 percent deoxycholate or sonication, and was not found in the soluble virion fraction of the culture supernatant. These findings suggest a possible role for nucleolin in capsid assembly and/or genome encapsidation and also point to the observation that the insoluble nuclear fraction of capsids is possibly of functional relevance for the assembly process.

In freeze-thaw lysates of A72 cells that had been transfected with an infectious clone of CPV, Yuan and Parrish (2001) observed not only monomers of VP1 and VP2 and assembled capsids but also a fraction of VP2 that sedimented in sucrose gradients at the position of a VP-trimer. In CPV assembly mutants no such product was found, suggesting that VP trimers could be an assembly intermediate.

Phosphorylation of parvovirus minute virus of mice (MVMp) capsid proteins does not seem to be involved in the capsid formation process (Santaren *et al.*, 1993). This

conclusion was supported by the fact that mutations in serine residues, which normally carry the bulk of the  $^{32}\text{P}$  label in assembled MVM particles, did not prevent capsid assembly (Maroto *et al.*, 2000). Interestingly, full virions showed a pattern of VP2 phosphorylation that was different to that observed for empty capsids, so it cannot be ruled out that phosphorylation plays a role in packaging and/or particle maturation.

## GENOME ENCAPSIDATION

### Visualization of parvoviral packaging

The current concept of parvovirus genome encapsidation postulates a process of translocation of replicated ss-genomes into preformed capsids. The electronmicroscopic pictures of nucleoprotein complexes present in an *in vitro* assembly/packaging reaction of Lu III viruses (Muller and Siegl, 1983) can be interpreted as the visualization of this encapsidation process. It provides some remarkable features: After fixation with formaldehyde, a network of nucleic acid strands could be observed to which particles of greatly varying diameters were attached. Some of them resembled disrupted capsids while others were larger than the fully packaged 110S particles. This suggests a rather dramatic reorganization of empty capsids in order to introduce the genome. The problem here is in discriminating between true packaging intermediates and possible preparation artefacts of fragile structures involved in the packaging reaction.

### Cellular localization of the encapsidation reaction

Genome encapsidation occurs in the cell nucleus and presumably at a site where capsids and replicated DNA co-localize. Initially, Hunter and Samulski (1992) observed that when AAV2 and Ad5 are used to co-infect D6 cells, Rep proteins and capsid proteins co-localized around 24-hours post infection in the same subnuclear clusters. Similar staining patterns have been obtained in HeLa, 4T29, and CaCo2 cells. It was later shown that Rep proteins and AAV-DNA co-localize together with the adenovirus (Ad) DNA binding protein in so-called replication centres (Weitzman *et al.*, 1996). At early stages of infection (12–16 hours post infection) when Rep proteins and DNA co-localize to give a punctate nuclear distribution pattern – presumably the AAV2 replication centers – no capsid protein synthesis was detectable in HeLa cells (Wistuba *et al.*, 1997). However, at later time points there was not only co-localization of Rep proteins and AAV2 DNA but also of capsid proteins, mostly forming large clusters in the nucleoplasm – similar to the clusters observed by Hunter and Samulski (1992) – whereas the nucleoli with large amounts of capsids were

almost devoid of both Rep proteins and DNA (Wistuba *et al.*, 1997).

For H1 it has been reported that high concentrations of unassembled capsid proteins surround the H-1 DNA synthesis centers, which are possibly derived from nucleolar fibrillar centres by disintegration (Singer and Rhode, 1978). Further characterization of H-1 replication centres showed the co-localization of NS1, viral DNA, PCNA, and the NS1 interacting small glutamine-rich TPR (SGT)-containing protein in structures designated as PAR-bodies (parvovirus-associated replication bodies; Cziepluch *et al.*, 2000). These structures are not nucleoli, nor coiled bodies, speckled domains, or promyelocytic leukemia (PML) bodies. The localization of capsid proteins or capsids relative to these replication centres was not analyzed. At an early stage in ADV infection, the VP proteins could be found at the periphery of nuclear inclusions in which non-structural (NS) proteins and viral DNA co-localized. The spatial organization of replication coincided only rarely with VP protein localization (Oleksiewicz *et al.*, 1996).

### Complexes of capsids with non-structural proteins

Several groups have observed capsids that were partially or fully packaged with DNA to which non-structural proteins were associated. Such a structure was first isolated from MVM-infected cells (Cotmore and Tattersall, 1989) and involves the NS1 protein covalently attached to the 5' termini of ss-genomes. The NS1 protein could be removed using nucleases and was also accessible to antibodies indicating that it is located outside the capsid and linked to DNA. The authors speculated that these structures could be formed as a result of the packaging process, which could be initiated by interactions of NS1 with preformed capsids. Similar observations were made for AAV2 (Prasad and Trempe, 1995; Wistuba *et al.*, 1995) by co-immunoprecipitation of capsids using antibodies to the Rep proteins. The fact that the peak of co-precipitation from extracts of Ad/AAV2 infected cells fractionated on sucrose gradients between 60S and 110S indicated that these complexes could represent intermediates of genome packaging. Prasad and Trempe (Prasad and Trempe, 1995) showed that the interaction was sensitive to nuclease and protease digestions, again indicating that the Rep protein is probably on the outside of the capsid and connected to the AAV genome.

These authors could further show that, in a productive infection, Rep78 is covalently linked to one of the 5' terminal thymidines of ss-DNA and remains associated with the AAV2 particle for up to 8 hours (Prasad *et al.*, 1997). The association of Rep with the capsids is at least partially maintained during CsCl gradient centrifugation confirming a stable, possibly covalent, interaction with packaged DNA. Another group also observed the association of the Rep78 protein with wt AAV2 virions and recombinant

AAV2 particles (Kube *et al.*, 1997). A large portion of these Rep proteins, however, was lost during CsCl density gradient centrifugation and a very small amount was considered to be inside the capsid based on indirect evidence. This result suggests that not all Rep proteins are associated with the capsid via covalent linkage to replicated DNA. In addition, co-immunoprecipitations, co-sedimentation and yeast to hybrid data indicated an interaction of Rep proteins with capsids in the absence of AAV2 DNA replication, supporting the idea of an additional, direct, and non-DNA-mediated association of Rep proteins with capsids at some stage in the assembly process (Dubielzig *et al.*, 1999).

## Initiation of encapsidation

Packaging signals can generally be regarded as *cis*-acting elements involved in the initiation of the genome packaging process. For AAV2 it has been shown that the inverted terminal repeats (ITRs) are necessary and sufficient for genome encapsidation and thus presumably contain a packaging signal (Samulski *et al.*, 1989). ITR deletion mutagenesis showed that the 20 nucleotide D-sequence, which immediately follows the 125 nucleotide long hairpin, is required for replication and encapsidation of genomes (Wang *et al.*, 1996). Further deletions revealed that only the 10 nucleotides proximal to the hairpin are necessary for these processes (Wang *et al.*, 1997). However, it was difficult to separate the effects of the deletion mutations on replication and on packaging. In a quantitative comparison, it was later suggested that deletion of the D-sequence affected packaging more than replication, supporting a role of the D-sequence in the packaging reaction (Srivastava *et al.*, 1996). Xiao and colleagues (1997) demonstrated that a 165 nucleotide sequence with one hairpin and two D-sequences is sufficient in *cis* for replication and packaging. Inefficient packaging has also been observed in the absence of ITRs and the D-element. Nony *et al.* (2003) showed that *rep* and *cap* genes comprising the Rep-binding site at the p5 promoter can be encapsidated with a low frequency of  $5 \times 10^{-4}$ . Deletion of a *cis*-acting replication element (CARE) abolished encapsidation. The CARE element contained the Rep-binding site (RBS) and nicking site (also called terminal resolution site, TRS), suggesting that these elements play not only an essential role in replication but also in packaging. It is noteworthy that instead of having a D-sequence, this packaging element has a Rep-binding site and an origin of replication. These data, together with the observation that Rep proteins interact with capsids (Dubielzig *et al.*, 1999), support the interpretation that the Rep proteins themselves could form part of the packaging signal.

Direct specific binding of ITRs to capsids as seen for the autonomous parvoviruses has not yet been shown for AAV2. For the 3' TR of ADV and MVM a specific binding to VP1 (Willwand and Kaaden, 1988; Willwand and Hirt, 1991) as well as to ADV empty capsids (Willwand and Kaaden, 1990)

has been reported. Similarly, the 3' TR of MVM bound to particles composed only of VP2 (Willwand and Hirt, 1993). Binding to VP1 does not seem to be necessary for genome packaging – at least for MVM – since it was later shown that VP1 is dispensable for packaging but plays a role in infectivity (Tullis *et al.*, 1993). VP2 of bovine parvovirus (BPV) binds to the first 290 nucleotides of the 3' terminus of the TR as shown in crude nuclear extracts of bovine fetal lung cells using a gel retardation assay (Metcalf *et al.*, 1990). The 5' end did not compete for binding of the 3' TR to the viral proteins.

Another way of studying the problem of packaging initiation is provided by the fact that genomes of some parvoviruses can be cross-packaged into parvoviral capsids of different serotypes or even different species. Cross-packaging was first observed for autonomous parvoviruses, when a Lu III-derived vector genome was packaged into capsids of MVMi, MVMP, H1, FPV, and CPV (Maxwell *et al.*, 1993, 1995; Spitzer *et al.*, 1996, 1997). It was later shown that recombinant MVM genomes could be pseudotyped with H1 capsids (Wrzesinski *et al.*, 2003). AAV2 genomes can be packaged into capsids of AAV1 to AAV8 (Gao *et al.*, 2002; Rabinowitz *et al.*, 2002; Grimm *et al.*, 2003), provided they are replicated by Rep proteins that are able to bind to the ITRs of the genome to be packaged and create a nick at the terminal resolution site. Similarly, genomes of AAV3 or AAV6 can be cross-packaged into capsids of AAV1 to AAV6 (Grimm *et al.*, 2003). More surprisingly, AAV2 vectors can even be packaged into B19 capsids in the presence of AAV2 replication proteins with relatively high efficiencies of up to  $10^9$  DNA-containing particles per ml (Ponnazhagan *et al.*, 1998). This suggests that AAV2 vector DNA replicated by Rep proteins can initiate packaging into B19 capsids.

More clues to the mechanism involved in the packaging initiation reaction came from the observation that some parvoviruses (MVM, Kilham rat virus, H-1, bovine parvovirus) specifically package only one of the two single-strand DNAs (+ or -; Salzman and Fabisch, 1979; Rhode and Klaassen, 1982; Astell *et al.*, 1983; Rhode and Paradiso, 1983; Chen *et al.*, 1986), whereas others (AAV or Lu III) package both strands in equal amounts (Mayor *et al.*, 1969; Rose *et al.*, 1969; Summers *et al.*, 1983; Bates *et al.*, 1984). In the case of AAV, this is easily explained by the presence of two identical ITRs; however, Lu III has two quite different ITRs (Diffoot *et al.*, 1989). Also both Lu III genomes could be cross-packaged into MVM or H1 capsids showing that no strand selection occurs on the capsid (Corsini *et al.*, 1995). In a recent publication it was suggested that preferred packaging of one strand in MVM is due to the varying efficiency of excision from replicated genomes and not to specific selection of negative versus positive strands, for example, by binding of specific sequences to the capsid (Cotmore and Tattersall, 2003).

A consequence of packaging initiation is the direction of translocation of the ss-genome into the capsid: either 3' to 5' or 5' to 3'. The DNA to capsid binding experiments of

autonomous parvoviruses (Metcalf *et al.*, 1990; Willwand and Hirt, 1993) suggest a 3' to 5' direction of packaging. When Southern blotted, packaged AAV2 genomes were hybridized with probes derived from different regions of the genome and it was found that the 3' portion could be detected in capsids containing both partially packaged and fully packaged genomes, whereas the 5' end was only detectable where full length genomes had been packaged (King *et al.*, 2001). The DNA, which had not entered the capsid, was degraded by DNase I treatment. This was interpreted as an indication that the 5' end of the genome enters the capsid last, giving rise to either a hybridization signal to the full length genome or no signal at all. The assumption for this interpretation is that DNase I protected DNA shorter rather than full length genome size and represents true packaging intermediates and not dead end products owing to interruption of the packaging process. Because in this study, end-labeled oligos were used that were specific for one of the strands (+ or -), only the encapsidation of half of the genomes was visualized. Hybridization with oligos detecting the opposite strand revealed the same preference for a 3' to 5' direction of packaging, but to some extent also partially packaged 5' ends were detectable (unpublished observation). In possible contrast to this interpretation is the observation of Faust and Ward (1979) that in a portion of incomplete MVM virions only 5' ends and short pieces of the genome were detectable. The 3' half of the MVM genome was absent, which would be consistent with the idea that in this fraction encapsidation had initiated at the 5' end of the virion DNA. The fraction of incompletely packaged virions was isolated on the basis of their sedimentation in sucrose gradients. Because of the selection of this fraction, it might be more likely that it contained defective interfering particles possibly generated by improper initiation of packaging. If packaging is functionally linked to strand-displacement DNA synthesis, it is difficult to reconcile it with a 3' to 5' packaging direction. A model in which packaging occurs in a 5' to 3' direction (Tattersall and Ward, 1976) is more plausible under this assumption. A tight correlation between Rep-dependent replication and packaging was observed in an *in vitro* packaging reaction (Zhou and Muzyczka, 1998). Inhibition of DNA replication prevented encapsidation. On the contrary, Myers and Carter (1980) reported that packaging can occur from a pool of replicated DNA under conditions in which replication is inhibited.

A packaging initiation hypothesis that integrates several aspects discussed so far has been put forward by Dubielzig and colleagues (Dubielzig *et al.*, 1999). According to these authors, packaging is initiated by interaction of Rep proteins with both the genome and the capsid, thereby bringing components of the packaging reaction together. They could demonstrate binding of large and small Rep proteins to empty capsids when they are co-expressed in the absence of DNA replication. Two different types of Rep–capsid interaction were postulated because of varying stabilities of the

Rep–capsid complexes in the presence of detergents. In addition, interactions of Rep proteins with each other (Rep78 with Rep68; Rep52 with Rep68 and to lower extent Rep52 with Rep40) provide the basis for the formation of multi-protein complexes involving both large and small Rep proteins at the capsid surface. The formation of these complexes occurred only when the components were present during the capsid assembly process, suggesting that they were formed in a co-assembly reaction and not via interaction with pre-formed capsids. Theoretically, this interaction could bring the 5' as well as the 3' ends of the genomes to the capsid surface. Introduction of the correct DNA end into the capsid could then be performed by additional DNA–capsid interactions. Although DNA–Rep and Rep–capsid interactions have been demonstrated, direct evidence for triple complexes in packaging initiation is still lacking. In particular, the broad range of cross-packaging is difficult to reconcile with a packaging initiation reaction involving a specific interaction of capsids with non-structural proteins. Nevertheless, the high functional homology of parvoviral non-structural proteins with phage proteins involved in genome encapsidation, for example the λ-terminase (Catalano, 2000) – including site-specific binding to the TRs, docking to the capsid, oligomer formation, adenosine triphosphatase (ATPase), helicase and endonuclease, respective nicking activity – supports the idea that the parvoviral proteins might play a similar role in the packaging process.

## Translocation of the genomes into the capsid

Very little is known about the mechanism of translocation of the parvovirus genome into the capsid following the initiation step. Chejanowsky and Carter (1989) reported that the small Rep proteins of AAV2 are required for the accumulation of ss-DNA in the cell, which has been interpreted as representing genome encapsidation. It was later shown that these two proteins are involved in completing the translocation of full length, capsid-associated genomes into capsids, and that their helicase activity is essential for this process (King *et al.*, 2001). It has been shown previously that the helicase/ATPase domains common to the large and small Rep proteins are also functional in Rep52 (Smith and Kotin, 1998). Although large Rep proteins are basically sufficient to package AAV2 genomes into capsids (Holscher *et al.*, 1995), their packaging efficiency is about 100-fold lower (based on infectious titers) than when in combination with the small Rep proteins. Analysis of capsid associated genomes showed that in absence of the small Rep proteins most full length genomes became only incompletely encapsidated. The authors postulated a model in which a capsid-immobilized helicase complex composed of large and small Rep proteins acts as a molecular motor to translocate the genome into the capsid. Such a mechanism would postulate a 3' to 5' direction of translocation reaction (King *et al.*, 2001). The recently determined atomic

structure of the Rep40 helicase/ATPase domain of Rep40 revealed it to be an AAA type ATPase which could theoretically assemble into a ring-like hexamer through which a genome could be translocated in an ATP dependent manner (James *et al.*, 2003).

## Termination of packaging

Phage packaging is commonly terminated by a terminase enzyme, which releases the packaged genome from a concatemeric replication intermediate (Fujisawa and Morita, 1997; Catalano, 2000). This means that a recognition sequence on the genome supplies the signal for packaging termination. Basically the same function could be performed in termination of parvovirus packaging by the NS1 respective Rep proteins. However, packaging of AAV2 vector genomes of different length showed that genomes smaller than half of the length of a normal genome could be packaged twice per capsid suggesting that recognition of the ITR and nicking is not sufficiently effective to terminate packaging correctly (Dong *et al.*, 1996; McCarty *et al.*, 2001). Furthermore, as measured by protection against DNase I digestion, genomes could be 'packaged' in a process that substantially exceeded wt genome length. These viruses were strongly impaired in their infectivity which may be due to capsid associated – but not encapsidated – DNA, which subsequently hinders decapsidation of these overpackaged virions. It is also possible that overpackaging of capsids prevents a maturation event from occurring that is required to obtain full infectivity. The limited data available indicate a 'head full' mechanism of AAV2 packaging termination; however, further investigation is needed to establish a clear model of parvovirus packaging termination.

## Maturation

As mentioned in the section describing the parvovirus assembly pathway, two density fractions of full length DNA containing capsids suggest a post-packaging maturation event. Such a maturation step could be documented for MVM (Richards *et al.*, 1977) and it correlates with proteolytic processing of VP2 to VP3 (Cotmore and Tattersall, 1987). In addition, maturation of MVM could be accompanied by a change of the phosphorylation pattern of capsid proteins (Maroto *et al.*, 2000). The existence of two density fractions ( $\rho = 1.41 \text{ g/cm}^3$  and  $\rho = 1.45 \text{ g/cm}^3$ ) containing a full length genome were also observed for wt AAV2 and later confirmed in viral vector preparations (Fisher *et al.*, 1996). The vectors of the  $\rho = 1.45 \text{ g/cm}^3$  fraction showed a 10–1000-fold lower infectivity than the  $\rho = 1.41 \text{ g/cm}^3$  fraction; however, evidence for a conversion from one fraction to the other is still lacking.

## SUMMARY

Our understanding of parvovirus capsid assembly and genome packaging is based on the concept that first empty capsids are formed into which replicated, strand-displaced ss-genomes are introduced. Efficient *in vitro* reconstitution of capsids from purified capsid proteins has not been possible and almost nothing is known about cellular proteins that may be involved in the assembly process that takes place in the nucleus. Interactions of parvoviral DNA with capsids either directly or mediated by non-structural proteins could initiate the packaging process. For complete translocation of ss-genomes into the capsid, the helicase function of non-structural proteins is required suggesting that they belong to the packaging machinery. Identifying the missing components of the assembly machinery is certainly a future challenge and will require better defined *in vitro* packaging systems in which, for example, capsid assembly, replication and genome encapsidation could be analyzed independently.

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PART **2**

# Pathogenesis and Biology of *Parvovirus* Infection

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# Pathogenesis of parvovirus infections

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## INTRODUCTION TO PARVOVIRUS INFECTIONS

This chapter will focus on the pathogenesis of infection with the autonomously replicating parvoviruses of vertebrates (i.e. those within the genera *Parvovirus* and *Erythrovirus*). Most of the knowledge on pathogenesis of infection relates to infection with canine parvovirus (CPV), feline panleukopenia virus (FPV), bovine parvovirus (BPV), porcine parvovirus (PPV), goose parvovirus (GPV), Aleutian mink disease virus (AMDV), mink enteritis virus (MEV), minute virus of mice (MVM), Kilham rat virus (KRV), rat H-1 virus (H-1) (genus *Parvovirus*), and parvovirus B19 (genus *Erythrovirus*).

## Transmission

Under epidemic conditions, parvoviruses are transmitted from a host (animal or human) who is undergoing an acute infection, which may be either asymptomatic or manifest by clinical symptoms. Generally, acutely infected host species have been found to shed parvoviruses in their feces, urine, saliva, and nasal secretions (Siegl, 1990). It is probable that following acute infection in a host species, infectious virus is shed for several weeks and possibly months (Csiza *et al.*, 1971a,b; Johnson and Collins, 1971; Johnson *et al.*, 1976; Siegl, 1990). In addition, it appears that following acute infection, the infected animal frequently becomes a carrier of the respective parvovirus, although this is not true in all cases, for example in CPV and FPV infections. In B19 infections, viral DNA can be detected months after resolution of an acute disease, but it is not known if this is persistence of the virus or simply of the genome. This phenomenon of

persistence may then have implications for the future health of that animal and also for further transmission of the virus to other naive hosts. For example, persistent infection with AMDV in mink is typically associated with acute symptomatic infection in susceptible animals, but constant virus shedding has also been demonstrated in subclinical infection (An and Ingram, 1978). Many other parvoviruses have been isolated from various tissues of their respective healthy immunocompetent hosts. Virus shedding in feces and, less frequently, in urine and semen, has been reported in asymptomatic animals for various parvoviruses, including FPV, CPV, MEV, BPV, PPV and the rodent viruses.

## Routes of virus entry

As parvoviruses are shed in feces, urine, saliva, and nasal secretions, routes of parvovirus transmission generally involve direct contact, the fecal-oral route or by inhalation of respiratory aerosols. In addition, as the viruses are highly stable to pH, temperature, solvents, detergents, and drying, they can also be transmitted by various fomites, for example dust particles and contaminated clothing. For example, it has been shown that it is difficult to prevent infection of susceptible host animals on introduction into clean housing within a colony of infected animals (Hammon and Enders, 1939; Kikuth *et al.*, 1940; Lawrence *et al.*, 1940; Robinson *et al.*, 1971). Transmission of AMDV within mink farms by vectors such as fleas and birds has also been suggested (Schofield, 1949; Wills, 1952; Lucas *et al.*, 1974). Transmission of CPV from infected to susceptible dogs on shoes of dog owners during dog shows has been suggested

to account for rapid spread following emergence of CPV in the late 1970s (Siegl, 1990). Sexual transmission has been demonstrated and is particularly recognized with PPV, which may result in death of the embryo (Mengeling, 1981). Vertical (transplacental) transmission is common during infection with several parvoviruses, including FPV, CPV (Kilham *et al.*, 1967; Csiza *et al.*, 1971b; Lenghaus *et al.*, 1980), AMDV (Padgett *et al.*, 1967; Porter and Cho, 1980), PPV (Mengeling, 1975), BPV (Storz *et al.*, 1972, 1978), rodent parvoviruses (Toolan, 1960; Ferm and Kilham, 1963, 1964; Toolan *et al.*, 1965; Kilham and Margolis, 1966a,b, 1975; Soike *et al.*, 1976) and human parvovirus B19 (Brown and Ritchie 1985; Mortimer *et al.*, 1985). Finally, parenteral transmission has also been documented for FPV and CPV (Truyen and Parrish, 2000) and for B19 (Heegaard and Brown, 2002).

## Tissue tropism

The tissue or cellular tropism of parvoviruses is determined by four factors:

- *The presence or absence of appropriate cellular receptors.* For example, cell entry of parvovirus B19 is restricted to those cells possessing both the erythroid cell receptor, globoside (Brown *et al.*, 1993a) and the co-receptor,  $\alpha 5\beta 1$  integrin (Weigel-Kelley *et al.*, 2003).
- *The particular phase of the cell cycle.* The autonomous parvoviruses have an almost absolute requirement for rapid cellular division as occurs during the S-phase of the cell cycle (Berns, 1984; Cotmore and Tattersall, 1987; Op De Beeck and Caillet-Fauquet, 1997).
- *Intracellular restriction of viral transcription and translation.* Parvovirus replication has been shown to require certain cellular factors. In the case of MVM, the minimum subset of which includes DNA polymerase  $\delta$ , proliferating cell nuclear antigen (PCNA), replication factor C (RFC), and replication protein A (RPA) (Bashir *et al.*, 2000).
- Parvoviruses require anatomical access to particular tissues within the body of the host animal.

## Clinical manifestations of infection

Clinical manifestations of infection with the autonomous parvoviruses in their natural hosts are extremely varied and dependent on the specific tissue tropisms of the viruses. The majority of animal parvoviruses are endemic within populations of their host species, infection occurring at an early age under the partial protection of maternal immunity and leading to a long-lasting immunity with minimal signs of disease. However, dramatic and even fatal disease may develop when a parvovirus is introduced into an immunologically naive animal population, for example epizootics

of feline parvovirus/panleukopenia virus (FPV) and goose parvovirus (GPV) infection in their natural hosts. Another example would be the emergence of the neonatal form of AMDV infection; in this case, infection occurs coincident with the birth of mink kits on AMDV-free mink farms (Alexandersen, 1986).

Fetal infection is a characteristic feature of parvovirus infections. In general, the extent to which a parvovirus is present in a host population determines the likelihood of immunity of the pregnant animal and therefore the likelihood of transplacental infection, the results of which will be determined by both susceptibility to virus replication and ability to mount an effective immune response (Siegl and Cassinotti, 1998). In general, infection occurring in the first and early second trimesters tends to be generalized and fatal. For example, the frequently fatal infection of cow and pig fetuses by bovine and porcine parvovirus, respectively, and the syndrome of hydrops fetalis which may result from intrauterine infection with parvovirus B19 in humans. During the third trimester, infection is less severe and is more organ-specific – for example, development of congenital cerebellar ataxia in the hamster, rat, ferret, kitten, and cow with infection of the dividing cells in the outer germinal layer of the cerebellum. There is also evidence from several case reports that this may also occur in humans with parvovirus B19 (Kerr *et al.*, 2002a). Puppies infected with CPV just before or just after birth may develop interstitial myocarditis and fatal congestive heart failure. The osteolytic syndrome may develop in rodents infected late in pregnancy or soon after birth, in the form of multiple congenital deformities affecting the head and skeleton with stunted growth occur due to viral replication in osteogenic tissues.

Encephalopathy has also been documented. For example, in addition to congenital cerebellar ataxia, brain hemorrhage and necrosis occurs in rats with reactivation of latent rat parvovirus infection during immunosuppression (Eldadah *et al.*, 1967). Encephalopathy also occurs in experimental FPV infection of cats and ferrets. Encephalomalacia and necrotizing vasculitis have been reported during a disseminated CPV infection in a dog (Johnson and Castro, 1984).

Enteritis is a common symptom of parvovirus infection and has been documented in rodents, chickens, cats, dogs, calves, goslings, mink, and raccoons. In general, the pathology is largely limited to the jejunum and ileum and the severity is proportional to the degree of necrosis of the epithelium. Symptoms range from lethargy and minimal signs of intestinal upset to frank watery diarrhea with or without blood-staining, dehydration, and death. Hepatitis is a well-recognized manifestation of parvovirus infection in many animals owing to viral replication within dividing hepatocytes. Anemia, leukopenia, and thrombocytopenia are typical features of animal parvovirus infection due to infection of the bone marrow, and they vary in frequency, severity, and duration. This occurs in cats (FPV), mink



**Figure 22.1** The rash of erythema infectiosum (from Susanne Modrow). See also Color Plate 22.1.

(MEV), dogs (CPV), cynomolgus monkeys (simian parvovirus – SPV), pig-tailed macaques (PmPV), rhesus monkeys (RmPV) and is more transient in pigs, calves and rodents.

Immune complex disease and vasculitis are prominent in the pathogenesis of Aleutian mink disease. This is a persistent and frequently fatal infection of mink by AMDV, which is characterized by plasmacytosis, hypergammaglobulinemia, glomerulonephritis, interstitial nephritis, polyarteritis, and splenomegaly. It is observed most frequently in mink of Aleutian genotype (Bloom *et al.*, 1994). Immune complex deposition is also thought to mediate the pathogenesis of erythema infectiosum caused by parvovirus B19 (Figure 22.1).

Acute interstitial pneumonitis may be caused by AMDV in newborn mink in which permissive viral replication occurs in alveolar cells and is analogous to preterm infantile respiratory distress syndrome (Bloom *et al.*, 1994).

## Histopathology of infection

In general, the pathology of parvovirus infections represents either the direct effect of viral replication in permissive cells or the effect of the immune response. Intestinal infection is characteristic of FPV and CPV infection of cats and dogs, respectively. Both viruses infect the actively dividing epithelial cells in the crypts of the intestinal villi within the jejunum and ileum. The clinical disease is thought to be related to the extent of damage the virus causes to the small intestine (Truyen and Parrish, 2000). FPV infection

of cats is characterized by panleukopenia with a decline in lymphocyte numbers and sparing of eosinophils, basophils, monocytes, and erythrocytes. FPV infects the lymphoid tissue, resulting in lymphocytolysis and cellular depletion, which is the basis of panleukopenia, and this is then followed by regeneration of lymphoid tissue in surviving animals. Viral replication and cellular necrosis occurs predominantly in the dividing cells of the germinal centres of lymph nodes and thymus cortex (Truyen and Parrish, 2000).

Infection of neonates with FPV and CPV results in entirely different disease manifestations from that in older animals, and is characterized by involvement of the cerebellum in kittens and of the heart in puppies. FPV infection of kittens *in utero* or soon after birth frequently results in virus replication in the neurones of the germinal epithelium of the cerebellum, which often results in cerebellar hypoplasia (Csiza *et al.*, 1971b; Kilham *et al.*, 1967, 1971). Infection of newborn puppies with CPV may be fatal as a consequence of myocarditis, which may occur up to 16 weeks of age (Robinson *et al.*, 1979). Myocardial and cerebellar manifestations of CPV and FPV, respectively, are age-restricted because active cell division occurs in these tissues only in neonatal animals. Depending on the age of the newborn, infection can result in generalized infection; *in utero* infection of kittens with FPV may result in death *in utero* or shortly after birth (Kilham *et al.*, 1967; 1971).

PPV infection in pigs results in stillbirth, mummification, embryonic death, and infertility (SMEDI syndrome). Other manifestations such as skin lesions and enteritis also occur, but at much lower frequency. PPV isolates vary in pathogenicity. A virulent strain may cause transplacental infection after acquisition via the upper respiratory tract; however, except for the most virulent strains (e.g. Kresse), the symptoms of PPV infection in adult pigs are minimal and self-limited.

AMDV was first described as an infection occurring in Aleutian mink, a particular variant of ranch mink with a modified, although uncharacterized genotype, which results in fur color variation and other genetically determined manifestations, such as Chediak–Higashi syndrome (Bloom *et al.*, 1994). Aleutian mink are particularly susceptible to disease following infection with AMDV and even to virus strains which are non-pathogenic to non-Aleutian mink. Classical Aleutian disease, which occurs in adults, is mediated by hypergammaglobulinemia and immune complex-mediated pathology of arteries and renal glomeruli; these antibodies are specific to AMDV proteins (Porter *et al.*, 1984). The disease is chronic and eventually fatal. In the newborn mink kits of AMDV-negative mothers, infection typically manifests as an acute interstitial pneumonia, which occurs as a result of virus infection of type-2 pneumocytes with cytopathology and aberrations in surfactant production (Alexandersen, 1986; Andersen *et al.*, 1987). It has been shown that AMDV targets mononuclear cells (lymphocytes and monocytes/macrophages) and type-2 alveolar cells (Mori *et al.*, 1991, 1994). Antibody-dependent enhancement

(ADE) of macrophage infection also occurs owing to monocyte/macrophage uptake of circulating immune complexes via the Fc receptor (Kanno *et al.*, 1993). In addition, it has been suggested that macrophage secretion of IL-6 is increased in Aleutian disease and significantly contributes to the pathogenesis by maintaining polyclonal B lymphocyte activation and differentiation with consequent enhancement of immunoglobulin production (Bloom *et al.*, 1994).

Rodent parvovirus-associated disease is specific to the particular virus, and also depends on age of the animal. MVM has historically been considered non-pathogenic. This assumption is correct in the case of adult animals, where the infection is self-limited and asymptomatic. However, in neonatal inbred mice MVMi (immunosuppressive variant) can cause renal infarcts and endothelial damage, which can be fatal (Brownstein *et al.*, 1991). The pathological outcome is dictated by the allotropic determinant encoded by the capsid gene as well as the expression level of non-structural protein (NS1) (Brownstein *et al.*, 1991; Ball-Goodrich and Tattersall, 1992; Colomar *et al.*, 1998). Mouse parvovirus (MPV-1), discovered only recently, is also lymphotropic in mice and causes immunosuppression with persistent virus shedding despite the production of specific antibodies; however, MPV-1 does not cause fatal infection in young mice. Kilham rat virus (KRV), the prototype of the rat parvoviruses, is virulent in newborn and fetal rats, the disease resulting in lesions of the liver, lung, kidney, central nervous system, and endothelium (Gaertner *et al.*, 1993). KRV has also been shown to persist and to modify lymphocyte function. Rat parvovirus (RPV-1), newly described, persistently infects adult rats (Ball-Goodrich *et al.*, 1998), is thought to be non-pathogenic and may be oncosuppressive (Jacoby *et al.*, 1996). Hamster parvovirus (HaPV) infection of the Syrian hamster is subclinical in weaning and adult animals, but is severe and frequently fatal in neonatal animals, with involvement and malformation of several organs, particularly the teeth (Gibson *et al.*, 1983).

Minute virus of canines (CnMV) infection maybe sub-clinical or cause mild diarrhea and/or reproductive failure in adult dogs, while more severe systemic infection results in newborn puppies (Carmichael *et al.*, 1994). Bovine parvovirus (BPV) causes diarrhea, mild respiratory symptoms, conjunctivitis, fetal death, and congenital cerebellar ataxia (Storz, 1990). Goose parvovirus (GPV) causes growth retardation (Derzsy's disease) in goslings which may be fatal. In adult geese, infection is persistent and subclinical but may also be associated with fetal death.

## HOST IMMUNITY

Protective immunity against parvovirus infections is generally mediated by neutralizing antibodies. In general, an immune response occurs to both the capsid and non-structural proteins of various parvoviruses in their respective

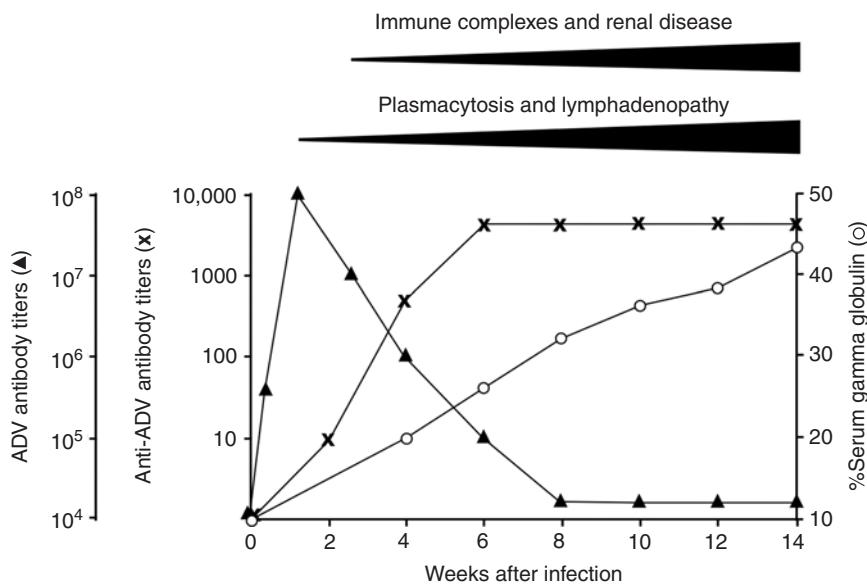
host species. As would be expected, the immune response to the capsid is more pronounced and occurs at an earlier stage than that to the non-structural proteins.

Passive immunization with specific antiviral antibodies can prevent infection, reduce severity of clinical disease and assist in the elimination of the virus. For example, in the case of CPV, parenteral administration of anti-CPV antibody protects against oral challenge and intestinal virus replication (Ishibashi *et al.*, 1983; Meunier *et al.*, 1985). In the case of B19, IgG antibody prevents *in vitro* infection of erythroid progenitor cells (Young *et al.*, 1984), and volunteer studies have shown it to be protective *in vivo* (Anderson *et al.*, 1985). It has been shown that individuals with persistent parvovirus infection have a specific defect in humoral immunity to B19 virus (Kurtzman *et al.*, 1989). In addition, intravenous administration of immunoglobulin (IVIG) is the only specific therapy for B19 virus and appears to be effective principally on the basis that it provides neutralizing antibodies (Anderson 1997). In AMDV infection, passive transfer of maternal antibody is protective to mink kits following birth as demonstrated by the fact that the characteristic pneumonia does not occur. In addition, mink kits from seronegative dams infected experimentally with AMDV and treated with anti-AMDV antibody develop an attenuated form of the adult disease but do not develop pneumonia (Alexandersen *et al.*, 1988). In AMDV infection, antibody has been shown to restrict intracellular replicative form DNA and mRNA 100- to 1000-fold (Alexandersen *et al.*, 1987, 1988). However, AMDV is an exception to other parvovirus infections in that specific IgG to the virus does not protect animals from infection but instead leads to an accelerated form of disease upon challenge (Porter *et al.*, 1972; Aasted *et al.*, 1998).

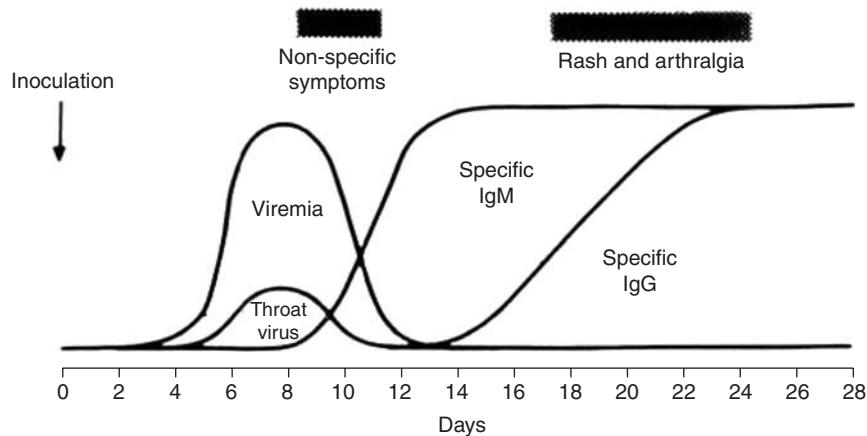
## Evolution of the immune response during and following infection

In general, the pattern of virus replication and antibody production is similar among the various parvovirus species. Following virus acquisition and after an incubation period, a viremia occurs followed by production of specific IgM. Some time later, specific IgG antibodies are produced (Figures 22.2 and 22.3). In the case of parvovirus B19 in humans, viremia occurs 6 days after infection and, following appearance of specific IgM on day 6, viremia begins to decline. The occurrence of symptoms of rash and arthralgia during acute infection are coincident with appearance of specific IgG approximately 10–14 days following the onset of viremia (Anderson *et al.*, 1985). Specific IgM persists for 3–6 months following infection while specific IgG persists for several years and lifelong in many individuals.

Cell-mediated immune responses have also been documented following parvovirus infections, although their significance remains largely undetermined. In AMDV infection, an increase of CD8+ T cells occurs in the periphery (Jensen



**Figure 22.2** Typical course of Aleutian mink disease. This diagram portrays the typical course of an adult sapphire mink infected intraperitoneally with ADV-Utah-1 (Reproduced from Bloom et al., 1994, with permission.).



**Figure 22.3** Time course of parvovirus B19 infection (Modified from Anderson et al., 1985.).

et al., 2003) along with infiltration of CD8+ T cells into tissues (Aasted, 1985, 1989) while CD4+ T-cell levels remain relatively normal (Chen and Aasted, 1997). In PPV infection, a weak cell-mediated immune response peaking on days 80 and 87 has recently been demonstrated (Ladekjaer-Mikkelsen and Nielsen, 2002). In B19 infection, proliferation of CD4+ T cells has been demonstrated in persons with past infection and in immunized volunteers (von Poblotzki et al., 1996; Franssilla et al., 2001). Other studies report elevated secretion of interferon- $\gamma$  and IL-2 (interleukin-2) in T-lymphocytes following *ex vivo* stimulation with B19 capsid antigen in persons with past infection, demonstrating a Th1-mediated cell-mediated immunity (Corcoran et al., 2003) and the importance of human leukocyte antigen (HLA) class I-dependent epitopes (Klenerman et al., 2002). Cellular immunity has also been demonstrated

in response to infection with KRV (Chung et al., 1997), MPV-1 (McKisic et al., 1998), and AMDV (Mori et al., 1994).

### Effect of immune response on clinical manifestations and pathology

Several clinical manifestations of parvovirus infection are mediated by the specific immune response. The most dramatic is that of AMDV infection in Aleutian mink, which results in a chronic immune complex disorder caused by persistent infection (Bloom et al., 1994). Infection of late gestation swine fetuses with certain PPV isolates causes an immunologically mediated vesicular skin rash (Kresse et al., 1985; Choi et al., 1987; Bergeron et al., 1996). An autoimmune response mediates KRV-induced diabetes in

diabetes-resistant rats (Chung *et al.*, 1997). The immune system has been implicated in the mediation of a number of clinical manifestations of parvovirus B19 infection, including rash and arthralgia (Anderson *et al.*, 1985), fatigue (Kerr *et al.*, 2001), glomerulonephritis (Takeda *et al.*, 2001), and arthritis (Lehmann *et al.*, 2003).

## Vaccination

Various live or inactivated vaccines have been developed to induce protection against CPV, FPV, and PPV in their respective host species (Parrish, 1995; Truyen and Parrish, 2000). In general, protection following vaccination correlates with the titer of neutralizing antibody. Attenuated CPV and FPV strains have been produced by repeated passage in cell culture and, although the particular mutations resulting in attenuation are not known, they were shed at lower rate in feces. In general, maternal antibody may persist for several months and can interfere with effective vaccination depending of the time of administration. For example, in sows maternal antibody may persist for several months (Paul *et al.*, 1982), and despite vaccination, when they are bred for the first time at approximately 6 months, some offspring may become infected during pregnancy.

As disease due to AMDV infection in mink is mediated by immune complex deposition, vaccination is not a feasible option, and therefore control is by eradication of infected mink, and this has been shown to be successful (Bloom *et al.*, 1994).

A vaccine against parvovirus B19 has been developed using recombinant empty capsids and in Phase 1 trials has been shown to induce B19 neutralizing antibodies in volunteers (Ballou *et al.*, 2003). It is likely that this vaccine may be used primarily to protect those with shortened red cell survival, for example, sickle cell disease sufferers, who are susceptible to B19 infection, in order to prevent the life-threatening aplastic crisis resulting from primary infection.

## VIRULENCE DETERMINANTS

### The virus

#### THE CAPSID PROTEINS

##### Mutations affecting host range and cell binding

It has been shown for various autonomous parvoviruses that small sequence changes in the structural proteins can significantly affect cell tropism and host range. The location of the critical amino acid positions has most frequently been shown to be in loops 3 and 4 and at the C-terminus of the capsid protein, all of which are near the surface of the virion, suggesting that they interact with a host cellular molecule. For example, infection of pigs with PPV strains, Kresse, and NADL-2, have a very different phenotype following

inoculation of the fetuses or after oronasal inoculation of the sow. Isolates of moderate to high virulence cause viremia and transplacental infection of the fetus with death of the non-immunocompetent fetus; for example, the Kresse strain is highly virulent causing death even in immunocompetent fetuses (Tijssen and Bergeron, 1994). However, isolates of low virulence, for example, the KBSH parvovirus, cause neither viremia nor transplacental infection (Molitor *et al.*, 1985). Study of these phenotypically variant PPV strains has shown they are closely related. Comparison of the Kresse strain with the non-virulent NADL-2 vaccine strain revealed a central BgIII fragment within the capsid protein gene that controls *in vitro* cell infection; the sequence of this fragment revealed three of the five amino acid differences known to reproducibly differentiate non-virulent from virulent strains (Bergeron *et al.*, 1996).

FPV has been recognized as a cause of disease in cats, and some other carnivores, such as raccoons, for decades (Hindle and Findlay, 1932); however, CPV is new, only emerging in the late 1970s (Parrish, 1990). As with other parvoviruses, the capsid proteins determine host range, with only small differences between CPV and FPV being sufficient to define the ability to replicate in dogs or cats and their cells *in vitro* (Parrish, 1995). Studies using chimeras of CPV and FPV revealed that two genomic regions are important; VP2 amino acids K80R, N564S, or A568G. These residues are adjacent in the conformation of the capsid at the top of the 2-fold depression where loops 1, 3, and 4 of three different monomers interact (Truyen and Parrish, 1995). It has also been shown that in chimeric viruses in which the genome of one parvovirus is inserted into the capsid of another, for example, the LuIII genome in CPV or FPV capsids, the chimera exhibits the tropism of the capsid and not the genome (Spitzer *et al.*, 1996).

In the case of MVM, the MVMp strain infects fibroblasts while the MVMi strain infects lymphocytes. The fibrotropic determinant has been mapped to amino acids 317 and 321 in MVMp, and mutations at these positions can mediate a conversion in MVMi from lymphotropic to fibrotropic (Ball-Goodrich *et al.*, 1991). However, two regions of MVMi (one non-structural segment and one structural segment) were needed to induce a lymphotropic phenotype to MVMp (Colomar *et al.*, 1998).

In AMDV, there is much more variability of the important determinants between different virus strains and this is particularly focused within a 25-nucleotide hypervariable region in the capsid gene. The AMDV-G strain is non-pathogenic for mink but replicates permissively in cell culture, whereas the AMDV-Utah 1 strain is highly pathogenic for mink but replicates poorly in cell culture. Using a full-length replication competent plasmid chimera of AMDV-G (non-pathogenic strain) with AMDV-Utah (pathogenic strain) (containing the sequence representing map units 15–88), it was shown that the region within 55–65 map units of AMDV-Utah inhibited replication in cell culture (Bloom *et al.*, 1993). Subsequently, using pathogenic

molecular clones of AMDV that were able to replicate both *in vivo* and *in vitro*, two additional regions were discovered (65–69 and 73–88 m.u. [map units]) that together could abolish *in vitro* growth (Bloom *et al.*, 1998).

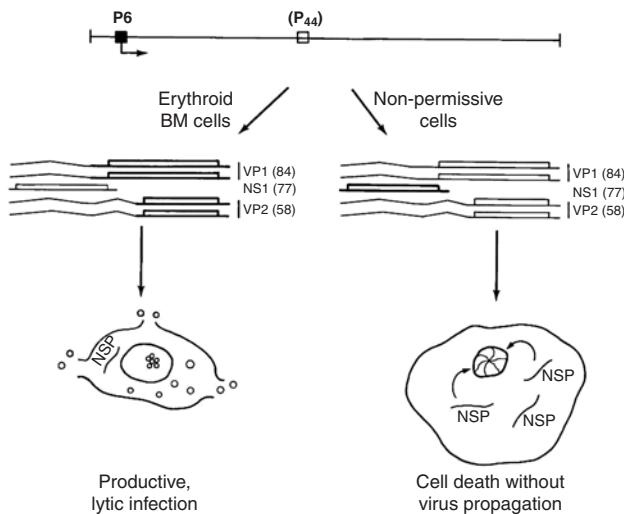
### Viral phospholipase activity

Sequence analysis has recently identified phospholipase A<sub>2</sub> (PLA<sub>2</sub>) motifs in the capsid proteins of various parvoviruses. It has also been shown that putative PLA<sub>2</sub> motifs from divergent parvoviruses can exhibit catalytic properties of secreted PLA<sub>2</sub> and this activity is essential for virus entry into cells in most parvoviruses (Zadori *et al.*, 2001). Mutations in these motifs markedly reduce this activity and also viral infectivity. However, such mutations do not affect attachment, entry, and endocytosis. It is apparent that PLA<sub>2</sub> activity is essential for efficient transfer of the viral genome from late endosomes/lysosomes to the nucleus prior to replication. Inhibition of this activity may represent a possible route to a new class of antiviral drug active against parvoviruses and their associated diseases (Zadori *et al.*, 2001).

### THE NON-STRUCTURAL PROTEINS

The non-structural protein of various parvoviruses performs various functions for the virus and also plays a role in the pathogenesis of infection. The pathogenesis of MVM infection depends on expression of non-structural proteins (Brownstein *et al.*, 1992). In cell culture, infection of transformed cells with H-1 or MVM results in cell lysis and death, a phenomenon that has been shown to be due to the NS1 protein. In the case of B19, cytotoxicity of the NS1 protein was first demonstrated by Ozawa and colleagues (1987) using cells transfected with an NS1 expression plasmid. Non-structural proteins of the autonomous parvoviruses contain a highly conserved nucleoside triphosphate (NTP)-binding motif which is essential for various functions. Mutations within this motif have been shown to abolish cytotoxicity of B19 NS1 protein (Momoeda *et al.*, 1994). The cytotoxicity of the B19 NS1 protein may have clinical consequences. For example, the pathogenesis of thrombocytopenia associated with acute B19 infection is thought to be explained by expression of the NS1 gene in the absence of replication (Pallier *et al.*, 1997). The cytotoxicity of the NS1 protein has also been proposed as a possible mechanism, which may account for certain other clinical manifestations of B19 infection including leucopenia and arthralgia (Heegaard and Brown, 2002) (Figure 22.4).

The viral NS1 protein has been described as a transactivator of both the viral p6 as well as a variety of cellular promoters. These include the promoter region controlling the expression of tumor necrosis factor (TNF)- $\alpha$  (Fu *et al.*, 2002) and IL-6 genes (Moffatt *et al.*, 1996). Elevated levels of TNF- $\alpha$  have been shown to be present in patients during the acute and convalescent phases of B19 infection (Kerr *et al.*, 2001). The prolonged or continuous presence of these pro-inflammatory cytokines during acute convalescent and persistent B19 infection respectively may result in the induction



**Figure 22.4** Permissive cells versus non-permissive cell infection Bloom and Young, 2001.

of long-lasting clinical symptoms and autoimmune reactions. IL-6 is also thought to be important in AMDV infection; this is consistent with the plasmacytosis that is the hallmark of Aleutian disease (Bloom *et al.*, 1994). The NS1 protein of AMDV may also play a role in a similar manner to that of B19 (Jensen *et al.*, 2003) (Figure 22.4).

### VIRAL GENOMIC CHARACTERS ASSOCIATED WITH PARTICULAR CLINICAL MANIFESTATIONS

AMDV infection in adult ranch mink has been associated with severe aseptic meningoencephalitis (Dyer *et al.*, 2000). Post-mortem examination of the brain in affected animals revealed an influx of lymphocytes and plasma cells with accompanying gliosis, satellitosis, and also a mild inflammation of the leptomeninges. AMDV was identified in mesenteric lymph nodes, spleen, brain, and liver using polymerase chain reaction (PCR). Sequence analysis of the AMDV isolate (TH5) revealed two unique amino acid residues in the particular genomic region that controls pathogenicity, suggesting that particular nucleotide sequence mutations could confer a propensity to cause neurological manifestations in infected mink (Dyer *et al.*, 2000).

Parvovirus B19 infection has also been associated with development of meningoencephalitis (Barah *et al.*, 2001). The mechanism by which parvovirus B19 causes CNS damage is not clear. Although B19 DNA has been detected by several groups in CSF of these cases (Cassinotti *et al.*, 1993; Okumura and Ichikawa *et al.*, 1993; Watanabe *et al.*, 1994; Yoto *et al.*, 1994), detection in brain tissue is rare (Isumi *et al.*, 1999), suggesting an immune-mediated pathogenesis rather than direct viral cytotoxicity. Markedly raised levels of inflammatory cytokines have been detected in four cases of B19-associated meningoencephalitis during the acute phase (IL-6, TNF- $\alpha$ , IFN- $\gamma$ , granulocyte-macrophage colony stimulating factor [GM-CSF], and

macrophage chemoattractant protein [MCP]-1) compared with normal controls (Kerr *et al.*, 2002a). Analysis of B19 virus strains isolated from cases of meningoencephalitis revealed a unique genotype, which was seen only in cases of meningoencephalitis (Umene and Nunoue, 1995).

## PERSISTENCE

Autonomous parvoviruses can persist in cells for long periods in the absence of lysis and in the presence of the requisite helper functions (Berns, 1984; Siegl, 1988). This has been documented both *in vitro* and *in vivo*. In the case of B19, viral DNA has been shown to persist in various sites including bone marrow (Kerr *et al.*, 1997), synovium (Kerr *et al.*, 1995), testis (Diss *et al.*, 1999), skin (Nikkari *et al.*, 1999), and peripheral blood mononuclear cells (PBMC) (Reed *et al.*, 2000). However, the mechanisms remain unclear. Despite repeated attempts to identify integration of the autonomous parvoviruses, evidence has been very scanty and remains highly controversial. However, recently MVM was shown to integrate in a site-specific manner into episomes (Corsini *et al.*, 1997), and B19 DNA has been found integrated into human DNA at low frequency in PBMC (Reed *et al.*, 2000). The biological relevance of these observations remains to be determined.

## The host

### PRESENCE AND DISTRIBUTION OF PARVOVIRUS RECEPTORS

Infection by parvoviruses begins with adsorption of the virion to specific receptors on the cell surface. Cell-surface receptors have been characterized for several autonomous parvoviruses. The feline transferrin receptor (TfR), is the primary receptor for both CPV and FPV on feline cells (Parker *et al.*, 2001). Cloned TfR can confer the ability to bind these viruses in hamster cells, which are normally resistant. Use of the TfR as the cellular receptor for these viruses on feline and canine cells is also closely correlated with their pathogenicity in nature, as the TfR is highly expressed on crypt cells in the intestinal epithelium and on hematopoietic cells, which are the main target cells of CPV and FPV in animals (Parrish, 1995).

Blood group P antigen or globoside has been shown to be the primary cellular receptor for human parvovirus B19 (Brown *et al.*, 1993a) and erythroid cells of the Amish people are resistant to B19 infection as they lack the globoside receptor (Brown *et al.*, 1994). Distribution of P antigen is widespread among human cell types and its profile has been suggested to account for several clinical manifestations of B19 infection (Cooling *et al.*, 1995). However, recently it has been shown that P antigen is necessary but not sufficient for parvovirus B19 binding but not sufficient for virus entry into cells, which also requires  $\alpha 5\beta 1$  integrins in their high-affinity conformation (Weigel-Kelley *et al.*,

2003). Mature red blood cells express high levels of P antigen, but not  $\alpha 5\beta 1$  integrins and facilitate B19 binding but not entry. While erythroid progenitor cells, which express high levels of both P antigen and  $\alpha 5\beta 1$  integrins, allow both binding and virus entry. The efficient and wide dissemination of B19 virus during an acute infection may result from their ability to bind mature erythrocytes (Weigel-Kelley *et al.*, 2003).

## STAGE OF FETAL DEVELOPMENT

The fetus is at particular risk from parvovirus infection as a consequence of the large number of cell groups in active mitosis. Many parvoviruses are capable of infecting the fetus in their respective host species. In general, infection occurring in the first and early second trimesters tends to be generalized and fatal, for example PPV, BPV, H-1, GPV, B19, and AMDV (Siegl, 1988), the frequently fatal infection of cow and pig fetuses by bovine and porcine parvovirus, respectively, and the syndrome of hydrops fetalis, which may result from intrauterine infection with parvovirus B19 in humans. During the third trimester, infection is less severe and is more organ-specific. For example, development of congenital cerebellar ataxia in the hamster, rat, ferret, kitten, and cow, with infection of the dividing cells in the outer germinal layer of the cerebellum. There is also evidence from several case reports that cerebellar infection and ataxia may also occur in human fetuses infected with parvovirus B19 (Kerr *et al.*, 2002a). Puppies infected with CPV just before or soon after birth may develop interstitial myocarditis and fatal congestive heart failure. The osteolytic syndrome may develop in rodents infected late in pregnancy or soon after birth; multiple congenital deformities affecting the head and skeleton with stunted growth occur due to viral replication in osteogenic tissues. Acute interstitial pneumonitis may be caused by AMDV in newborn mink in which permissive viral replication occurs in alveolar cells and is analogous to preterm infantile respiratory distress syndrome (Bloom *et al.*, 1994).

## AGE

In general, parvovirus infection occurring in newborns may be severe and lead to severe sequelae, while infection occurring at some time later during childhood/adolescence may be mild or asymptomatic. For example, a particular strain of MVM causes infection in renal vascular endothelial cells and lymphocytes in newborn mice (Jacoby and Ball-Goodrich, 1995). AMDV causes infection of type 2 pneumocytes in newborn mink kits whereas AMDV infection in adult mink is restricted at the level of the individual cell, and an immune complex disease results (Bloom *et al.*, 1994). PPV infections are generally asymptomatic in adult swine, but have been implicated in severe dermatitis and enteritis in young swine (Kresse *et al.*, 1985; Dea *et al.*, 1985). B19 infection in human children causes a characteristic rash illness, erythema infectiosum, and is generally

mild and self-limited. However, B19 infection in adults is more severe because of the increased incidence of arthritis, which may last for several months.

### IMMUNE COMPETENCE

B19 infection in humans with immunosuppression may result in a persistent low titer viremia accompanied by pure red cell aplasia (PRCA). Various syndromes predispose to persistent viremia including acquired immunodeficiency syndrome (AIDS), chemotherapy for acute lymphoblastic leukemia (ALL), chronic myelomonocytic leukemia, chemotherapy for cancer, organ transplantation, steroid treatment, and congenital immunodeficiency (Heegaard and Brown, 2002). Persistent B19 infection is thought to result from a defect in the humoral immune response to B19 structural proteins, which does not neutralize the virus (Kurtzman *et al.*, 1989).

AMDV was first described as a cause of infection in Aleutian mink, which have a particular but uncharacterized genotype resulting in a degree of immunosuppression. For example, Aleutian mink suffer from Chediak–Higashi syndrome, which is a lysosomal storage disorder resulting in impaired immune function. These animals are particularly susceptible to AMDV infection and develop an eventually fatal disease even with virus strains which are non-pathogenic for non-Aleutian mink (Bloom *et al.*, 1994).

Parvoviruses may reactivate from a state of latency under certain circumstances, and factors that promote reactivation of a latent parvovirus infection are generally those which impair the ability of the immune system in the persistently infected animal. The mechanism by which the immune system controls persistence is deserving of additional attention.

### DURATION OF RED CELL SURVIVAL

The first clinical symptom to be associated with B19 infection was a transient aplastic crisis (TAC) in patients with sickle cell disease (Pattison *et al.*, 1981). Previous to this, TAC had been well recognized as a precipitous drop in hemoglobin associated with cessation of reticulocyte production against a background of chronic hemolytic anemia. B19 infection is now recognized to cause TAC in association with shortened red cell survival owing to a variety of conditions. These include sickle cell anemia (Pattison *et al.*, 1981),  $\alpha$  and  $\beta$ -thalassemia, autoimmune hemolytic anemia, glucose-6-phosphate dehydrogenase deficiency, hereditary spherocytosis, hereditary stomatocytosis, iron deficiency anemia, pyruvate kinase deficiency, sideroblastic anemia, congenital dyserythropoietic anemia, pyrimidine-5'-nucleotidase deficiency, malaria, and paroxysmal nocturnal hemoglobinuria. It has also been shown that blood loss may also represent a predisposition to TAC upon B19 infection (Heegaard and Brown, 2002).

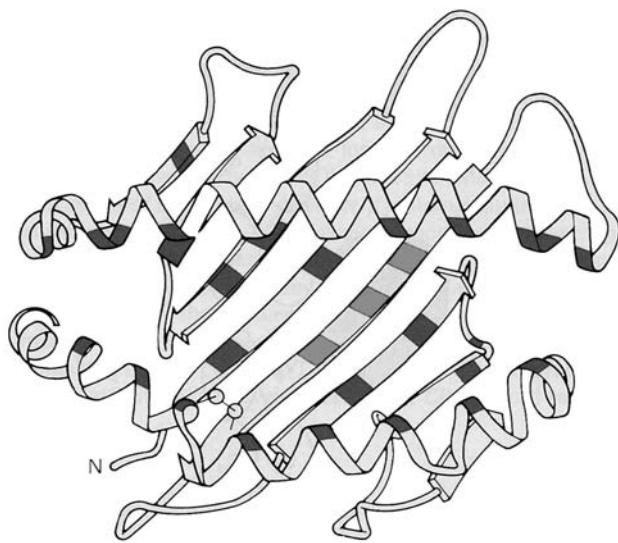
### SEX

A difference in clinical manifestations of parvovirus infection between the sexes has been reported only for human parvovirus B19 in which B19-associated arthritis is significantly more common in infected adult women than it is in men (Kerr, 2000). It is interesting also to note that chronic fatigue syndrome (CFS), which has been reported to follow acute B19 infection at low incidence (Jacobsen *et al.*, 1997; Kerr *et al.*, 2002b), is also more common in women. Both these manifestations almost certainly have an immune pathogenesis. The humoral immune response, at least, is known to be enhanced by estrogens and suppressed by androgens (Cutolo *et al.*, 2002) and this may account for the sex difference in clinical manifestations of B19 infection.

### GENOMIC DETERMINANTS OF IMMUNE RESPONSE

Various groups have investigated the possibility that genomic polymorphisms within the major histocompatibility complex (MHC) may have a bearing on the clinical manifestations of B19 infection, particularly arthritis or persistence of arthritis. Several studies have implicated the role of HLA-DR4 in the pathogenesis of parvovirus B19 arthritis (Klouda *et al.*, 1986; Gendi *et al.*, 1996; Kerr *et al.*, 2002c); however, this is inconsistently reported (Dyckmans *et al.*, 1986; Woolf *et al.*, 1989). Inconsistencies may be due to use of older antigen-based methods as compared with molecular typing, size of the studies, and the important issue of population mixing.

The largest study in this area was performed by Kerr *et al.* (2002c), which compared acute symptomatic parvovirus-infected cases (positive for serum anti-B19 IgM) ( $n = 36$ ) with those in control subjects from the north west of England ( $>900$ ). Symptoms of B19 infection consisted predominantly of rash, arthralgia, fatigue ( $n = 32$ ), intrauterine death without preceding maternal rash ( $n = 1$ ), thrombocytopenia ( $n = 2$ ) and transient aplastic crisis ( $n = 1$ ). The frequency of each of HLA-DRB1\*01 ( $P = 0.016$ ), DRB1\*04 ( $P = 0.007$ ), and DRB1\*07 ( $P < 0.0001$ ) alleles was significantly higher in the parvovirus B19 patients than in control subjects. In the parvovirus group, 63.9 percent carried the rheumatoid arthritis-associated shared epitope sequence, compared with 45 percent of control subjects (odds ratio [OR], 2.2; 95 percent CI 0.97–4.8;  $P = 0.04$ ), and carriage was associated with fatigue during the acute phase (OR 4.2; 5 percent CI 0.8–23.9;  $P = 0.047$ ). HLA-DRB1\*01, \*04 and \*07 carry a neutrally charged glutamine at position 10 and a positively charged lysine at position 12 of the first hypervariable region, resulting in an overall positive charge in this region compared with other molecules (Figure 22.5). Other HLA-DRB1 alleles with charge similarity to these alleles in the first hypervariable region include HLA-DRB1\*09 (rare allele), \*15 and \*16. DRB1\*15 and \*16 are also associated with clinical manifestations of B19 infection in the absence of DRB1\*01, \*04 and \*07 alleles in the setting of B19-associated meningoencephalitis (Kerr *et al.*, 2002a).



**Figure 22.5** Predicted location of the polymorphic amino acid residues of the MHC class II molecules mapped onto the outline structure of a class I molecule. Residues that may be important in parvovirus B19 infection are coloured green. (Adapted from Brown *et al.*, 1993, with permission.) See also Color Plate 22.5.

These residues do not contribute directly to contact of the binding groove with bound peptide (Stern *et al.*, 1994; Brown *et al.*, 1993b). However, they may influence the binding ability of adjacent residues such as the tryptophan at position 9, which has been associated with development of dermatomyositis-specific Mi-2 autoantibodies and susceptibility to demyelinating polyneuropathy in plasma cell dyscrasia (Kerr *et al.*, 2002c). The role of the HLA-B locus has also been studied (Woolf *et al.*, 1989; Jawad, 1993; Kerr *et al.*, 2002), again with conflicting results. HLA-B27, important in spondyloarthropathy, has been associated with persistent B19 arthritis (Jawad, 1993). Kerr *et al.*, (2002c) found an association of symptoms with HLA-B49, which was independent of the HLA-DRB1 locus, but did not confirm the previously reported association with HLA-B27. Woolf *et al.* (1989), found no association between symptomatology and polymorphism at the HLA-B locus.

The role of cytokine polymorphisms has been examined by Kerr *et al.* (2003). In this study, which examined the same patients as in the previous HLA study (Kerr *et al.*, 2002), the authors reported a reduced frequency of the TNF- $\alpha$ -308\*A allele in the parvovirus group (13.9 percent) compared with controls (27 percent) ( $P = 0.02$ ), and an association of the transforming growth factor (TGF)- $\beta$ 1 +869 T allele with development of skin rash at acute infection ( $P = 0.005$ ), and an association of the IFN- $\gamma$  +874\*T allele with development of anti-B19 NS1 antibodies at follow-up (mean 22.5 months) ( $P = 0.04$ ). Taken together, these results suggest that inherited variability in antigen presentation and cytokine responses may affect the likelihood of developing symptoms during parvovirus infection. However, the role of these polymorphisms remains to be confirmed.

The phenotype of Aleutian disease in mink is determined by inheritance of an uncharacterized genotype, which results in the desirable blue-gray fur but also Chediak-Higashi syndrome, a rare autosomal recessive lysosomal storage disorder that results in hypopigmentation of the skin, eyes, and hair, prolonged bleeding times, easy bruising, recurrent infections, impaired phagocytic function, abnormal natural killer (NK) cell function, and peripheral neuropathy (Ward *et al.*, 2002). In humans, Chediak-Higashi syndrome has been mapped to chromosome 1q42–43 (Barrat *et al.*, 1996).

#### DEFECTIVE NUCLEOTIDE EXCISION REPAIR (NER)

DNA damage within a cell may occur from a number of insults, including UV- and gamma-irradiation, and accumulation of metabolic end-products. DNA lesions as well as NER processes are implicated in changes in cellular metabolism, which may influence cell-cycle progression and arrest, genetic instability, and apoptosis; these factors may promote mutagenesis and carcinogenesis. NER machinery recognizes and repairs various helix-distorting lesions and is the most versatile eukaryotic DNA repair mechanism (Costa *et al.*, 2003). NER defects in Chinese hamster ovary (CHO) cell lines are known to facilitate enhanced replication efficiency of MVM, which in normal CHO cells is interrupted at an early stage before conversion of the single-stranded viral DNA into the double-stranded replicative form (Tauer *et al.*, 1996).

Several human syndromes result from defective NER, including Cockayne syndrome, xeroderma pigmentosum, and trichothiodystrophy, and other syndromes may exhibit a lesser NER defect, for example, Down syndrome and Turner syndrome. In a recent study of CNS infection caused by parvovirus B19, two patients with Cockayne syndrome and Turner syndrome, respectively, had a particularly fulminant illness that was fatal (Barah *et al.*, 2001).

#### VIRULENCE MECHANISMS

Virulence mechanisms are the means by which the virus may cause disease once it has been acquired by the host.

#### Viral replication and lytic infection

Viral replication may occur in various tissues, depending on anatomical access, the presence of appropriate cell receptors, the target cells being in S-phase of the cell cycle, and a lack of intracellular restriction. For example, the bone marrow (B19, FPV, CPV, MEV), gut epithelial cells (CPV, MEV, FPV, GPV, rodent PVs), fetal/neonatal osteogenic tissue (rodent PVs), outer germinal layer of the

cerebellum (rat PVs, CPV, FPV), cardiac myocytes (CPV, B19), hepatocytes (various animal, B19) lymph nodes (AMDV, CPV, FPV, MEV).

## Apoptosis and cytotoxicity

Apoptosis is a feature of infection with various human and animal parvoviruses, including parvovirus B19 (Moffatt *et al.*, 1998), FPV (Ikeda *et al.*, 1998), parvovirus H-1 (Ohshima *et al.*, 1998), CPV (Bauder *et al.*, 2000), MVM (Segovia *et al.*, 1999), and AMDV (Best *et al.*, 2002). The TNF- $\alpha$  signaling pathway has been shown to be important in apoptosis from parvoviruses H-1 (Rayet *et al.*, 1998) and B19 virus (Sol *et al.*, 1999). In the case of parvovirus H-1, virus-infected and NS1-expressing U937 promonocytic cells showed activation of CPP32 interleukin-1 beta-converting enzyme (ICE)-like cysteine protease with resultant apoptotic changes, in a manner similar to that resulting from exposure of these cells to TNF- $\alpha$  (Rayet *et al.*, 1998). In the case of parvovirus B19, virus-infected and NS1-expressing erythroid cells were sensitized to TNF- $\alpha$ -induced apoptosis (Sol *et al.*, 1999). Activation of caspases 3, 6, and 8 was induced by B19 NS1 in UT7/Epo cells (Moffatt *et al.*, 1998). In the case of AMDV, caspase activation is required for permissive virus replication (Best *et al.*, 2002) and mutation of two particular amino acid residues within the NS1 protein renders the virus replication defective (Best *et al.*, 2003).

In contrast to the destruction of erythroblasts during parvovirus B19 infection, which follows viral replication, the pathogenesis of thrombocytopenia associated with acute B19 infection is thought to be explained by expression of the left side of the viral genome in the absence of replication (Pallier *et al.*, 1997). The cytotoxicity of the NS1 protein was first demonstrated by Ozawa and colleagues (1987) using cells transfected with an NS1 expression plasmid. The cytotoxicity of the NS1 protein has also been proposed as a possible mechanism accounting for certain clinical other manifestations of B19 infection, including leucopenia and arthralgia (Brown *et al.*, 1994).

## Upregulation of cytokines

The viral NS1 protein has been described as a transactivator of both the viral p6 as well as a variety of cellular promoters. These include the promoter region controlling the expression of TNF- $\alpha$  (Fu *et al.*, 2002) and IL-6 genes (Moffatt *et al.*, 1996). Elevated levels of TNF- $\alpha$  have been shown to be present in patients during the acute and convalescent phases of B19 infection (Kerr *et al.*, 2001). The prolonged or continuous presence of these proinflammatory cytokines during acute-convalescent and persistent B19 infection respectively may result in the induction of long-lasting clinical symptoms and autoimmune reactions.

Upregulation of cytokines occurs both in the circulation in the case of B19 infection (Kerr *et al.*, 2001) and at other sites, for example lymph nodes in AMDV infection (Bloom *et al.*, 1994). In AMDV infection, Fc receptor-mediated antibody-dependent infection of macrophages may be responsible for cytokine-induced immune abnormalities.

## Autoantibody production

Some clinical features of both acute and chronic B19 infection are very similar to those of autoimmune connective tissue diseases, a fact which led to investigations into the possibility of autoimmunity and autoantibodies. Various autoantibodies have been documented during and following acute B19 infection. Detection of antinuclear antibody (ANA) (both homogenous and speckled pattern) and rheumatoid factor (RF) appears to be common (Kerr *et al.*, 2002b; Scroggie *et al.*, 2002) and one study found an association between development of chronic arthralgia and presence of ANA during the acute phase (Kerr *et al.*, 2002b).

It has been shown that in patients with skin rashes, RA and chronic B19 arthritis, anti-VP1 IgG that has been affinity-purified using a synthetic B19 VP1 peptide reacts specifically with human keratin, collagen type II, single-stranded DNA, and cardiolipin (Lunardi *et al.*, 1998). The main reactivity was against keratin and collagen type II, and there was a correlation between the clinical features and the main autoantigen specificity; immunoglobulin from patients with arthritis reacted preferentially with collagen II, while immunoglobulin eluted from patients with skin rashes reacted preferentially with keratin. As type II collagen is a target antigen for autoantibodies and clonally-expanded T cells in the rheumatoid arthritis (RA) synovium (Sekine *et al.*, 1999), this finding may have considerable significance for the link between B19 infection and rheumatic disease.

Recently an association between persistent parvovirus B19 infection and the production of antiphospholipid antibodies in pediatric and adult patients with rheumatic disease has been described (von Landenberg *et al.*, 2003). The antiphospholipid syndrome (APS) (Cervera *et al.*, 2002) is, like some parvovirus B19 infections, characterized by a wide variety of hemocytopenic and vaso-occlusive manifestations. Additionally, recurrent fetal loss and the association with autoantibodies directed against negatively charged phospholipids and protein cofactors, mainly  $\beta$ 2-glycoprotein-I are important features of the APS. The hypothesis regarding a common pathogenicity of the antiphospholipid syndrome and the autoimmunity features observed in B19 infection is supported not only by the close association between the presence of antiphospholipid antibodies and parvovirus B19 infection but also by the similarity in the presenting of clinical symptoms in patients with parvovirus B19 infection and patients with the APS. Furthermore, in a study by Loizou *et al.*, a close

similarity in the specificity of antibodies against different phospholipids induced by parvovirus B19 infection and antibodies found in systemic lupus erythematosus (SLE) patients could be shown (Loizou *et al.*, 1997). Molecular mimicry was suggested to be the major cause for the formation of antiphospholipid antibodies. This mechanism was strikingly demonstrated by Blank and coworkers in a recently published experimental study, using peptides from different bacteria for the induction of the antiphospholipid syndrome in a mouse model (Blank *et al.*, 2002).

One of the pathogenic mechanisms involved in triggering the production of antiphospholipid antibodies might be the phospholipase-A2-like activity observed in the VP1-unique region of the structural protein VP1 of parvovirus B19 (Dorsch *et al.*, 2002). This enzyme activity may contribute to the inflammatory processes induced by the production of leukotrienes and prostaglandins, but may also lead to the generation of unnatural cleavage products from cellular phospholipid compounds that may induce anti-phospholipid (aPL)-antibodies in combination with a distinct genetic background.

## Invasive cell phenotype

Ray *et al.* (2001) established an experimental *in vitro* system in which normal primary human synovial fibroblasts were tested for their ability to degrade reconstituted cartilage matrix. They showed that treatment of fibroblasts with parvovirus B19 containing human sera resulted in an increase in invasion of up to 248 percent compared with untreated fibroblasts. In addition, pre-incubation of viremic serum with a neutralizing antibody to B19 abrogated the effect. This work provides a significant biologic link between exposure to B19 and phenotypic changes in human synovial fibroblasts, and supports the proposed link between B19 and rheumatoid arthritis. Confirmation of this work will be required before final conclusions can be reached.

## Immune complex deposition

Experimental infection of human volunteers led to the elucidation of the pathogenesis of B19 infection (Anderson *et al.*, 1985; Potter *et al.*, 1987). These studies revealed that production of specific immunoglobulin G (IgG) following infection was coincident with appearance of the B19-related skin rash, arthralgia, and peripheral neuropathy. On this basis, immune complex deposition was proposed to account for these clinical manifestations (Anderson *et al.*, 1985). This hypothesis is further supported by the fact that rash and arthralgia are known to occur in chronically infected patients following administration of immunoglobulin (Pattison, 2000). This is also likely to be of prime importance in the pathogenesis of B19-associated vasculitis (Finkel *et al.*, 1994). AMDV infection in adult mink results

in classical Aleutian disease, the hallmark of which is hyper-gammaglobulinemia and immune complex deposition in arteries and glomeruli and tubules of the kidney (Bloom *et al.*, 1994). Perhaps surprisingly, immune complex disease is not a marked feature of other parvovirus infections.

## ACUTE VERSUS PERSISTENT INFECTION

The determinative factors in the pathogenesis of parvovirus infections vary depending on the stage of infection, and specifically, whether this is acute (i.e. primary) or persistent.

### Acute infection

#### TARGET CELL POPULATIONS AND DISEASE SYNDROMES

Acute infection with several parvovirus species in their respective host animals is characterized predominantly by infection of specific target cell types with the characteristic disease syndromes resulting directly from effects on these target cells. Therefore, during the acute phase of infection, the propensity for a parvovirus to cause disease in a particular host animal is determined by which cell types are undergoing active division at the time of infection. Actively dividing lymphoid and intestinal epithelial cells are the main target cells for FPV, MEV, and CPV, infection resulting in an enteritis, and the severity of infection is in part determined by the turnover rate in these cells (Parrish, 1995). Panleukopenia is a consistent and marked feature of FPV infection in cats, which is characterized by a decline in lymphocyte numbers while cell numbers of eosinophils, basophils, monocytes, and erythrocytes are only minimally affected (Truyen and Parrish, 2000). Panleukopenia may also occur during acute infection with PPV and BPV in their respectively adult hosts. AMDV infection of mink kits born to AMDV negative mothers typically results in an acute and fatal interstitial pneumonia owing to a cytopathic infection of type-2 pneumocytes, which results in reduced production of surfactants and then in an abnormally high surface tension in the alveolae with consequent impairment in breathing capacity (Alexandersen *et al.*, 1986, 1987). Osteolytic syndrome may develop in rodents infected during or soon after pregnancy with H-1 parvovirus and KRV infection, which leads to multiple skeletal abnormalities as a result of virus replication in osteogenic tissue. The target cell for B19 virus in humans is the erythroid progenitor and infection of these cells results in cessation of erythropoiesis typically lasting 7–10 days. Although this is of little consequence in normal persons whose erythrocytes have a normal life span, in those with shortened red cell survival (owing to, for example, hereditary spherocytosis) a life-threatening drop in hemoglobin may result that requires hospital admission and blood transfusion.

## DEPENDENCE OF DISEASE PATTERNS ON AGE

The spectrum of disease manifestations of parvovirus infection varies with the age of the host. This is due to several factors including the number of dividing cells (maximal in the fetus and young animal), the efficiency of the immune system (reduced in the fetus), and the presence or absence of specific maternal antibodies. The majority of animal parvoviruses are endemic within populations of their host species, infection occurring at an early age under the partial protection of maternal immunity and leading to a long-lasting immunity with minimal signs of disease. However, if infection occurs in susceptible dams, this may lead to intrauterine infection with fetal disease, which may be fatal. With waning or absent antibody responses, susceptibility to infection again increases (see above for specific examples).

## Persistent infection

### THE VARIETY AND BASIS OF PERSISTENT PARVOVIRUS INFECTIONS

Viral persistence may be an important determinant in chronic symptoms following infection with certain parvoviruses. Long-term virus persistence is a particular feature of infection with the parvoviruses and this may be achieved by different mechanisms depending on the species. However, the means by which the autonomous parvoviruses are able to persist are incompletely understood. Integration is known to occur with AAV (Kotin *et al.*, 1989, 1990a,b), MVM (Corsini *et al.*, 1997), and rarely in B19 infection (Reed *et al.*, 2000), and has the potential to occur with other autonomous parvoviruses. If this occurs in a non-site-specific manner, then this has the potential to disrupt gene function. Persistent parvovirus infection, as opposed to virus persistence per se, results from an inability of the immune system to resolve the active infection and the 'inadequate' immune response then continues alongside the virus infection and one or both may be responsible for the resultant clinical manifestations.

Persistent infection (as opposed to virus carriage) is a characteristic of several parvoviruses. In AMDV infection of adult mink, classic Aleutian disease results (Bloom *et al.*, 1994). Persistent B19 infection in adult humans may have several consequences including pure red cell aplasia (PRCA), virus-associated hemophagocytic syndrome (VAHS) (Heegaard and Brown, 2002), arthritis (Lehmann *et al.*, 2003), and chronic fatigue syndrome (CFS) (Kerr *et al.*, 2002b). Persistent infection with KRV causes autoimmune diabetes in diabetes-resistant (DR)-BB rats without infection of the beta cells in the pancreas, whose deficient production of insulin is the cause of diabetes. MVMi persistently infects the bone marrow in severe combined immunodeficient (SCID) mice (Bosma *et al.*, 1983).

## THE ROLE OF THE IMMUNE RESPONSE IN THE PATHOGENESIS OF PERSISTENT INFECTIONS

The immune response plays a key role in the pathogenesis of disease caused by persistent parvovirus infections. The most dramatic example of this is classic Aleutian disease in AMDV-infected adult mink (Bloom *et al.*, 1994). Although specific antibody is produced in Aleutian disease that can neutralize infectivity for cell culture, the same antibodies fail to eliminate virus *in vivo*. Anti-AMDV antibody also enables AMDV to infect macrophages, which are the target cells for persistent infection, via Fc-receptor-mediated antibody-dependent enhancement (ADE) (Bloom *et al.*, 2001).

KRV infection results in disruption of the immune balance of Th1-like CD45RC+CD4+ and Th2-like CD45RC-CD4+ T cells, which results in the selective activation of beta-cell-cytotoxic effector T cells (Jun and Yoon, 2001). MVMi persistently infects the bone marrow in SCID mice (Bosma *et al.*, 1983), and this leads to a lethal leucopenia and dysregulated erythropoiesis and megakaryopoiesis (Lamana *et al.*, 2001; Segovia *et al.*, 1999). *In vitro*, MVMi infection suppresses the lymphoid and myeloid repopulating capacity of hemopoietic stem cells (Segovia *et al.*, 2003).

The immune response plays a significant role in a number of B19-associated syndromes. Persistent B19 infection in humans may lead to VAHS, which is an exaggerated and prolonged overactivity of phagocytic histiocytes (tissue macrophages) with marked release of pro-inflammatory cytokines. Persistent B19 infection may also be one of the causes of CFS, a multisystem disease that may result from a prolonged and inadequate immune response to an infectious agent. B19-associated CFS is characterized by upregulated expression of circulating TNF- $\alpha$  and IFN- $\gamma$  (Kerr *et al.*, 2001), similar to idiopathic CFS (Patarca *et al.*, 1994; Rasmussen *et al.*, 1994; Moss *et al.*, 1999). These immune parameters return to normal following IVIG therapy in B19-associated CFS (Kerr *et al.*, 2003), the only specific treatment for B19 virus. However, IVIG has only limited success in idiopathic CFS. Arthritis may be associated with persistent B19 infection and this has been shown to be associated with autoimmune phenomena (Lehmann *et al.*, 2003). B19 virus infection may also cause meningoencephalitis (Barah *et al.*, 2001) in which virus is detectable in CSF. An immune pathogenesis is supported by the rarity of detection of B19 DNA in brain tissue of fatal cases, raised levels of proinflammatory cytokines in CSF of cases (Kerr *et al.*, 2002a), and documentation of improvement following therapy with corticosteroids (Barah *et al.*, 2001). B19 infection in children has been complicated by chronic lymphocytic myocarditis in which high levels of cytokines were detected (Nigro *et al.*, 2000). The role of the immune system in mediation of the common symptoms of B19 infection is further supported by the fact that certain HLA-DRB1 types appear to be significantly associated with development of commonly associated symptoms in B19 infection (rash and arthralgia), while other alleles do not (Kerr *et al.*, 2002c).

## INFECTIONS IN IMMUNE COMPROMISED HOSTS

Classic Aleutian disease, caused by AMDV infection in adult mink, likely results from the immunosuppressive Chediak–Higashi syndrome, which occurs in Aleutian mink and which is characterized by impaired function of phagocytic cells (Bloom *et al.*, 1994).

Persistent B19 infection occurs predominantly in immunosuppressed persons such as those with AIDS, congenital immune deficiency, chemotherapy, immune suppressive therapy, and cancer (Heegaard and Brown, 2002). However, it has recently become apparent that normal persons may also be susceptible to persistent B19 infection and it may be that these persons have a subtle immune deficiency, which becomes apparent only under certain circumstances and upon infection with this pathogen. Persistent B19 infection is thought to result from defective humoral responsiveness to B19 capsid proteins, in that specific antibodies do not neutralize the virus (Kurtzman *et al.*, 1989). The only specific treatment is human normal immunoglobulin, providing the neutralizing antibodies, which are lacking.

## CONCLUSIONS

The autonomous parvoviruses are responsible for a diverse menu of disease patterns. This variety of clinical presentations arises both from direct viral cytopathology, as well as immune mediated phenomena. B19, the only known pathogenic human parvovirus, causes disease by both mechanisms. Many of these infections have not been examined with contemporary analytical methods of immunology and host gene expression. Such studies would probably be extremely informative because of the relatively simple genetic structure of parvoviruses.

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# Parvovirus host range, cell tropism and evolution – studies of canine and feline parvoviruses, minute virus of mice, porcine parvovirus, and Aleutian mink disease virus

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Parvoviruses are found in many different species of animals from insects to humans, and it is likely that there are many more viruses to be discovered, particularly those not associated with animal disease. Many of the parvoviruses that have been isolated have tightly controlled host ranges and tissue tropisms, while others infect many hosts and many tissues. Understanding the controls of host range and cell and tissue tropism will result in a better knowledge of the pathogenesis of the viral diseases, while in gene therapy applications an understanding of tissue tropism would allow the therapeutic delivery of genes to be better controlled. Here we review the variation and evolution of these viruses, and summarize what is known about the viral and cellular mechanisms that control the ability to infect particular cells and animals. In the case of canine parvovirus (CPV) and its close relative feline panleukopenia virus (FPV), the viruses have shifted their host ranges in the recent past, while for Aleutian mink disease virus (AMDV), minute virus of mice (MVM), and porcine parvovirus (PPV), virus strains show differences in the tissue tropism associated with changes in the growth of the viruses in cell lines or in animals. These differences in viral properties allow us to define a number of critical steps involved in the cell recognition, uptake, and infection by the autonomous parvoviruses, while determining the origins and evolutionary processes that gave

rise to CPV as a new virus of dogs increases our understanding of disease emergence.

## **CPV AND FPV – ACQUISITION OF EXTENDED HOST RANGE BY MULTISTEP ADAPTATION TO A NEW RECEPTOR**

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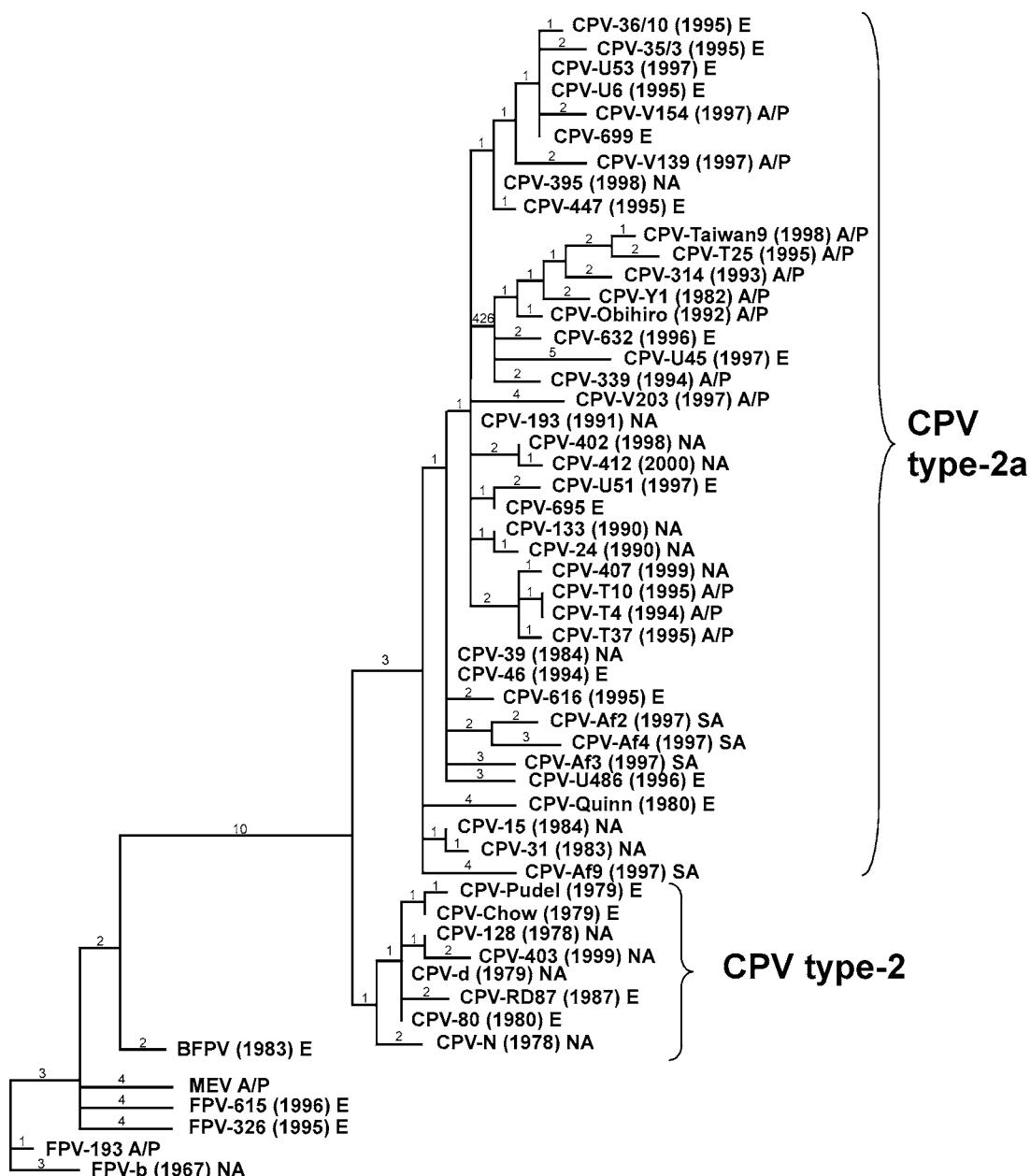
In 1978 two new diseases were observed among dogs in many parts of the world, characterized by puppies dying acutely of congestive heart failure or with acute gastroenteritis. Neither disease had been seen in dogs before 1978, but by the end of that year reports indicated that the virus had spread to virtually all the inhabited areas of the world, and serological testing also showed that the virus had become widespread among wild dogs worldwide (reviewed in Parrish, 1990). The enteric disease seen in the dogs was very similar in histopathologic examination to that seen in cats infected with the FPV, and it was soon determined that the virus causing the new canine diseases was antigenically closely related to FPV. Subsequent DNA sequencing showed that the CPV genome is >99 percent identical to that of FPV, but many studies show that those viruses differ in several properties, including host range *in vivo* and *in vitro*, antigenicity,

and in the ability to hemagglutinate erythrocytes at pH 6.2 or pH 7.5, a measure of sialic acid binding.

Phylogenetic analysis of DNA sequences showed that the CPV isolates from dogs were all derived from a single common ancestor, which most likely emerged in the mid-1970s (Figure 23.1). When compared with the almost complete genomes of several viruses, the CPV ancestor was seen to differ in only a few nucleotides (0.4 percent of the genome) from the most recent common ancestor among the FPV-like

viruses, and most of those changes were conserved in the CPV isolates after 1978.

Host range was clearly a key difference between the viruses, and this property proved to be quite complex, as the evolution of CPV proceeded in multiple steps. The first virus to spread worldwide was termed the CPV type-2 strain (to distinguish it from the previously described parvovirus of dogs, the minute virus of canines). However, by 1979 an antigenically variant strain of CPV was observed in dogs in



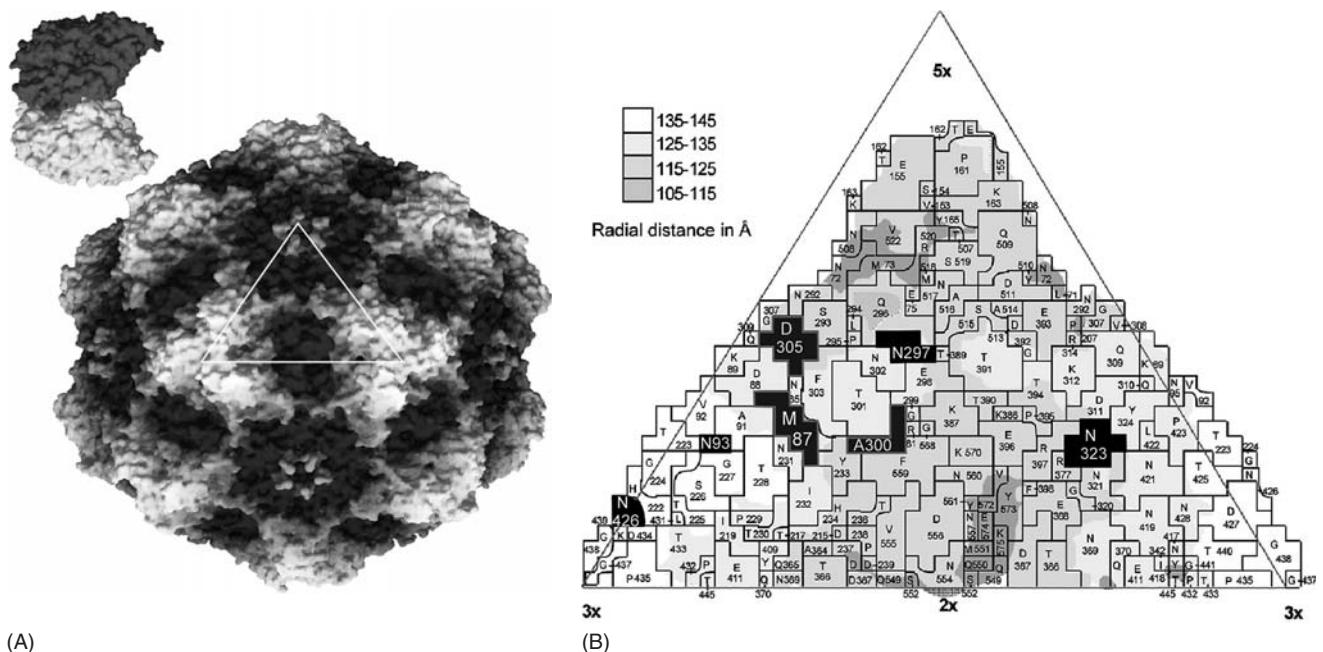
**Figure 23.1** Maximum likelihood phylogeny prepared from the VP2 sequences of FPV and CPV isolates collected during various years and in various locations around the world. The FPV, MEV, and fox virus (BFPV) isolates all from one clade, while the CPV isolates (from either dogs or cats) for a separate clade. The CPV clade is subdivided into the CPV type-2 and the CPV type-2a subclades. Numbers indicate the number of changes on each branch. The year of isolation is shown, along with the origin of the virus (A/S, Asia/South Pacific; E, Europe; NA, North America; SA, South Africa).

many parts of the world, and that was named CPV type-2a (Parrish *et al.*, 1991). All of the viruses from either cats and dogs replicated efficiently in feline cells, but only the CPV isolates infected dogs and cultured dog cells (Truyen and Parrish, 1992). When examined in detail, the host ranges of the viruses show significant variations, particularly in animal infections. FPV had the ability to infect cells in the thymus of dogs after both parenteral and oral inoculation (Truyen and Parrish, 1992). The feline host ranges of the viruses also differed over time, since the CPV type-2 isolates did not replicate in cats, but the more recent CPV strains were able to replicate efficiently in cats, and indeed were isolated from cats with clinical disease in several different countries (Ikeda *et al.*, 2000, 2002; Truyen *et al.*, 1996).

The host range properties of CPV and FPV for both dogs and for cats were controlled by residues in three separate regions on or around a raised region of the capsid structure – the 3-fold spike. By recombination mapping and mutational analysis, the primary control of canine host range of CPV was seen to be determined by combinations of the sequences at VP2 positions 93 and 323, which acted together. Changing those residues in FPV simultaneously to the CPV sequences introduced the ability to infect canine cells, but neither change alone altered the host range (Chang *et al.*, 1992; Horiuchi *et al.*, 1994; Strassheim *et al.*, 1994; Llamas-Saiz *et al.*, 1996; Parker and Parrish, 1997; Hueffer *et al.*, 2003a). Certain changes in residues close to 299 and

300 on the surface of the capsid near the 3-fold spike blocked the ability of the virus to infect dog cells, and changes in that region also appeared to control the *in vivo* feline host range of CPV (Truyen *et al.*, 1994). The natural variant of CPV (termed CPV type 2a), which replaced the original CPV virus and which had also regained the ability to infect cats, contained changes of residues 87, 300, and 305, which are all found on the shoulder region of the 3-fold spike of the capsid (Truyen *et al.*, 1995, 1996). The locations of the changes in the structure important for host range are shown in Figure 23.2. Since 1978 the CPV viruses have continued to evolve, with changes being found in at least two regions of the capsid, including changes of VP2 residues 426 and 297 (near residues 93 and 300, respectively, on the 3-fold spike) (Parrish *et al.*, 1991; Ikeda *et al.*, 2000; Buonavoglia *et al.*, 2001). A number of additional changes in CPV have also been detected in various parts of the world, but most are found in only a minority of viruses (Ikeda *et al.*, 2000). Interestingly some changes that became widespread were synonymous codon (non-coding) changes, suggesting that there was selection for DNA sequence, or that those changes hitch-hiked along with changes in other parts of the genome.

Some of the host ranges of CPV and FPV in animals are recapitulated in tissue culture, where it is now clear that the block to infection by FPV in dog cells is primarily due to the lack of a functional cell surface receptor for that virus. That deficiency could be overcome by expression of the feline



**Figure 23.2** (A) Surface rendered model of the CPV capsid showing the 2-fold dimple, the 3-fold spike, the canyon, and the 5-fold cylinder. One asymmetric unit of the capsid is indicated by the triangle. A model of the ectodomain human transferrin receptor is shown at the same scale, to indicate the relative size of the virus and its ligand on feline or canine cells. (B) A close-up view of one asymmetric unit of the CPV capsid, with the surface-exposed residues indicated. VP2 residues that control specific functions are highlighted: canine host range of CPV (93 and 323); CPV type-2a specific differences (87, 300, 305) and the variation that has been selected worldwide in variant viruses (297 and 426). (Modified from Hueffer *et al.* with permission. Copyright 2003, the American Society for Microbiology. All rights reserved.)

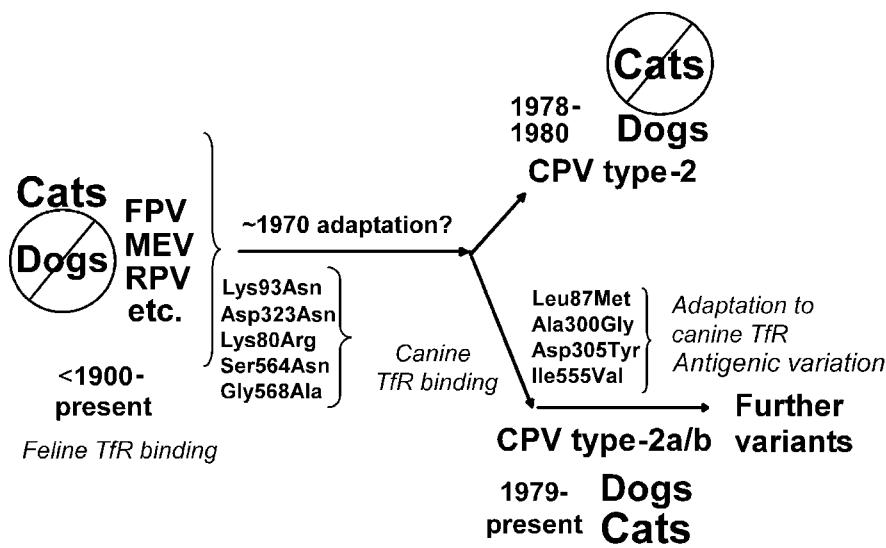
transferrin receptor (TfR) in the canine cells, where those cells became susceptible to FPV binding and infection (Hueffer *et al.*, 2003b). A difference in the binding of CPV and FPV capsids to the canine TfR controls the canine host range, and that binding was determined by changes in all three regions of the capsid that control host range control (Figure 23.2) (Hueffer *et al.*, 2003b).

The functions of some mutations have been defined. VP2 residues 93 and 323 together control virus binding to the canine TfR and thereby allow infection of canine cells (Hueffer *et al.*, 2003a). It is not yet clear what the precise functions are of the naturally selected changes in the vicinity of residue 300. One group of changes (of VP2 residues 87, 300, and 305) improves the ability of the virus to use the canine TfR for infection (Hueffer *et al.*, 2003b). However, other mutations in that same region selected by growth of the virus in cat cells appear to adapt the virus back to the feline cells and in some cases cause the concomitant loss of the canine TfR binding, as are some *in vitro* prepared mutants that affected canine cell infection and canine TfR binding (Llamas-Saiz *et al.*, 1996; Parker and Parrish, 1997).

The current model of CPV evolution is summarized in Figure 23.3. After emerging in dogs through the acquisition of the initial series of changes that allowed the virus to infect dogs, the subsequent evolution of CPV in the dogs has led to the acquisition of further mutations in the capsid protein at one of the host range-determining regions. Those changes together resulted in more efficient usage of the canine TfR for cell infection. These findings all indicate

that TfR binding and efficient usage of the canine TfR for infection are central events in the evolution and pathogenesis of CPV (Hueffer *et al.*, 2003b). Use of the TfR as the cellular receptor for these viruses on feline and canine cells is also closely correlated with their pathogenicity in nature, as the TfR is highly expressed on crypt cells in the intestinal epithelium and hematopoietic cells, which are the main target cells of CPV and FPV in animals (Parrish, 1995).

In parallel with the alterations in host range and TfR binding, many of the viruses also showed differences in antigenic structure, which were determined by the same substitutions in the capsid protein. Those were seen between FPV and CPV, where the CPV-specific epitope was due to the Lys-Asn substitution of VP2 residue 93, and between CPV type-2 and CPV type-2a where the group of changes in the capsid protein (VP2 residues 87, 300, and 305) determined the loss of CPV type-2 epitopes and the gain of an epitope specific for CPV type-2a (Strassheim *et al.*, 1994). A subsequent change of Asn 426 to Asp was widely selected in a CPV type-2a background, and changed an antigenic epitope of the virus. Later on a further change of residue 426 to Glu was also observed in viruses from Europe and Asia. The relative importance of the antigenic selection compared with the host range selection owing to these changes is not known. These viruses cause acute infections of less than a week, invoking immunity that prevents re-infection by the same or by other strains of virus, so that the immune selection is unlikely to occur in the recovered animals. However, most infections occur in 2–4-month-old puppies



**Figure 23.3** A model of CPV evolution and host range adaptation. CPV evolved from FPV or a closely related parvovirus. FPV-like viruses cause disease in cats but not dogs, and infect feline cells by binding the feline TfR, but they are unable to bind the canine TfR. Changes in the capsid protein gave rise to an ancestral CPV around 1970, and some form of that virus could bind the canine TfR and infect canine cells and cause disease in dogs, emerging worldwide in 1978. Although it could bind the feline TfR and infect feline cells *in vitro*, the original CPV type 2 did not infect cats. CPV type-2 was replaced by a more evolved virus by 1980 (CPV type-2a), and that virus contained several changes in its capsid gene, which led to a more efficient infection of canine cells. CPV type-2a and subsequent viruses had regained the ability to efficiently infect cats and they now cause disease in both dogs and cats. Evolution is still ongoing for these viruses as can be seen by the global emergence of new virus sequences.

with waning circulating maternal antibodies, and under those conditions of low and declining levels of antibody, infection of puppies with different antigenic types than the virus that infected the mother to induce maternal immunity may be selected, leading to replacement by the antigenically variant viruses.

In summary, the emergence and adaptation of CPV has been a complex process. The acquisition of the ability to infect dogs by the ancestor of CPV probably occurred in the early 1970s, and allowed the virus to spread between dogs, resulting in the pandemic spread of CPV type-2 in 1978. The later steps of the adaptation appear to have involved a variety of receptor and antibody selections, which have given rise to the better adapted virus of today, and the viruses containing those variants have become globally distributed. It is likely that there is still undergoing selection and that the final form of the virus is yet to be seen.

## CONTROLS OF CELL AND TISSUE TROPISM – THE EXAMPLES OF MINUTE VIRUS OF MICE, PORCINE PARVOVIRUS AND ALEUTIAN MINK DISEASE VIRUSES

### Minute virus of mice (MVM)

The MVM was first recognized as a contaminant of an adenovirus preparation and grown in cultured mouse cells. A clone of that virus termed prototype MVM (MVM[p]) was derived by plaqueing on mouse A9 fibroblast cells (Crawford, 1966). A second strain of MVM was later isolated from the EL-4 murine lymphoma cells, and that virus proved to be a T-cell-adapted virus which was named MVM(i) for its immunosuppressive properties. Those viruses differ in their tropism for differentiated cells in culture, since MVM(p) efficiently infects fibroblasts but not lymphoid cells, while the reverse is true for MVM(i) (Engers *et al.*, 1981; Tattersall and Bratton, 1983). The block to infection was apparently due to some intracellular step in replication, as the incoming virus genome was seen to be converted to a double-stranded form (Spalholz and Tattersall, 1983). Hybrids between the lymphoid and fibroblast cells were susceptible to infection, indicating that the block to infection is due to lack of some factor required for infection or replication in the resistant cell type (Tattersall and Bratton, 1983). The MVM(p) and MVM(i) also differ in their replication and pathogenesis *in vivo* after infection of mice (Brownstein *et al.*, 1991, 1992). The differences in cell tropism between the two different strains were mapped to the capsid gene (Gardiner

and Tattersall, 1988b), and the change of the tissue tropism of MVM(i), which allowed infection of fibroblasts, was due to combinations of the sequences at VP2 residues 317 and 321 (Ball-Goodrich *et al.*, 1991, 1992). When those residues were Ala and Glu respectively, the viruses were lymphotropic, and when they were Thr and Gly the virus was fibrotropic (Ball-Goodrich and Tattersall, 1992). Both residues are found on the side of the 3-fold spike of the virion, and VP2 residue 321 aligns with the host range-controlling residue 323 of CPV (Agbandje-McKenna *et al.*, 1998). Other VP2 residues influencing cell tropism were selected by passage of single mutants of MVM(i) in the restrictive host cells. When the virus containing the Ala317 to Thr mutation was grown in fibroblasts, compensating mutations included those of residues Asp339 to Gly or Ala, and Asp533 to Asn. For the Glu321 to Gly mutations the compensating mutations were Ser460 to Ala and Tyr558 to His (Agbandje-McKenna *et al.*, 1998) (Table 23.1). All of those changes are located on the side of the 3-fold spike of the virus, and are predicted to reduce the stability of the capsid structure, suggesting a change in the capsid cell entry, genome uncoating, or assembly process.

The functions of those MVM capsid residues in controlling host range are not completely understood. Radioactively-labeled capsids of the viruses were able to bind to cells in which they were unable to replicate, and double-stranded DNA production was seen, but little viral protein or production of new capsids occurred (Gardiner and Tattersall, 1988a; Previsani *et al.*, 1997). Interestingly, changes in the non-structural 2 (NS2) protein influenced the results seen for the allotrophic infection. Differences in the capsid proteins of the viruses and their ability to complete their replication cycles in human cells correlated with changes in the sequence of the NS2 protein of the virus (Rubio *et al.*, 2001), and the NS2 proteins also acted along with the allotrophic determinants to alter the pathogenesis of the virus in mice (Brownstein *et al.*, 1992). The NS2 protein has been associated with the assembly and nuclear export functions of the capsids in cells from different hosts (Cotmore *et al.*, 1997; Eichwald *et al.*, 2002; Miller and Pintel, 2002), and therefore the capsid changes that affect host range may need to be compensated for by changes in NS2, which influence the assembly or nuclear transport functions of the cell.

### Porcine parvovirus (PPV)

The PPV normally causes a subclinical infection of adult pigs, where it appears to replicate primarily in cells of the lymphoid system (Oraveerakul, 1993). Less common

**Table 23.1** The mutations selected in the genome of single mutants of MVM(i) after growth on fibroblast cells. The top row shows the mutation in the virus, and the bottom row shows the mutations selected after culturing in the cells. (From Agbandje-McKenna *et al.*, 1998 with permission.)

Site-directed mutation	Ala317Thr	Ala317Thr	Ala317Thr	Glu321Gly	Glu321Gly	Glu321Gly
Selected forward mutation	Asl339Gly	Asp339Ala	Asp533Asn	Ala317Thr	Ser460Ala	Try558His

infections are a dermatitis that results after infection with some PPV strains, and a severe disease can result from the dual infection with both PPV and porcine circovirus (Choi and Chae, 2000). When a pregnant pig is infected by PPV, the virus may invade the uterus and cause fetal death after viral replication in many tissues. The determinants of tissue tropism between the attenuated NADL-2 strain of PPV and the Kresse strain isolated from a case of dermatitis have been mapped to a single region in the capsid gene (Bergeron *et al.*, 1993). The residues that control the tissue culture cell tropism of the viruses are all exposed on the surface of the virus capsids (Simpson *et al.*, 2002). One amino acid difference between the two strains in this region (VP2 residue 436) is on the 3-fold spike of the virus capsid, while two other VP2 residues (378 and 383) are on the edge of the canyon surrounding the 5-fold cylinder (Bergeron *et al.*, 1993; Simpson *et al.*, 2002). The mechanisms of the host range restriction are still unknown, but changes in receptor binding and infection are likely to be involved.

### Aleutian mink disease virus (AMDV)

Wild-type AMDV isolates readily infect mink, but do not replicate well in cultured cells. The AMDV-G strain was selected in cultured feline cells by growth at low temperature, and that virus replicates efficiently in cultured cells but not mink (Bloom *et al.*, 1998). The altered cell tropism of the AMDV was mapped to a fragment of the capsid gene (Bloom *et al.*, 1993; Stevenson *et al.*, 2001). Coding differences between the two strains within this fragment map to a region predicted to be on or near the surface of the 3-fold related structures of the capsid (Bloom *et al.*, 1993; McKenna *et al.*, 1999; Stevenson *et al.*, 2001). However, the mechanisms for the block to infection in tissue culture by the wild-type virus or the altered infection of animals by the culture adapted viruses are still not understood.

### EXPERIMENTAL STUDIES OF VIRUS SEQUENCE VARIATION AND EVOLUTION

The evolution of CPV during the process of serial passage leading to attenuation of a vaccine strain was examined and showed a pattern of change consistent with mutation followed by recombination, rather than sequential selection of individual mutations. Several of the changes observed during the attenuation process were identical or at the same positions as changes observed in the natural evolution of CPV and FPV (Badgett *et al.*, 2002).

During a study investigating the emergence of antibody escape mutants of MVM both *in vitro* and in immunodeficient SCID mice inoculated with antibodies, it was seen that even without selection a high degree of heterogeneity develops in MVM populations during the *in vitro* and *in vivo*

replication of the virus (Lopez-Bueno *et al.*, 2003). Once antibody selection was applied neutralization escape mutants were rapidly selected and dominated the viral populations. These findings have broad consequences for the development and safety of live attenuated viral vaccines in general and indicate that the adaptability and heterogeneity of DNA viruses may be underestimated.

### ANTIGENIC STRUCTURE AND VARIATION

Apart from being important in determining tropism of parvoviruses, the 3-fold spike is also a major antigenic site. Neutralizing antibodies against this region in the capsid have been described for CPV and FPV (Strassheim *et al.*, 1994; Wikoff *et al.*, 1994), as well as for MVM (Lopez-Bueno *et al.*, 2003). Antibodies also bind those structures of AMDV, although those can lead to immune enhancement of the disease, an important feature of AMDV infection and pathogenesis (Bloom *et al.*, 2001).

### CONCLUSIONS

The various models described above indicate that for many parvovirus cell tropisms, host ranges and modes of pathogenesis are controlled by structural elements of the capsids, which appear to cluster around the 3-fold axes of symmetry. Those mechanisms are in some cases modified by the activity of the viral NS2 protein. The atomic models of the viruses, and the understanding of the receptors and antibodies that interact with them show that small changes in the viruses can have profound effects on the virus properties. Although similar structural elements of the different viruses are involved in several host and tissue tropisms, each appears to be controlled through a different mechanism. Host ranges of CPV and FPV are primarily controlled by receptor binding, while the replication of MVM mutants is restricted at later intracellular steps. The emergence of CPV as a well-documented case of host range shifts will be better understood when the results from related parvoviruses allow the development of a more general understanding of host range control and evolution of small non-enveloped DNA viruses.

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# Immunology of human erythrovirus infection

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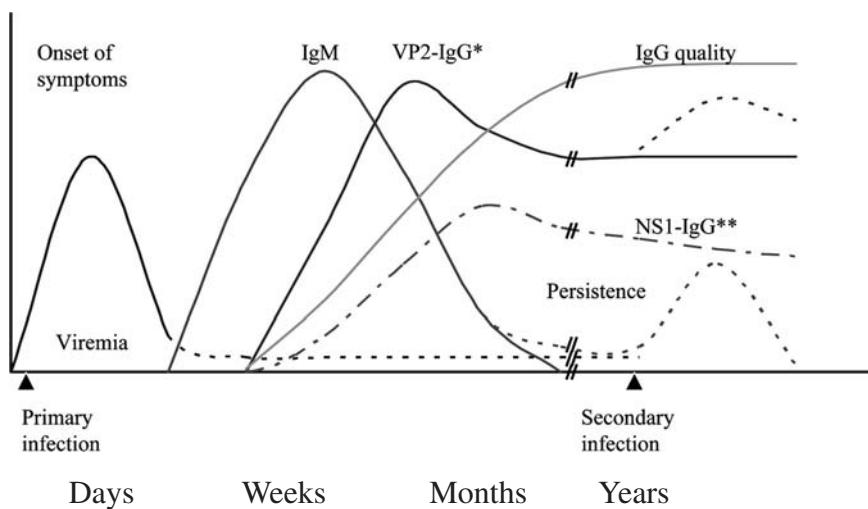
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## HUMORAL IMMUNITY

### Capsid protein IgG and IgM

B-cell immunity (Figure 24.1) is thought to constitute the major defense against the B19 virus. The B19 capsid-IgM (immunoglobulin M) and immunoglobulin G (IgG) antibodies are produced both in experimental (Anderson *et al.*, 1985) and natural (Saarinen *et al.*, 1986) infection. After inoculation, the virus-specific IgM appears during the

second week and IgG by the end of the third week (Anderson *et al.*, 1985). The IgM levels in natural infection begin to decline during the second month of clinical onset, yet may occur for several months (Anderson *et al.*, 1986). The capsid-protein IgG as a rule persists for life and protects against secondary infection (Brown *et al.*, 1994). However, in a volunteer with little B19 IgG, an asymptomatic reinfection occurred, with a brief viremia and an anamnestic antibody response (Anderson *et al.*, 1986). Even if prior immunization would not in each case prevent reinfection, it is likely to



\* Conformational-VP2-IgG level

\*\* Prevalence of NS1-IgG

**Figure 24.1** B-cell response in erythrovirus infection. See also Color Plate 24.1.

prevent disease. Chronic B19 infections are treated with immunoglobulins containing neutralizing antibodies (Kurtzman *et al.*, 1989; Koch *et al.*, 1990; Koduri *et al.*, 1999). However, chronic infections have occurred also in some apparently immunocompetent subjects (Faden *et al.*, 1992; Hemauer *et al.*, 2000); and virus clearance is not always complete even after repeated immunoglobulin infusions (Frickhofen *et al.*, 1990; Tang *et al.*, 1994). These data suggest that humoral immunity is a necessary though not always sufficient defense for the B19 virus. Furthermore, in immunocompetent individuals the presence of neutralizing B19 immunity does not overrule B19-DNA-PCR-positivity in bone marrow (Lundqvist *et al.*, 1999a) or in solid tissues (see below).

During a major epidemic, the prevalence of B19 IgM positivity as measured by a high quality enzyme-immunoassay (EIA) reached 29 percent, compared with the baseline prevalence of 1–3 percent (Jensen *et al.*, 1998). Among US blood donors the B19 IgM baseline prevalence (by using an FDA-approved, high quality EIA) was 1 percent (Doyle *et al.*, 2000).

Also, B19 antibodies of the immunoglobulin E (IgE) class have been detected in acute and recent B19 infection (Bluth *et al.*, 2003). The predominant IgG subclass for VP1 and VP2 at all stages after infection is IgG1, and IgG3 is associated with acute infection. On the other hand, IgG4 was observed to appear long after infection, and was restrictively specific for VP1. The ratio of VP1-IgG3 and VP1-IgG4 levels provided an assay for primary infection, with a high diagnostic sensitivity and specificity (Franssila *et al.*, 1996). Another group, however, found no evidence of the VP1-IgG3/IgG4 subclass switch (Corcoran *et al.*, 2000).

An international standard (IS) for parvovirus B19 IgG antibody (NIBSC 93/724) has been issued by the Expert Committee on Biological Standardization of the World Health Organization. The preparation is a freeze-dried pool of sera from six UK blood donors, and has been assigned a unitage of 100 IU per ampoule. It is useful, for example, in calibration of in-house or commercial antibody assays, and in reporting of IgG results in IU/ml (Ferguson *et al.*, 1997; Searle *et al.*, 1997).

## IgG avidity and IgG epitope-type specificity

The avidity (functional affinity) of B19-IgG has been measured successfully with the protein-denaturing IgG-avidity assays (Hedman *et al.*, 1993; Korhonen *et al.*, 1999; Pereira *et al.*, 2001), i.e. immunofluorescence assay (IFA) or EIA in which the antigen-complexed IgG is exposed to urea, and the proportion of urea-resistant relative to total bound IgG is determined (Gray *et al.*, 1993; Söderlund *et al.*, 1995a). With EIA, a fusion-protein of VP1u (unique part of VP1), or baculovirus-expressed virus-like particles containing both structural proteins (VP1 and VP2) gave rise to avidity-EIAs of very good diagnostic performance. By contrast, VP2-alone

recombinant capsids were irreversibly damaged by the protein denaturant, as well as by mere adsorption (without linker) onto plastic, rendering VP2 unsuitable for use in the protein-denaturing assays (Söderlund *et al.*, 1995a).

The avidity-EIA studies revealed another intriguing characteristic of the quality (of the paratope) of VP2-specific IgG. While the VP2 capsids immobilized 'gently' (via linker) on solid phase readily detected IgG in patients with acute infection, as well as in subjects with past immunity, denatured VP2 capsids did not detect IgG in the latter cohort. Yet, the denatured VP2 capsids detected – very well – IgG in the patients with acute infection. By deduction, the B-cell clones that express IgG for the VP2 linear epitopes must become deleted or anergized in just a few weeks (or even more rapidly?) after their activation; whereas, in the same patients, the B-cell clones that express IgG for the VP2 conformational epitopes remain viable and functional for years, decades or life.

The molecular and cell biological mechanisms of this challenging 'epitope-type specificity (ETS)' phenomenon (i.e. conformational dependence of VP2 B-cell response) are still by and large unknown. In general, VP2 epitopes recognizing acute-phase IgG have been located to VP2-amino acids 65–75 (Fridell *et al.*, 1989; Kaikkonen *et al.*, 1999) and 266–273 (Sato *et al.*, 1991b; Kaikkonen *et al.*, 1999) (Figure 24.2); and in particular to the immunodominant heptapeptide KYVTGIN at VP2-amino acids 344–350 (corresponding to VP1-amino acids 571 to 577) (Kaikkonen *et al.*, 1999). Isolated VP2 molecules studied in Western-blots (in denaturing conditions) show results similar to the denatured VP2 capsids in EIA (Söderlund *et al.*, 1995b), adding proof that the antigen–antibody interactions essential for this phenomenon occur at intra- rather than supramolecular levels (with respect to VP2). The simplest explanation, cryptic residence, appears unlikely as the KYVTGIN sequence has been located externally on the surface protrusions between the 2-fold and 3-fold symmetry axes of the VP2 capsid (Chipman *et al.*, 1996; Kaufmann *et al.*, 2004).

Söderlund *et al.* (1995b) hypothesized that the VP2 primary structure might resemble a 'self' antigen for which a breach of immunologic tolerance could occur transiently during the acute phase. Such an hypothesis is indirectly supported by the findings of Lunardi *et al.* (1998), whose patients with chronic B19 infection had an autoantibody-like IgG for an acute-phase epitope (VP2 amino acids 65–88). However, cross-reactive antibodies have not been documented for the other VP2 epitopes. Kaikkonen *et al.* (1999) reviewed additional examples, among different viruses, of kinetically distinct IgG responses for their linear and conformational epitopes. A transition from linear-epitope to conformational-epitope specificity of IgG has been observed with human immunodeficiency virus (Cole *et al.*, 1998) and equine infectious anemia virus (Hammond *et al.*, 1997); furthermore, a linear epitope in the envelope glycoprotein of Sin Nombre virus appears to be specific for acute-phase IgG (Hjelle *et al.*, 1997). Indeed, the B19-ETS phenomenon may represent a molecular property of many unrelated

viruses, or their structural components, which we are only beginning to understand.

The survival of B cells recognizing conformational VP2 epitopes was recently suggested to be associated with their better (hypothetical) ability to receive T-cell help, stronger B-cell receptor cross-linking or 'maintenance signals' as compared with linear VP2 epitopes (Corcoran *et al.*, 2004). Yet, these theories also fail to explain the relatively long IgG persistence for the VP1u primary structure (Söderlund *et al.*, 1995b; Kerr *et al.*, 1996; Musiani *et al.*, 2000). Interestingly, the B19 capsid is much more susceptible to disintegration by low pH than are capsids of the parvovirus genus (e.g. MVM) (Boschetti *et al.*, 2004).

Antibodies for the primary structure (linear epitopes) of VP1u and VP2 are functionally important. Some neutralizing antibodies target linear epitopes in VP1u (Gigler *et al.*, 1999), and others in VP2 (VP2 amino acids 328–344) (Sato *et al.*, 1991a). Also immunization of rabbits with oligomeric peptides has revealed neutralizing epitopes within the N-terminus of VP1u and within the VP1–VP2 junction area (Saikawa *et al.*, 1993). A linear neutralizing epitope in the N-terminal region of VP2 has been identified by a monoclonal antibody for the native virus (Yoshimoto *et al.*, 1991). On the other hand, some of the conformational VP2 epitopes also target neutralizing antibodies, as shown by murine (Yoshimoto *et al.*, 1991) or human (Arakelov *et al.*, 1993; Gigler *et al.*, 1999) monoclonal antibodies (MAbs). Neutralizing human MAbs bind a 'conformationally defined epitope' spanning VP1 residues 30–42 (Dorsch *et al.*, 2001). The antibodies recognized this epitope with similarly high affinity in wild-type virions and recombinantly expressed virus-like particles. One conserved and denaturation-resistant neutralizing VP2 epitope that has received much attention is the binding site of the monoclonal R92F6. That MAb has found use in B19-IgM serology (O'Neill and Coyle 1992; Loughrey *et al.*, 1993) and in detection of B19 antigens from serum or paraffin-embedded tissues (Morey *et al.*, 1992; Kerr *et al.*, 1995; Essary *et al.*, 1998).

Animal immunization studies emphasize the importance of the VP1u in B19-virus B-cell immunity. Neutralizing antibodies were generated in rabbits immunized with fusion proteins containing VP1u (Rosenfeld *et al.*, 1992) or polypeptide sequences of it (Saikawa *et al.*, 1993). The same holds for baculovirus-expressed capsids containing both VP1 and VP2 (Bansal *et al.*, 1993; Kajigaya *et al.*, 1991). For comparison, rabbits immunized with VP2 polypeptides (Saikawa *et al.*, 1993) or VP2-alone capsids (Bansal *et al.*, 1993; Kajigaya *et al.*, 1991) generated strong antibody responses, yet with little or no virus-neutralizing activity (Bansal *et al.*, 1993; Saikawa *et al.*, 1993; Kajigaya *et al.*, 1991).

## NS1 antibody response

The initial reports suggested that the occurrence of NS1 (C-terminal region) IgG, would be confined within patients

with prolonged B19 arthropathy (Von Poblotzki *et al.*, 1995a) or with persistent B19 infection (Von Poblotzki *et al.*, 1995b). Subsequently, this theory has been challenged (Searle *et al.*, 1998b; Venturoli *et al.*, 1998; Jones *et al.*, 1999; Mitchell *et al.*, 2001). Three NS1 antigenic regions (amino acids 191–206, 271–286, and 371–386) reacted equally with sera of patients with persistent B19 infection or of healthy, remotely infected controls (Tolfvenstam *et al.*, 2001a). While some data permissive for the early view have been presented (Hemauer *et al.*, 2000); NS1 antibodies have been seen more often in chronic B19 arthropathy than in acute B19 arthropathy (Kerr and Cunniffe, 2000). In all, the prevalence of IgG for (denatured) NS1 among non-symptomatic adult controls is only ~20–36 percent (Venturoli *et al.*, 1998; Searle *et al.*, 1998b; Jones *et al.*, 1999; Hemauer *et al.*, 2000; Ennis *et al.*, 2001). In pregnant women, the NS1-IgG was first observed 6 weeks or more post infection (Searle *et al.*, 1998a). On the other hand, more recent studies with non-denatured NS1 (from another recombinant source) showed IgG in many samples of both remotely and recently infected patients (Heegaard *et al.*, 2002b).

Recognition of protein conformation in general appears to be essential for NS1-IgM (von Poblotzki *et al.*, 1995b; Hemauer *et al.*, 2000; Ennis *et al.*, 2001); and the same appears to hold for NS1-IgG (Heegaard *et al.*, 2002b). High-quality NS1 assays could turn out to be useful in monitoring the efficacy of the (forthcoming) capsid-based B19 vaccines, as the presence of NS1 antibodies would indicate vaccine failure (Heegaard *et al.*, 2002b).

## Autoantibodies in erythrovirus infection

B19 infection of constitutionally healthy individuals gives rise to a transient autoimmune state with expression of anti-ds and anti-ss DNA antibodies and cytotoxic antilymphocyte IgM (Soloninka *et al.*, 1989), and antineutrophil cytoplasmic antibody (ANCA) and anticardiolipin (aCL) antibody (IgM) (Chou *et al.*, 2000). In a long follow-up (26–85 months) after B19 infection, many more patients than controls maintained one or more serum autoantibodies (antinuclear antibody, antismooth muscle antibody, gastric parietal cell antibody, antireticulin antibody, antimitochondrial antibody, rheumatoid factor) at a titer of  $\geq 40$  ( $P = 0.004$ ). Several test patients and no controls had serum autoantibody at a titer  $\geq 160$  (Kerr and Boyd, 1996).

Human antibodies binding to and affinity-purified with a 24-amino acid VP2 synthetic peptide were reported to cross-react with an amazingly wide spectrum of autoantigens (keratin, collagen type II, ssDNA, and cardiolipin); and to elicit, via immunization in mice, antibodies with the same reactivities (Lunardi *et al.*, 1998). Such findings clearly warrant and await confirmation and extension.

IgG for negatively charged phospholipids, cardiolipin and phosphatidyl serine occur commonly in B19 acute infection, and show similarities in binding specificity with

the phospholipid antibodies found in systemic lupus erythematosus (SLE) (Loizou *et al.*, 1997). In general, the clinical significance of phospholipid antibodies in patients with viral infections (besides B19, also hepatitis C virus, HIV, cytomegalovirus, varicella zoster, Epstein–Barr virus, adenovirus) is poorly known. Their persistence in some patients has given rise to discussions on whether the infections can trigger the phospholipid antibodies occurring in autoimmune diseases (Uthman and Gharavi 2002). Also, cross-reactivity between a recombinant chlamydial lipopolysaccharide (LPS) antigen and parvovirus was observed, and may have diagnostic implications (Persson and Haidl 2000). However, the anticardiolipin antibodies occurring in pregnant women with acute B19 infection did not correlate with an adverse maternal or perinatal outcome (Gratacos *et al.*, 1995).

Of note, B19 infection has been shown to be an important etiology for the ‘anticomplementarity’ that frequently impairs viral (and some other microbial) diagnosis by the complement fixation test (Barton *et al.*, 1995).

## Serodiagnosis and diagnostics

The IgM response was initially shown in patients with aplastic crisis by an IgM-antibody capture radioimmunoassay (MACRIA) (Anderson *et al.*, 1982). Besides DNA hybridization and (immuno)electron microscopy (Anderson *et al.*, 1984; Plummer *et al.*, 1985), the early B19 diagnostics based on IgG and IgM detection used natural virus antigen (Paver and Clarke, 1976; Cohen *et al.*, 1983; Okabe *et al.*, 1984; Shiraishi *et al.*, 1985; Anderson *et al.*, 1986; Cohen and Buckley, 1988; Brown *et al.*, 1989; Yaegashi *et al.*, 1989; Cohen, 1997). The early man-made antigens were VP1 recombinant proteins (Morinet *et al.*, 1990; Rayment *et al.*, 1990; Söderlund *et al.*, 1992; Schwarz and Jager, 1994; Yaegashi *et al.*, 1995; Schwarz *et al.*, 1997) or a VP2 synthetic peptide (Fridell *et al.*, 1989; Patou and Ayliffe, 1991; Skjoldebrand-Sparre *et al.*, 1996).

The B19-IgM antibodies target both the VP1 unique portion (VP1u) (Söderlund *et al.*, 1992) and VP2 (O'Neill *et al.*, 1995). Commercial B19 IgM assays suffered for a long time from variable diagnostic quality (Helftenbein *et al.*, 1994; Bruu and Nordbo, 1995; Sloots and Devine, 1996; Manaresi *et al.*, 2002).

Recombinant capsids (VP2 or VP1-VP2) expressed in eukaryotic (insect or vertebrate) cells provide an excellent source of antigen for the detection of B19 IgG and IgM antibodies (Kajigaya *et al.*, 1989; Brown *et al.*, 1990; Salimans *et al.*, 1992; Yoto *et al.*, 1995; Koch 1995; Wang and Erdman, 1995; Heegard *et al.*, 2002a). In sensitivity the high quality VP2-EIAs equalled or exceeded that of VP1-IFA (Gray *et al.*, 1993; Tolfsenstam *et al.*, 1996). In comparative studies between commercial EIAs employing a baculovirus-derived (conformational) VP2-capsid antigen and a prokaryotic (denatured) VP1 antigen, the former produced fewer

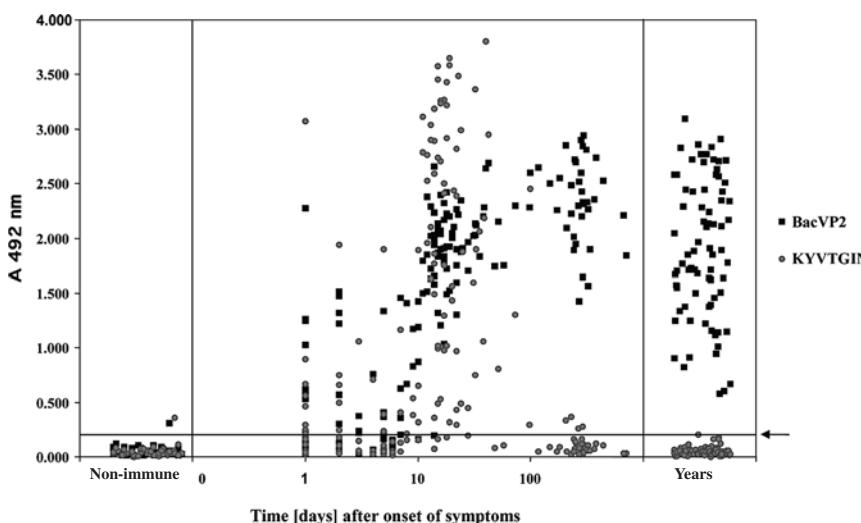
equivocal IgM and IgG results, and correlated more closely to the confirmatory VP1 IFA (Jordan, 2000). VP1-IgG and IgM, with either natural or recombinant virus proteins as antigen, are detectable also by immunoblotting (Palmer *et al.*, 1996). However, sensitive detection of VP2-IgM by recombinant-antigen EIA calls for conformational antigens; while the denaturing Western blot merely qualifies for a confirmatory test, and then requires a VP1 antigen (Kerr *et al.*, 1999; Manaresi *et al.*, 2001; Zuffi *et al.*, 2001).

For improved B19 diagnosis, the combination of IgM and DNA-PCR has been recommended (Hoebe *et al.*, 2002). In primary infection, the diagnostic utility of B19-PCR is greatest at the immediate onset (before antibody appearance) and during convalescence (after IgM clearance) (Gallinella *et al.*, 2003). However, the viremic persistence of B19 DNA among some immunocompetent subjects without symptoms complicates the interpretation of PCR positivity.

The conformational dependence and the acute-phase restriction of B-cell immunity for denatured VP2 (Söderlund *et al.*, 1995b) are now generally accepted (Manaresi *et al.*, 1999; Corcoran *et al.*, 2000, 2004), and diagnostic antibody assays (of two generations) using this ‘ETS’ phenomenon have been set up (Kaikkonen *et al.*, 1999, 2001). In the second-generation ETS-EIA the IgG activity for the KYVTGIN epitope (a linear synthetic peptide antigen) is plotted against the corresponding IgG activity for the native VP2 capsid (a conformational recombinant antigen), and the resulting ‘ETS index’ tells the time of primary infection (Figure 24.2). According to analysis with sera (from three European countries) of 489 patients with B19-related symptoms or asymptomatic controls, the second-generation epitope-type EIA had a diagnostic sensitivity of 98 percent and a diagnostic specificity of 94 percent. By more accurate timing of B19 primary infection, this approach greatly increases the reliability of B19 serodiagnosis (Kaikkonen *et al.*, 2001). Furthermore, an immunochromatographic modification of the KYVTGIN test showed promising performance as a point-of-care (bedside) test (Kaikkonen *et al.*, 2002).

The assays for B19-IgG quality, i.e. VP1-IgG avidity and VP2-IgG ETS (conformational dependence), used either separately or together, provide a valuable means for timing of B19 infection – or distinction of primary from secondary/memory response (Söderlund *et al.*, 1995a,b; Lundqvist *et al.*, 1999b). The secondary immune responses (or their analogues) occur in medical conditions of several types, impairing conventional (IgM and IgG) serodiagnoses. Of note, the genuine secondary responses are distinct from the IgG ‘pseudo-seroconversions’ that frequently occur among transplant recipients owing to iatrogenic administration of immunoglobulins or even thrombocyte concentrates in large doses (passive immunization) (Azzi *et al.*, 1993; Söderlund *et al.*, 1997a). Also the latter may cause diagnostic confusion.

The measurement of VP1-IgG avidity and VP2-IgG ETS allowed for analysis of the type (primary versus secondary) of adoptively transferred B-cell immunity among recipients



**Figure 24.2** IgG binding to VP2 capsid versus KYVTGIN epitope before and after B19 infection. (From Kaikonen *et al.*, 1999.) See also Color Plate 24.2.

of allogeneic bone marrow stem cell grafts. Acute B19 infections in recipients with B19-seronegative donors showed primary response (low avidity; low ETS ratio), whereas B19 infections in recipients with B19-seropositive donors showed secondary response (high avidity; high ETS ratio), irrespective of the recipients' own serostatus before transplantation (Söderlund *et al.*, 1997a).

Even high quality B19 IgM assays show false-positive or clinically inappropriate results in certain other virus infections (Jensen and Vestergaard, 1997). Owing to the simultaneous occurrence of IgM reactivity against more than one virus, reliance on specific IgM results alone for diagnosis of measles, rubella, and B19-virus infections has been warned against (Thomas *et al.*, 1999). Furthermore, each assay, no matter how good, sometimes yields false- (or clinically inappropriate) positive results. The relative frequency of such misleading results depends greatly on factors of at least three categories:

- assay technology (quality);
- pathogen-patient biology (interrelation; variance);
- infection/disease incidence (temporal and geographical; health-care infrastructural).

For example, if an IgM assay of exquisite quality, with a 99 percent diagnostic sensitivity and 99 percent diagnostic specificity, is used for B19 diagnosis during epidemiologically silent times, with a B19 infection incidence in the local laboratory environment of, say, 1.0 percent; then (by elementary statistics) only half of the IgM positive test results are correct, i.e. the predictive value of the positive result is only 50 percent! However, if that same IgM assay is accompanied (or followed) by a confirmatory assay; e.g. one for IgG quality (Figure 24.2), with an inherent sensitivity and specificity of ~95 percent (Kaikonen *et al.*, 2001),

the predictive value of the (double-) positive result (in the same demanding conditions) rises to ~94 percent. Analogous combinations of IgM/IgG-quality assays turned out superior in a pan-European multicentre evaluation of serodiagnostic strategies for toxoplasma (Roberts *et al.*, 2001). The assays for IgG quality (VP1-IgG avidity; VP2-IgG ETS) have shown their value in disclosing the remoteness of B19 infection history (longevity of immunity) also in analysis of tissue persistence of genomic B19-DNA (Söderlund *et al.*, 1997b; Hokynar *et al.*, 2000, 2002, 2004).

### Additional clinical correlates and diagnostic approaches

A B19 virus hemagglutination assay and an hemagglutination inhibition (HI) assay for B19 antibodies have been set up (Hilfenhaus *et al.*, 1993). HI positivity, however, does not equal virus neutralization (Wakamatsu *et al.*, 1999). B19-neutralizing antibodies (in naturally infected humans or experimentally immunized animals) may be measured by reverse transcriptase polymerase chain reaction for viral transcripts in a B19-permissive cell line (Bostic *et al.*, 1999). A VP1-VP2 recombinant vaccine (MEDI-491; MedImmune) was recently evaluated in a randomized, double-blind, phase 1 trial, and was found to be immunogenic and safe. All volunteers ( $n = 24$ ) developed neutralizing antibodies that were sustained through the study of 364 days (Ballou *et al.*, 2003). The importance of such studies becomes augmented by recent observations that antibodies against a single VP2 peptide on mink parvovirus (AMDV) capsid can mediate both virus neutralization *in vitro* and Fc-receptor-mediated infection persistence

and illness enhancement *in vivo*; this possibly explains the failure of capsid-based vaccines against that parvovirus (Bloom *et al.*, 2001).

In saliva of acutely infected patients, B19-IgM could be detected in 11 of 20 (55 percent) samples by an M-capture EIA and in 15 of 18 (83 percent) samples by an M-capture hemagglutination test (Cubel *et al.*, 1996). In another study with saliva, B19-IgM detection had a sensitivity of 60 percent and a specificity of 98 percent; and B19-IgG detection had a sensitivity of 100 percent and a specificity of 95 percent (Rice and Cohen, 1996). For salivary diagnosis and surveillance, three oral fluid collection devices (OraSure, Omni-SAL, and Oracol) were compared in terms of the quality of oral fluid collected and acceptability by participants. Each of the devices gave qualitative results acceptable for surveillance and epidemiological studies of rubella and parvovirus B19 (Vyse *et al.*, 2001).

In prenatal diagnosis of intrauterine B19 infection (Koch *et al.*, 1993), maternal blood (or fetal blood) B19-DNA-PCR has been reported to be more sensitive than IgM serology, and to be essential in cases of doubtful serological results (Dieck *et al.*, 1999). Interestingly, fetal anemia due to B19 infection could be detected non-invasively by Doppler ultrasonography, by increase in peak velocity of systolic blood flow in the middle cerebral (fetal) artery (Cosmi *et al.*, 2002). Following maternofetal parvovirus B19 infection no serological evidence of persistent infection could be detected (Dembinski *et al.*, 2003).

Of note, some data suggest that among (B19-IgM-negative) patients with hemophilic arthritis, IgG against denatured VP2 can be detected by immunoblotting more often than among controls (Azzi *et al.*, 2004).

## INNATE AND CELLULAR IMMUNITY

Cellular immunity provides antiviral immune responses by secreting soluble antiviral cytokines, by direct killing of virus-infected cells and by providing help for virus-specific B-cell immunity. Two categories of cellular, as well as humoral immunity exist: the innate and the adaptive immunity.

### The cells of the innate immune system in B19 infection

Of innate immunity, granulocytes are short-lived phagocytic cells, which are very important effectors against various bacterial infections. The possible role (if any) of granulocytes in B19 immunity is currently unknown. Neutrophilia is not commonly associated with B19 infection, whereas mild neutropenia was reported among all voluntary test subjects after experimental infection (Anderson *et al.*, 1985).

### NATURAL KILLER CELLS

Natural killer (NK) cells are the population of lymphocytes that can be activated into cytotoxicity and production of high levels of chemokines and antiviral cytokines, such as tumor necrosis factor (TNF)- $\alpha$  and IFN- $\gamma$ , for important early defense against viruses and tumors (Biron *et al.*, 1999; Papamichail *et al.*, 2004). The NK-mediated cytotoxicity and antiviral cytokine expression peaks at several hours to days after primary infection, whereas adaptive T- and B-cell responses take more than a week to develop (Biron *et al.*, 1999).

At present, only two studies have appeared on the role of NK cells in B19 immunity. Wagner *et al.* (1995) noted the vigorous IFN- $\gamma$  mRNA response in peripheral blood mononuclear cells (PBMC) during acute B19 infection. Bluth *et al.* (2003) determined the percentages of NK and T cells (in PBMC) in an allergic pediatric patient with acute B19 infection: virtually no change in NK-cell and T-cell numbers were found at 0, 14, and 210 days after onset.

The role of natural killer T (NKT) cells in B19-specific immunity is currently unknown (Moody *et al.*, 1999).

### MACROPHAGES

Macrophages are long-lived phagocytic cells that can circulate in the blood or reside in different organs and tissues, and have an important role in the control of intracellular bacterial, protozoan, and fungal infections. Macrophage activation also provides important antiviral defense by expressing cytokines with antiviral activity, such as interferon (IFN)- $\gamma$ , -TNF- $\alpha$  and nitric oxide as well as others with indirect immunoregulatory functions (e.g. interleukins -1, -6, -8, -10, -12, and -18, and granulocyte-macrophage-colony stimulating factor [GM-CSF]) (Guidotti and Chisari, 2001).

Macrophages are likely to be important in B19-specific immune response, both in antigen presentation to T cells and as a source of proinflammatory cytokines. Vigorous production of IL-1 and IL-6 mRNA has been observed in peripheral blood monocytes of a patient with acute B19 infection (Wagner *et al.*, 1995). Circulating IL-1, IL-6, and IL-8 (Nigro *et al.*, 2000; Kerr *et al.*, 2001, 2004) have been detected in B19-infected patients. IL-8 is an important mediator of chemotaxis for lymphocytes and granulocytes. IL-1 and IL-6 are important inducers of acute-phase proteins in liver. IL-1 and IL-6 synergistically stimulate B-cell and T-cell proliferation. Whereas IL-6 is not an IgG subclass switch factor, it enhances production of all IgG subclasses (IgG1–4) in class-switched B-cells. IL-6 is thought to be an important mediator in B19 infection; as direct B19 infection of macrophages has been claimed to enhance IL-6 and TNF- $\alpha$  production (Takahashi *et al.*, 1998). In cell lines, B19 NS1 has been shown to enhance IL-6 (Moffatt *et al.*, 1996) and TNF- $\alpha$  (Fu *et al.*, 2002) promoters, leading to increased secretion of cytokine proteins (Moffatt *et al.*, 1996; Fu *et al.*, 2002).

## DENDRITIC CELLS

Dendritic cells (DCs) are highly specialized in capturing and presenting antigens to naive T cells, for production of antigen-specific cytotoxic or helper T cells. Because of these functions, DCs are thought to be the key modulators of the adaptive immune responses against viral infections. Dendritic cells can also activate resting NK cells by triggering their NKp30 receptor (Ferlazzo *et al.*, 2002). Viruses may stimulate dendritic cells to secrete a variety of immunoregulatory cytokines, such as IFN- $\gamma$ , IL-6, TNF- $\alpha$ , IL-1, -12, and -18.

The role of DCs in B19-specific immunity is currently undefined, but they presumably are important in initiation of B19-specific Th-cell responses. The possible role of DCs in priming B19-specific cytotoxic T cells is more uncertain as, classically, *de novo* or endogenous synthesis of proteins has been considered as a prerequisite for MHC-I-restricted cytotoxic T-cell responses. DCs are not known to be infected by B19. However, increasing evidence suggests that the 'classical' pathway is not the only mechanism for MHC-I restricted antigen presentation. Via alternative pathways, collectively denoted as cross-priming or cross-presentation, DCs have been shown to capture and present exogenous viral antigens under MHC-I restriction. This mechanism has been shown to operate with murine DCs primed with porcine parvovirus-like particles (Morón *et al.*, 2002). Importantly, an NS1-specific cytotoxic B19 T-cell response has been described (Tolfvenstam *et al.*, 2001b). As NS1 induces apoptosis (Moffatt *et al.*, 1998), these cytolytic T-cells (CTLs) might have been induced via DCs cross-primed by B19-infected apoptotic cells containing NS1. Whether empty B19 capsids could induce cross-presentation similar to papillomavirus or porcine parvovirus capsids clearly needs to be studied.

## Antiviral cytokines in B19 infection

To date, IFN- $\gamma$  and TNF- $\alpha$  are the most thoroughly studied antiviral cytokines in B19 infection. CD4 $^+$  T cells, CD8 $^+$  T cells, and NK cells provide the main sources of IFN- $\gamma$  whereas TNF- $\alpha$  is also secreted by macrophages, as well as by NK and T cells.

IFN- $\gamma$  mRNA has been detected in PBMC of a B19-infected adult (Wagner *et al.*, 1995) and a child (Bluth *et al.*, 2003). Also the serum levels of IFN- $\gamma$  were elevated in majority of B19 (as well as measles or rubella) virus-infected patients (Hari *et al.*, 1999). Elevated levels of circulating IFN- $\gamma$  and TNF- $\alpha$  have been detected among pediatric patients with B19-associated myocarditis (Nigro *et al.*, 2000) and recently infected adults (Kerr *et al.*, 2001). Importantly, among patients with chronic fatigue the IFN- $\gamma$  and TNF- $\alpha$  levels remained elevated through followup (Kerr *et al.*, 2001). In addition, circulating TNF- $\alpha$  has been detected among adults with recent B19 infection and hemophagocytic syndrome (Tsuda *et al.*, 1994; Watanabe

*et al.*, 1994) and pediatric patients with self limiting infection (Barash *et al.*, 2003). At present, no studies on the effects of IFN- $\gamma$  and TNF- $\alpha$  on B19 replication have been published, even if these cytokines have been implicated in pathogenesis of B19-associated diseases (Watanabe *et al.*, 1994; Wagner *et al.*, 1995; Nigro *et al.*, 2000; Kerr *et al.*, 2001). Among animal parvoviruses, raised levels of serum TNF- $\alpha$  have been associated with canine parvovirus (CPV) enteritis (Otto *et al.*, 1997), and both IFN- $\gamma$  and TNF- $\alpha$  have a role in Kilham rat virus-induced autoimmune diabetes in rats (Chung *et al.*, 1997).

TNF- $\alpha$  has been assigned particular importance in B19-associated inflammation (Fu *et al.*, 2002) or even B19-induced rheumatoid arthritis (Takahashi *et al.*, 1998), as transfected B19-NS1 can enhance TNF- $\alpha$  secretion by activation of TNF- $\gamma$  promoter (Fu *et al.*, 2002). Also, direct B19 infection of macrophages leading to enhanced TNF- $\alpha$  production has been claimed (Takahashi *et al.*, 1998).

## Cytotoxic T-cell immune responses in B19 infection

Cytotoxic T cells may kill virus-infected cells by delivering apoptotic signals via Fas/FasL or granule exocytosis pathway. The Fas/FasL system is mainly responsible for activation-induced cell death, but it also may have a role in virus clearance. The granule exocytosis pathway uses perforin to target granzymes A and B to appropriate locations in the target cells, where they cleave critical substrates that initiate DNA fragmentation and apoptosis (Russell and Ley, 2002). Cultured human CD4 $^+$  and CD8 $^+$  T cell lines can use both the Fas/FasL and perforin/granzyme pathways.

In B19 infection, NS1-specific CD8 $^+$  T cells may have importance, at least among human leukocyte antigen (HLA) B35 positive individuals. Vigorous cytolytic response has been shown in an HLA-B35-positive subject against an optimized 9-mer NS1 epitope. In order to determine the number of these NS1-specific CTLs, IFN- $\gamma$  Elispot and HLA-B35 tetramer staining (using the optimized epitope) experiments were carried out: IFN- $\gamma$  Elispot experiments showed a frequency of NS1-specific CD8 $^+$  T cells as high as ~300 spot forming cells/10 $^6$  PBMC, unexpectedly high relative to the HLA-A2 restricted influenza- and Epstein–Barr virus-specific CTLs (Tolfvenstam *et al.*, 2001b). At present, it is not known whether the other HLA class I molecules can present epitopes of NS1, or whether the structural proteins VP1 or VP2 contain CTL epitopes. Therefore, in order to fully understand the nature of B19-specific cytotoxic T-cell immunity, larger groups of subjects (with more HLA-class I alleles) need to be studied, also with B19 structural proteins.

## T-helper cells

T-helper or CD4 $^+$  T cells are essential in antiviral immunity, by participating both directly (cytokines and possible

cytotoxicity) and indirectly (help for B-cells and CTLs) (Guidotti and Chisari, 2001). All T-helper lymphocytes start out as naive IL-2 secreting Th0 cells, which, after being activated, are able to differentiate into mature Th0 cells secreting both IFN- $\gamma$  and IL-4, which can, in the presence of polarizing factors, become increasingly Th1 or Th2-oriented effector cells. In humans this division is not as stringent as in inbred mice, as some human Th1 cells may secrete IL-10.

Th1 cells are the key regulators of cellular immunity, and their main proinflammatory cytokine is IFN- $\gamma$ . Th2 cells, on the other hand, support humoral and regulate Th1-oriented immunity. They are essential in generation of B-cell memory, antibody class switch and affinity maturation. Little or no T-cell help seems to be needed for B-cell memory maintenance, whereas their activation into IgG-secreting cells, again, is Th-cell dependent. The Th2 cytokine IL-10 is particularly important in B19-specific humoral immunity, being a class-switch factor for IgG1, the predominant IgG subclass for B19 (Franssila *et al.*, 1996; Corcoran *et al.*, 2000).

To date, most data on the possible role of B19 specific T-cell functions in clinical manifestations such as arthropathy are indirect, such as detection of cytokine mRNAs or their protein products. IL-4 and IL-10 (Bluth *et al.*, 2003) and IL-1, IL-6, and IFN- $\gamma$  (Wagner *et al.*, 1995; Mitchell, 2002) mRNA expression has been observed in PBMC from recently B19 infected patients. Placentas from women with B19 infection during pregnancy (Anand *et al.*, 1987), contained higher numbers of positive IL-2 CD3 $^{+}$  T cells than did placentas from women with uncomplicated pregnancy (Jordan, 2000). Transforming growth factor-beta gene polymorphism has been correlated with the occurrence of rash (Kerr *et al.*, 2003). Symptomatic B19 infection was found to be associated with HLA-DRB1\*01,\*04, \*07, and HLA-B49 alleles (Kerr *et al.*, 2002). The HLA-B35 allele, reported to be important in presentation of a defined 9-mer NS1 epitope to CTLs (Tolfsenstam *et al.*, 2001b), was carried by 4 of 36 patients with symptomatic B19 infection; however, it was not over-represented in the parvovirus group compared with controls (Kerr *et al.*, 2002).

### NS1-SPECIFIC TH-CELL RESPONSES

Von Poblotzki *et al.* (1996) studied NS1-specific Th-cell proliferation among 10 remotely B19-infected adults, and observed proliferation in only two healthy NS1-seropositive subjects who had extensively worked with this protein.

Mitchell and coworkers (2001) studied remotely, as well as recently B19-infected subjects, and concluded that NS1-specific lymphocyte proliferation correlated with the time of B19 infection rather than with the development of B19 arthropathy. Interestingly, NS1-specific responses were also found among two B19-exposed individuals without VP2 seroconversion and among one VP2-seronegative rheumatology patient (Mitchell *et al.*, 2001).

### TH-CELL RESPONSES AGAINST STRUCTURAL B19 PROTEINS

VP1, VP2, and VP1u-specific Th-cell proliferation was studied among 10 remotely infected adults, and 60 percent showed the strongest proliferation with VP2 (Von Poblotzki *et al.*, 1996). Corcoran *et al.* (2000) studied B19-specific Th-cell responses among children with fifth disease and remotely infected adults. Among the latter, IFN- $\gamma$  responses with VP2 and VP1 were readily detectable, and were  $\sim 3$  times stronger with VP2 than with VP1. By contrast, among the recently infected children the IFN- $\gamma$  responses with the two antigens were equally weak, whereas T-cell proliferation was stronger with VP1 than with VP2. Th2-like cytokines IL-4 and IL-5 were not detected (Corcoran *et al.*, 2000). In another study from this group, B19-specific IFN- $\gamma$  responses of pregnant women were suggested to be attenuated (Corcoran *et al.*, 2003).

Franssila *et al.* (2001), by using sterile, endotoxin-tested recombinant capsids containing VP2 and VP1 in the ratio (2:1) recommended for vaccine use (Bansal *et al.*, 1993), showed vigorous proliferation of CD4 $^{+}$  T cells from both recently and remotely infected adults. Some remotely infected subjects with particularly vigorous responses were classified as 'top responders'. In a subsequent study, the VP1/2 capsids and the VP2-alone capsids induced Th cells from remotely B19 infected subjects to proliferate, and to secrete interferon IFN- $\gamma$  and interleukin IL-10 (Franssila and Hedman, 2004). In most subjects IFN- $\gamma$  secretion was predominant, i.e. the VP-specific Th-cell cytokine responses resembled those described for Th0 clones (Yssel *et al.*, 1992). However, 20 percent B19-seropositive subjects responded with IL-10 secretion alone, i.e. showed Tr1-like responses (McGuirk *et al.*, 2002). No Th-cell activity within VP1u was observed among the remotely infected subjects. The conclusion was that, whereas VP1u contains important epitopes for B cells, VP2 contains the main epitope(s) for Th-cell proliferation, as well as for IFN- $\gamma$  and IL-10 responses (Franssila and Hedman, 2004). Interestingly, VP2-specific Th-cell proliferation has been shown also with rat (Ball-Goodrich *et al.*, 2002) and canine parvoviruses (Rimmelzwaan *et al.*, 1990; Langeveld *et al.*, 1994).

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# Parvovirus oncosuppression

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The frequent isolation of parvoviruses from tumor cells, as contaminants of tumor viruses, from tumor-bearing animals or from animals treated with physical-chemical agents, initially suggested a role of these viruses as etiological agents of cancer (Siegl, 1984; Toolan, 1990). Yet, against this expectation, parvoviruses could never be shown to cause malignant transformation *in vitro* or *in vivo*. On the contrary, some of these agents proved to interfere with the development of tumors in infected laboratory animals, a phenomenon known as oncosuppression (reviewed in Toolan, 1990; Rommelaere and Cornelis, 1991). Parvovirus-associated oncosuppression refers to the *in vivo* situation and can be revealed by any of the following effects:

- prevention of tumor establishment;
- reduction or arrest of tumor growth;
- regression of established tumors;
- diminished take of transplantable tumor cells;
- prolongation of the life of tumor-bearing animals.

Hence, the inhibition of tumor cell growth in culture will not be considered as an oncosuppressive event.

While the antitumor effects of recombinant parvoviruses will be presented in another chapter devoted to parvoviral vectors, the present review will deal more particularly with observations that are relevant to wild-type parvovirus-induced oncosuppression and were published from 1990 onwards. If not further specified, the term parvovirus will refer to the vertebrate autonomous parvoviruses. Although a possible contribution of the immune system was often put forward, the selective killing of malignant cells *in vitro* (oncolysis) was formerly considered as the main cause of autonomous parvovirus-induced oncosuppression (Toolan,

1990). This view is challenged by more recent results providing strong evidence to suggest that, in at least some tumor models, immunomodulation plays a major role in parvovirus oncosuppression (McKisic *et al.*, 1996). In this chapter, we will first describe our current knowledge on the oncolytic and oncotropic properties of certain rodent parvoviruses. Recently reported parvovirus oncosuppressive effects will then be described and contrasted with the oncolysis working hypothesis. Observations that may shed light on alternative parvovirus oncosuppressive mechanisms will finally be discussed, with emphasis on immune reactions. On the basis of these data as a whole, possible explanations for the parvovirus-mediated oncoprotective effects will be proposed in the light of recent progress in the fields of parvovirology and of tumor biology and immunology. From a practical point of view, the continuing investigation of parvovirus-mediated oncosuppression should help not only to assess the potential use of these agents as antitumor medicines, but also to identify the cellular factors that are targets for parvoviruses and may lead to development of novel cancer therapies.

## ONCOTROPISM AND ONCOLYSIS

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A major step in the understanding of the interference of parvoviruses with tumor growth was made by comparing the interactions of rodent parvoviruses, in particular the H-1 virus (H-1PV) and minute virus of mice (MVM), with normal versus (pre)neoplastic cells under *in vitro* culture conditions. The notion of parvoviral oncolysis, already put forward in the early days of parvovirus research (Toolan and Ledinko,

1965), was substantiated in this way by showing that MVM was able to kill simian virus (SV)40-transformed derivatives of mouse fibroblasts which were normally resistant to this virus (Mousset and Rommelaere, 1982). It was subsequently found that a variety of primary tissue cultures or even immortalized cell lines of normal origin resisted parvovirus infection, whereas their equivalents originating from tumors or transformed *in vitro* by genotoxic treatments or specific (viral or cellular) oncogenes were sensitive to the viral cytopathic effects (Rommelaere and Cornelis, 1991). It was therefore hypothesized that at least part of the parvovirus oncosuppression observed *in vivo* was due to a viral toxic activity directed more particularly against tumor cells as compared with their normal progenitors.

### The parvoviral life cycle stimulated in response to malignant transformation

By comparing pairs of normal and transformed cells, it was shown that malignant transformation can have a dramatic influence on the parvovirus life cycle. In particular, viral DNA amplification and gene expression were found to be often increased in transformed cells that were eventually killed as a result of virus infection (Cornelis *et al.*, 1988b). This cell transformation-associated stimulation of parvovirus replication is referred to as oncotropism (Rommelaere and Cornelis, 1991). It is altogether not surprising that viruses such as the autonomous parvoviruses, which strongly rely on the S-phase for their replication, are subject to, and may take advantage of, the cell-cycle perturbations that are characteristic of neoplastic tissues. This parvoviral oncotropism can be explained, at least in part, by the fact that the replication and expression of the viral genome are controlled by cellular factors, which are activated in response to cell transformation.

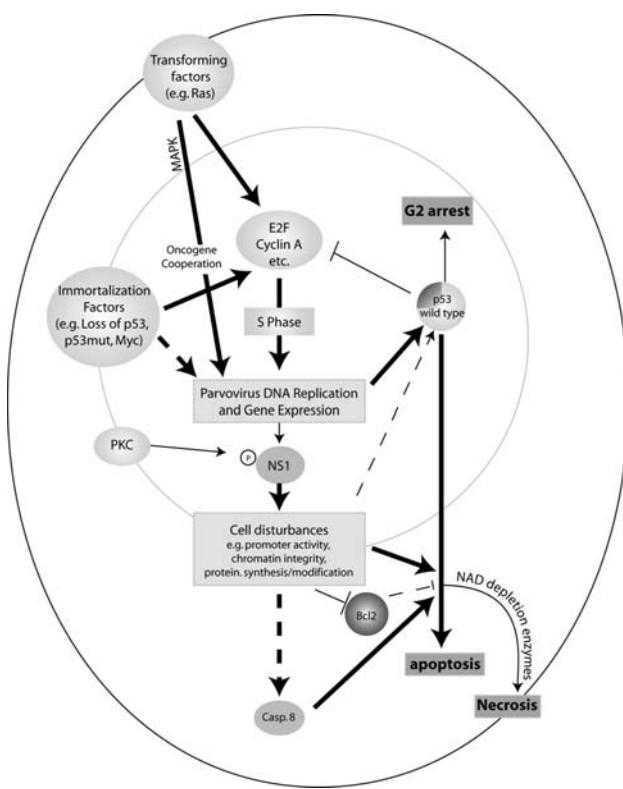
The pivotal parvoviral promoter P4 appears to integrate multiple signals that cooperate in the induction of cell transformation. Activated oncogenes can induce resting cells to enter the mitotic cycle. The G1/S transition follows up the release of high levels of active E2F transcription factors. These were found to be limiting for the activity of the P4 promoter, thereby representing one of the elements responsible for the S-phase-dependence of parvoviruses (Deleu *et al.*, 1998). Yet E2F factors are also activated in normal proliferating cells and are not sufficient to account for the parvovirus oncotropism. Additional mediators were identified through the analysis of the influence of the Ras oncoprotein on the viral life cycle. Ras activation results in the stimulation of multiple cellular pathways. Among these, the MAPK signaling cascade activates transcription factors of the Ets and ATF/CREB families. These transcription factors were shown to bind to specific elements within the early promoter P4 of MVM (Perros *et al.*, 1995; Fuks *et al.*, 1996). These binding sites are conserved between the related H-1PV and MVM viruses. Accordingly, viral gene expression was stimulated to a significant extent in Ras-transformed cells (Spegelaere

*et al.*, 1991). Furthermore, the P4 promoter comprises binding sites for other transformation-sensitive transcription factors, such as USF and NF-Y that are targets for c-Myc and SV40 large T, respectively (Plaza *et al.*, 1991; Gu *et al.*, 1995, 1999). It should also be stated that promoter P4 can be engineered so as to become responsive to transcription factors that are preferentially produced in certain tumors. The substitution of  $\beta$ -catenin/Tcf-binding sites for genuine P4 elements was recently shown to result in a parvovirus construct that is specifically expressed and replicated in colon cancer cells in which the Wnt signaling pathway is activated (Malerba *et al.*, 2003).

Besides gene expression, parvoviral DNA amplification is stimulated as a result of the malignant transformation of various host cells. An oncogene-responsive step in the parvoviral DNA amplification pathway appears to lie in the processing of multimeric DNA replicative intermediates, as shown more particularly in SV40-transformed cells (Kuntz-Simon *et al.*, 1999). The cellular factors involved in this stimulation remain, however, to be identified. It is noteworthy that the conversion of the incoming single-stranded viral DNA into a double-stranded monomeric replicative form, a prerequisite for further viral DNA replication amplification is strictly dependent on cyclin A (Bashir *et al.*, 2000). Given that cyclin A is induced concomitantly with cell entry into S-phase, this dependence can be considered as a primary determinant of the coupling of viral and cellular DNA replication.

As a result of these various regulations, the parvoviral life cycle is strictly dependent on cell proliferation and further up-modulated by specific oncoproteins that induce hyperproliferative and/or other cellular conditions stimulating virus replication and expression (see Figure 25.1). It is unclear, however, whether the striking difference in permissiveness for parvovirus infection, as displayed by some pairs of transformed versus normal proliferating cells, can be fully assigned to the transcriptional and replicational controls discussed above. While the binding of input virions to cell surface receptors was not found to be significantly modulated by transformation in any of the systems analyzed so far, subsequent early steps, such as the intracellular delivery and uncoating of the viral genome, can limit the onset of parvovirus replication and deserve to be further tested for their responsiveness to cell transformation. Even though some of its components remain to be unravelled, the parvoviral oncotropism is marked enough in some systems to result in a significantly greater fraction of transformed (compared with normal) cells that are able to accumulate a threshold amount of parvoviral cytotoxic proteins, as seen at the single-cell level.

This enhanced viral replication and/or expression should not be considered, however, as a universal feature of transformed cells, as it cannot be achieved by all oncogenes (Salome *et al.*, 1990) nor in all target cells (Chen *et al.*, 1986; Van Hille *et al.*, 1989). These variations may be ascribed to differences in the modes of action of distinct oncogenes and to the occurrence of transformation-insensitive barriers to the ongoing parvoviral life cycle. In particular, early



**Figure 25.1** Known positive (blue) or negative (red) cellular regulators of parvovirus DNA replication, gene expression, and cytopathic effects. The parvoviral NS1 protein is regulated through phosphorylation (P) and is essential for parvovirus-induced cell killing, yet its mode(s) of action remain(s) poorly understood. MAPK, mitogen activated protein kinase signaling pathway. →, stimulation; —, inhibition; broken line, hypothetical reaction. See also Color Plate 25.1.

events restricting the onset of parvovirus replication appear to be dependent on the tissue origin of host cells (Tattersall and Bratton, 1983; Wrzesinski *et al.*, 2003). It is noteworthy in this regard that the activation of promoter P4 involves tissue-specific components, at least in the context of transgenic mice harboring P4 in an integrated form (Davis *et al.*, 2003). Therefore, multiple limitations can affect parvovirus replication, some of which may not be overridden by malignant transformation and account for the poor permissiveness and/or sensitivity of some tumor cells to virus infection. Finally, it should also be stated that the oncospecificity of parvoviruses may be controlled not only at the quantitative level (extent of virus replication and expression) but also at the qualitative level (functioning of viral proteins), as discussed below.

## Malignant transformation reveals the cytotoxic potential of parvoviruses

Cell transformation can have dramatic effects not only on the replication and expression of parvoviruses but also on

their cytotoxicity. The identification of the molecular determinants that render a cell sensitive to parvovirus-induced lysis, and of the death pathways that are activated, remain a major challenge for further research. This issue is likely to be of central importance for the understanding of parvovirus oncosuppression. Indeed, cancer cell death will obviously reduce the tumor burden, but it may also initiate the cross-presentation of tumor-associated antigens (TAA) released from dying tumor cells by naive dendritic cells (DCs), a process that is required to achieve antitumor immunity (Arina *et al.*, 2002).

A number of oncogenes, but not all of them were found to sensitize immortalized rat fibroblasts to the lytic activity of parvoviruses (Salome *et al.*, 1990; Legrand *et al.*, 1992). The impact of oncogenes on the susceptibility of low-passage cell cultures to parvovirus infection appears to be more complex. Thus, c-Myc and Ha-Ras applied either alone or in combination, are not sufficient to sensitize rat embryo fibroblasts (REFs) to H-1PV virus-induced killing, even if the combined treatment leads to cell transformation. These oncogenes can only sensitize REFs when they are expressed together with a dominant negative (DN) mutant form of p53 (Telerman *et al.*, 1993); p53 mutations, naturally occurring in more than 80 percent of human tumors, may therefore contribute to make cells vulnerable to parvovirus infection. Indeed, mutations in one or both p53 alleles, which are characteristic of Li-Fraumeni patients and derived tumors, respectively, proved to correlate with the progressive sensitization of human fibroblasts to H-1PV (Cornelis *et al.*, unpublished data). Myc and mutant p53 are both able to immortalize REFs and cooperate with Ras in the transformation of these cells. In contrast, only p53DN can cooperate with Ras in making REFs targets for parvovirus-induced killing. Therefore, mutant p53 functions other than cell immortalization appear to be necessary to increase the susceptibility of host cells to parvoviral cytopathic effects.

Though being an important determinant, the p53 status is certainly not the only clue to parvoviral oncolysis. For example, immortalized human keratinocytes and their tumorigenic ras-transformed derivatives carry mutations in both p53 alleles and are similarly sensitive to H-1PV virus, albeit to a significantly lesser extent than squamous carcinoma cells (Chen *et al.*, 1989). Therefore, additional factors that are associated with oncogenesis and remain to be identified, cooperate with mutant p53 in order to sensitize human skin tumor cells to parvovirus attack. The activity of some of these factors may depend on extracellular signals. In particular, hormones are key players in the outcome of various cancers, and may also have an impact on parvovirus-induced cell death. It is worth noting in this respect that expression of estrogen receptors correlates with an enhanced sensitivity of human mammary carcinoma cells to H-1PV virus (Van Pachterbeke *et al.*, 1997). Moreover, MVM-induced death of Ha-Ras-transformed fibroblasts was found to be interconnected with the thyroid hormone signaling pathway (Vanacker *et al.*, 1993).

Cells that are sensitive to the cytopathic effects of parvoviruses express high levels of viral non-structural proteins. The major non-structural (NS) product NS1 is a multifunctional protein that is required not only for virus replication and expression but also for cytotoxicity (see below). As schematized in Figure 25.1, NS1 is endowed with various activities that may contribute to cell intoxication, yet its exact role(s) in the induction of cell death remain(s) to be unraveled. The accumulation of NS1 proteins in cells bound to die of parvovirus infection, suggests that the above-mentioned cellular determinants of sensitivity may act at least in part by modulating the production of NS proteins (see previous section). Although it is likely to be necessary, this regulation does not appear, however, to be sufficient to bring about cell death. Indeed, NS1 proteins proved to be more or less innocuous to normal cells, even when expressed in amounts that resulted in the death of oncogene-transformed derivatives thereof (Mousset *et al.*, 1994). The intrinsic cytotoxic activity of NS1 therefore appears to be stimulated in response to cell transformation.

One possible explanation may be found in the fact that the replicative and cytotoxic functions of NS1 are both activated through phosphorylation driven by protein kinases of the protein kinase C (PKC) family (Nuesch *et al.*, 1998a, 1998b; Corbau *et al.*, 2000). Interference with NS1 phosphorylation through amino acid substitutions for PKC target residues of NS1 led to the isolation of hypo- and hypertoxic variants of MVMp (Daeffler *et al.*, 2003). It may thus be speculated that the hypertoxicity of NS1 for oncogene-transformed versus non-transformed cells results, at least in part, from differences in the phosphorylation state of the viral product. Although PKC activities are known to be altered in response to cell transformation, proliferation or differentiation, the role of these kinases in the oncoselectivity of NS1 toxic functions remains to be demonstrated.

Another clue to the success of cancer virotherapy lies in the ability of incoming virions to multiply and spread within the tumor mass, thereby allowing the oncolytic effect to be propagated and eventually hit all neoplastic cells. This spreading of the parvoviral oncolytic activity represents another level of heterogeneity among different tumors. Indeed, high sensitivity to parvovirus-mediated killing does not always correlate with high capacity for progeny virus production, as exemplified by the human myeloid leukemia cell line U937 (Lopez-Guerrero *et al.*, 1997; Rayet *et al.*, 1998). Upon infection at relatively low multiplicities mimicking the *in vivo* situation, such sensitive but poorly productive cell cultures undergo a growth delay resulting from the death of primarily infected cells, but are subsequently overgrown with the cells that escaped the initial inoculum and run little risk of secondary infection. This is, for example, the case of many colon carcinoma cell lines, which were found to be very poor wild-type H-1PV producers (Malerba *et al.*, 2003). Corresponding tumors *in vivo* are expected to be somewhat retarded by parvovirus infection, but eventually relapse owing to the failure of virus to be propagated, unless the

primary oncolytic effect triggers an antitumor immune response that can relay the virotherapeutic treatment. The greater the capacity of target tumor cells for producing parvovirus bursts, the higher the probability that a significant tumor suppression can be achieved. Among the few low passage tumor cells tested so far, human gliomas appear to represent a promising system since all samples analyzed proved to be sensitive to H-1PV virus-induced killing, while some were competent for virus production (Herrero *et al.*, 2004). This analysis should be extended to a wide spectrum of gliomas and other tumors in order to identify the most suitable cancer targets for parvovirus-mediated therapy.

### Multiple cellular death pathways can be activated by parvovirus infection

Parvoviruses were found to exert both cytostatic and cytotoxic effects. A number of molecular disturbances were detected in parvovirus-sensitive cells expressing NS1 proteins, which may contribute in a way or another to impair cell proliferation and survival. These perturbations include the transregulation of cellular promoters (Legendre and Rommelaere, 1992; Vanacker *et al.*, 1993), the induction of single-stranded DNA breaks in the host cell genome (Op De Beeck and Caillet-Fauquet, 1997), and the alteration of cellular protein synthesis and phosphorylation (Anouja *et al.*, 1997). The respective roles, if any, of these molecular disturbances in the eventual killing of infected cells, remain to be assessed. Another open question is whether the specificity of these NS1-induced cellular perturbations for parvovirus-sensitive (transformed) cells results from the modulation of NS1 activity (see above) or of some of the cellular targets of the viral product.

Parvovirus infections have been shown to induce apoptosis or apoptosis-like cell death (Ohshima *et al.*, 1998; Rayet *et al.*, 1998; Ran *et al.*, 1999; Sol *et al.*, 1999; Ueno *et al.*, 2001). Oncogene activation is tightly linked with the regulation of this type of cell death, and transformed cells often protect themselves from apoptotic stimuli. Two major apoptotic pathways have been reported. The mitochondrial pathway is triggered by DNA damage and/or induction of p53, requires caspase 9 activation and is inhibited by Bcl2. The second pathway is activated by binding of a death-ligand to its death-receptor, and is mediated by caspase 8. These two processes are not exclusive, and activation of the death-ligand pathway can be amplified by the mitochondrial one. Both pathways converge to activate the death effector caspase 3. As depicted in Figure 25.1, evidence was obtained to indicate that parvoviruses can induce either pathway, depending on the target cells.

Through the structure of their genome, parvoviruses have been suggested to be potential triggers of a DNA damage response. Genotoxic stress activates p53, which induces either cell cycle arrest – allowing DNA repair to occur – or apoptosis, depending on the extent of damage. Neutralization of

p53 protects most cancer cells from this pathway, unless damage is too important and cells die from an abnormal mitotic division. In agreement with the view that parvoviruses cause and/or are recognized as DNA damage, infection of rodent cells with MVMp results in a cell-cycle arrest that is p53-dependent (Op De Beeck *et al.*, 2001). Interestingly, rat parvovirus infection of thymic lymphoma cells results in the down-modulation of Bcl 2, an inhibitor of p53-dependent apoptosis, and cells resistant to this virus are also resistant to gamma radiation-induced DNA damage (Ueno *et al.*, 2001). Furthermore, H-1 virus infection of C6 glioma cells – which carry functional p53 – induces caspase 3-dependent apoptosis (Ohshima *et al.*, 1998). Unfortunately the role of p53 in parvovirus-induced cell death was not investigated in the latter studies. Recent work on the related group of defective adeno-associated viruses (AAV) is worth mentioning in this context. In the absence of expression of any viral proteins, AAV genomes in high copy number were hypothesized to provide structures that presumably mimic damaged DNA signals and induce host cells to either stop growing or undergo apoptosis, depending on whether they contain functional p53 or not, respectively (Raj *et al.*, 2001). Although this response may also take place to some extent in cells infected with autonomous parvoviruses, it is unlikely to represent a major component of the cytotoxic effects discussed above. Indeed, cell killing by autonomous parvoviruses was observed at relatively low multiplicities of infection, and could be reproduced through the expression of viral NS proteins out of the context of the viral genome (Caillet-Fauquet *et al.*, 1990). It can thus be assumed that the cytotoxic NS proteins play a major role in the activation of the death program in permissive cells infected with autonomous parvoviruses. Whether these viral proteins act by damaging chromatin (Op De Beeck and Caillet-Fauquet, 1997), inducing pro-apoptotic factors or down-regulating inhibitors of apoptosis, is presently a matter of speculation. It is noteworthy that NS1 was reported to interact with a p53 co-activator and may in this way modulate the death response by affecting the transcriptional activity of p53 (Ohshima *et al.*, 2001). Besides p53, the c-Myc oncoprotein promotes apoptosis through the mitochondrial, caspase 9-mediated pathway (Juin *et al.*, 1999), while being also required for some caspase 8-dependent processes (Aza-Blanc *et al.*, 2003; Klefstrom *et al.*, 2002). Whereas c-Myc was found to sensitize immortalized rat fibroblasts to the killing effect of parvoviruses (Salome *et al.*, 1990), it failed to do so in low-passage cultures of ras-transformed rat embryo fibroblasts (Telerman *et al.*, 1993). Furthermore, c-Myc is down-regulated upon H-1 virus infection of highly sensitive leukemic cells (Lopez-Guerrero *et al.*, 1997; Rayet *et al.*, 1998), questioning its contribution to parvovirus-induced death. There is thus need for a careful investigation of the impact of p53, Myc and other mediators of apoptosis on the death program activated by parvoviruses.

Even in the absence of functional p53, tumor cells are particularly sensitive to the induction of apoptosis via

the death-receptor pathway. Activation of this caspase 8-dependent pathway by the death-ligand TRAIL can lead to the killing of tumor cells that are resistant to the genotoxic stress-induced death response (Sheridan *et al.*, 1997). In some cell systems, parvoviruses have been shown to activate the caspase 8-mediated pathway independently of the binding of death-ligands (e.g. TNF, Fas ligand, TRAIL) to death-receptors (Ohshima *et al.*, 1998; Rayet *et al.*, 1998; Sol *et al.*, 1999; Ueno *et al.*, 2001). Although the underlying activation mechanism is presently unknown, this function of parvoviruses is intriguing as it may account for some of their oncolytic properties. It should also be stated that the induction of apoptosis in parvovirus-infected cells may end up in a necrotic cell death, in particular under conditions in which the intracellular nicotinamide adenine dinucleotide (NAD) pool gets depleted (Ran *et al.*, 1999).

The outcome of the infection of susceptible cells with parvoviruses is therefore complex, with varying contributions of growth arrest, apoptosis, and necrosis, depending on the host cells and experimental conditions used. Tumors often develop mechanisms allowing them to escape death-ligand or damage-induced killing. The capacity of parvoviruses to activate distinct death pathways is interesting in this respect, as it should decrease the risk of these viruses being affected by resistance mechanisms. Moreover, parvoviruses may be able to overcome some of the death escape processes acquired by tumors, or to induce alternative death pathways. Further work is necessary to determine whether parvoviruses are endowed with such properties and can indeed cause the lysis of tumor cells that resist conventional anticancer treatments.

## ONCOSUPPRESSIVE EFFECTS OF PARVOVIRUSES

Parvoviruses were found to protect laboratory animals from tumor development under four types of experimental conditions:

- Animals infected with parvoviruses at birth show a much reduced incidence of both spontaneous and virally- or chemically-induced tumors during their life span. The extent of protection achieved and large numbers of animal used make these data highly significant.
- Parvovirus-carrying animals become resistant to syngeneic or heterologous tumor grafts.
- There is a lower probability that neoplastic cells, infected *in vitro* with parvoviruses, form tumors after implantation in recipient animals.
- Established tumors can be suppressed through the infection of tumor-bearing animals with parvoviruses.

Relevant observations made during the last decade concern the latter three conditions and are listed in Table 25.1. For earlier reports, readers are referred to previous reviews (Toolan, 1990; Rommelaere and Cornelis, 1991).

**Table 25.1** Interference of wild-type parvoviruses with tumor formation and growth.\* Experimental conditions

Parvovirus	Infection route	Target	Recipient	Antitumor effects	References
<b>A. Infection of animals prior to tumor grafting</b>					
MPV1	ip	Myeloma	Mice	Rejection	(McKisic <i>et al.</i> , 1993)
MPV1	ip + on	Sal allogenic sarcoma	Balb/c mice	Accelerated rejection	(McKisic <i>et al.</i> , 1996)
RPV1	on	Leukemia	Rats	Reduced growth, milder disease	(Ball-Goodrich <i>et al.</i> , 1998)
<b>B. Ex vivo infection of tumor cells</b>					
H-1PV	<i>in vitro</i>	HeLa human cervical carcinoma	NUDE Swiss CD1 mice	Reduced incidence	(Haag <i>et al.</i> , 2000)
MVMp	<i>in vitro</i>	B78 syngenic melanoma	C57B1/6 mice	Delayed onset	(Giese <i>et al.</i> , 2000)
MVMp	<i>in vitro</i>	HSV syngenic endothelioma	C57B1/6 mice	Slower occurrence of primary lesions and reduced incidence of metastases	(Giese <i>et al.</i> , 2002)
MVMp	<i>in vitro</i>	P815 syngenic mastocytoma	DBA/2 mice	Reduced incidence and induction of antitumor immunity	(Lang, 2003)
<b>C. Infection of individuals/animals bearing pre-established tumors</b>					
H-1PV	it	Cutaneous metastases	Cancer patients	Not significant	(Le Cesne <i>et al.</i> , 1993)
H-1PV	it	HeLa human cervical carcinoma	SCID Balb/c mice	Dose-dependence regression	(Faisst <i>et al.</i> , 1998)
MVMp	it	B78 syngenic melanoma	C57B1/6 mice	Growth retardation	(Giese <i>et al.</i> , 2000)
MVMp	it + ip	HSV syngenic endothelioma	C57B1/6 mice	Not significant	(Giese <i>et al.</i> , 2002)
MVMp	it	P815 syngenic mastocytoma	DBA/2 mice	Growth retardation	(Lang, 2003)
H-1PV	iv (virus or carrier cells)	MH syngenic lung metastases	ACI rats	Reduced incidence	(Raykov <i>et al.</i> , 2004)

\* Data reported from 1990 onwards.

Abbreviations: it, intra-tumoral; ip, intraperitoneal; iv, intravenous; on, oronasal.

## Protection of parvovirus carriers from tumor grafts

As indicated in Table 25.1A, recently identified lymphocytotropic parvoviruses of mouse (MPV1) and rat (RPV1) origin were found to interfere with the take of tumor grafts in pre-infected animals by inducing or accelerating the rejection of implanted cancer cells, and preventing these from developing into neoplastic lesions. More importantly, allogeneic sarcoma cells, which were rejected more efficiently *in vivo* as a result of the preinfection of recipient mice with MPV1, proved to be fully resistant to this virus under *in vitro* conditions (McKisic *et al.*, 1996). Although it cannot be ruled out that the tumor cells became sensitive to MPV1 in the animal context, this observation provides strong evidence to suggest that in some systems, mechanisms other than direct oncolysis play a major role in parvovirus oncosuppression, such as the modulation of host anticancer immune responses. This interpretation was substantiated by showing that the enhanced rejection of tumors (and also syngeneic

skin transplants) by MPV1-infected mice is T cell-dependent (McKisic *et al.*, 1996; McKisic *et al.*, 1998).

## Reduced tumorigenic potential of neoplastic cells infected *ex vivo* with parvoviruses

A number of cancer cells were tested for their ability to form tumors in recipient animals after being infected (or not) with parvoviruses immediately prior to implantation. As shown in Table 25.1B, this *ex vivo* application of parvoviruses interferes with oncogenesis, yet to various extents ranging from a mere delay in the onset of tumor development to a long-lasting suppression of tumor appearance. A correlation can be made between these anticancer effects and the toxicity of involved parvoviruses for their respective tumor cell targets. It should be stated, however, that the parvoviral oncolytic activity is unlikely to be the only key to these oncosuppressive effects. It may indeed be speculated that by intoxicating neoplastic cells, parvoviruses will promote the release

of tumor-associated antigens and immunomodulating cytokines, thereby priming innate and/or acquired immune responses that are directed against cancer cells and can relay the initial viral oncotoxicity (see section below). In support of this possibility, tumor remnants from H-1PV-infected HeLa cells implanted in nude mice were found to express markers (e.g. granzyme A) that are indicative of the recruitment of natural killer (NK) cells in which the death program is activated (Haag *et al.*, 2000). This represents the first evidence to suggest that parvovirus infection of tumor cells may not only result in direct oncolysis, but also elicit bystander effects through the activation of components of the immune system. This conclusion was substantiated by analyzing the consequence of the infection of H5V endothelioma cells with MVMp for their tumor-forming ability in syngeneic mice. The H5V system is especially interesting in that it is under the tight control of the immune system, which leads primary lesions to first regress before they take over and recur concomitantly with the appearance of multiple distant metastases. *Ex vivo* infection of H5V cells with MVMp results in the long-lasting suppression of primary lesions recurrence and, more importantly, of metastases formation, together with the infiltration of stagnating tumors with activated T and NK cells (Giese *et al.*, 2002). These observations argue again for the involvement of an immune component in parvovirus oncosuppression. A further evidence in favor of this possibility lies in the fact that immunocompetent mice remaining tumor-free after implantation of MVMp-infected Ehrlich ascitic cells (Guetta *et al.*, 1986; our unpublished results) or P815 mastocytoma cells (Lang, 2003) become resistant to challenges with naive tumor cells. It can thus be concluded that at least in some of the systems tested, the *ex vivo* parvovirus infection protocol mimics an autologous tumor cell vaccination procedure, whereby the patient's own neoplastic cells are manipulated *in vitro* before getting reinjected in the donor in order to raise immunity against the residual primary tumor and its metastases (Ward *et al.*, 2002). The failure to achieve a long-term protection against certain tumors (e.g. B78/H1 melanomas) through their *ex vivo* treatment with parvoviruses may be assigned to their low infectivity and/or immunogenicity (Giese *et al.*, 2000). Yet, the use of recombinant parvoviruses transducing appropriate cytokines should help to induce antitumor immune responses under conditions in which the wild-type virus is inefficient (see Chapter 44).

### Suppression of established cancers through parvovirus infection of tumor-bearing individuals

Parvoviruses can also inhibit the growth or even induce the regression of pre-existing tumors after *in vivo* administration (Table 25.1C). In some of the tumor models tested, such as syngeneic ascites in immunocompetent mice (Guetta *et al.*, 1986; Cornelis *et al.*, unpublished data), HeLa xenografts in

severe combined immunodeficient (SCID) mice (Faisst *et al.*, 1998) or allogeneic gliomas in immunocompetent rats (Geletneky *et al.*, unpublished data), spectacular regressions were observed. However, in several other systems, intraperitoneally or intratumorally injected wild-type parvoviruses proved to have a more limited protective effect in immunocompetent animals, causing at best a transient slowing down of tumor growth but no permanent cure (Kimsey *et al.*, 1986; Giese *et al.*, 2000, 2002). Similarly, no significant modification of the size of cutaneous metastases was achieved through injection of H-1PV in cancer patients, as part of a phase I clinical study aimed to assess tolerance for this virus (Le Cesne *et al.*, 1993). The probability that the parvovirotherapy will fail, increases with the size of tumors at the time of virus injection, indicating that the oncolytic and immunostimulating activities of parvoviruses can be overwhelmed when the tumor burden is too big. For instance, the outcome of MVMp infection of B78/H1 melanoma-bearing mice consists in the regression, transient growth arrest or continuing development of neoplastic lesions, depending on whether the tumors are small (<2 mm in diameter), medium (2–5 mm) or large (>5 mm) when animals get infected, respectively (Giese *et al.*, 2000, and unpublished data). Similarly, the rate of cure of human mammary carcinoma xenografts in nude mice treated with H-1PV was found to drop when the virus treatment was delayed until tumors reached a large size (Dupressoir *et al.*, 1989). Therefore, parvoviruses are not always winning in the race between the proliferation of tumor cells and the multiplication of viruses or mobilized defense cells.

The varying susceptibility of distinct tumors to parvovirotherapy may be assigned to differences in their permissiveness for virus replication and cytopathic effect (see above) and/or in their immunogenicity. In addition, the intratumoral dissemination of viruses, the accessibility of neoplastic cells to immune effector cells, the infiltration of tumors with non-malignant cells trapping free viruses, and the generation of virus-specific neutralizing antibodies, may all represent limiting factors for a successful parvovirus-based cancer therapy and vary from one system to the other (Jacoby *et al.*, 1996; Ball-Goodrich *et al.*, 2002; Lang *et al.*, 2002). For example, the inefficiency of MVMp in suppressing pre-established H5V endotheliomas (Giese *et al.*, 2002) may be due, at least in part, to the recruitment of a majority of host non-malignant cells in these opportunistic lesions, which may sequester viruses and prevent them from reaching tumor cells. Mouse P815 mastocytoma cells and Ehrlich ascites tumor cells are both very sensitive to MVMp infection and immunogenic, yet repeated parvovirus infections into established subcutaneous P815 tumors only retarded their growth, whereas the intraperitoneal injection of MVMp resulted in the full regression of ascites tumors (Guetta *et al.*, 1986; Lang, 2003; Cornelis *et al.*, unpublished data). This difference may tentatively be ascribed to the higher accessibility of ascites tumor cells to infecting viruses, and the presence of large numbers of leukocytes in ascitic

fluids. It should be stated that the oncosuppressive effect of MVM on ascites tumors requires infectious virus that is endowed with a cell tropism allowing it to replicate in these cells (Guetta *et al.*, 1986). It is worth noting that parvoviruses can be administered to tumor-bearing animals not only in the form of free particles but also through carrier cells. It was recently reported that lethally irradiated cells sustain a prolonged production of parvoviruses and can be used to deliver virions locally, in particular to sites of tumor formation (Raykov *et al.*, 2004). The interest of this approach lies in the fact that carrier cells may protect viruses against circulating antibodies and be endowed with homing properties, which lead them to deliver viruses to nascent metastases. Indeed, the carrier cell-mediated administration of H-1PV proved to be more efficient than the direct virus injection, as regards suppression of hepatoma cell metastases in the lungs of immunocompetent rats (Raykov *et al.*, 2004).

## **CONTRIBUTION OF ONCOLYSIS TO PARVOVIRUS-MEDIATED ONCOSUPPRESSION**

As stated above, parvovirus infection of tumor-bearing animals induces in certain cases the regression of neoplastic lesions. This cure is accompanied by the destruction of neoplastic tissues, which may be indicative of the *in vivo* occurrence of the parvoviral oncolytic effects observed in cell cultures (see previous section). However, this interpretation is complicated by the fact that under *in vivo* conditions, infected tumors become infiltrated with non-malignant cells, in particular immune effectors such as macrophages, NK, and cytotoxic T cells. Indeed, the lymphoid cell death program was found to be activated in parvovirus-infected human carcinoma (Haag *et al.*, 2000) and mouse endothelioma (Giese *et al.*, 2002) cell implants in nude and immunocompetent mice, respectively. These observations raise the question whether dead neoplastic cells, seen in necrotic areas of tumor remnants, were killed as a result of their infection with parvoviruses and/or their interaction with effectors cells of the innate or acquired immune system. The overall tumor regression is likely to involve both virus and cell-mediated cytolytic components in proportions that depend on the target tumor and host animal. Viral oncolysis can be assumed to be more particularly important in immunodeficient animals (SCID and nude mice) carrying parvovirus-sensitive tumors (Dupressoir *et al.*, 1989; Faisst *et al.*, 1998). Yet, even in this situation, host innate defense mechanisms may play a significant role, preventing the contribution of direct parvoviral oncolysis from being definitely assessed. Indications of the role of viral cytoidal effects in oncosuppression are therefore indirect and primarily based on the above-mentioned sensitization of many transformed cells to the lytic activity of parvoviruses. Furthermore, H-1PV replication and gene expression (in particular production of cytotoxic NS proteins) have been

demonstrated in human tumor xenografts from infected immune-compromised mice (Dupressoir *et al.*, 1989; Faisst *et al.*, 1998) and in metastases from infected cancer patients (Le Cesne *et al.*, 1993). Given that these viral parameters can be correlated with cytotoxicity *in vitro* (Cornelis *et al.*, 1988a; Chen *et al.*, 1989; Salome *et al.*, 1990), their detection in neoplastic lesions constitute strong evidence to suggest that parvovirus oncolysis takes place at least to some extent in infected tumors.

## **INTERACTION OF PARVOVIRUSES WITH THE IMMUNE SYSTEM: CONTRIBUTIONS TO ANTITUMOR IMMUNITY**

The immune response against viruses is generally governed by strong innate as well as cellular immune reactions. A series of specific immune effector mechanisms, together with non-specific defense systems, are called into play to eliminate infecting viruses. Antibodies specific for viral surface antigens are often crucial in containing virus spread during acute infection and protecting against reinfection. However, cell-mediated responses are also important in order to limit virus replication. Yet, viruses are not only targets but also potential modulators of the immune system. In the following paragraphs, the findings of the last few years pointing to a cellular and innate immune component particularly in rodent parvovirus-mediated oncosuppression and possible mechanisms will be discussed. We will summarize the milestones regarding the effects of parvovirus infection on the immune system and end with some data supporting the intriguing possibility of parvoviruses to be used as specific inducers of antitumor immunity.

Many parvoviruses target cells of the hematopoietic system, namely lymphoid, myeloid, and erythroid committed progenitors (Studdert, 1990), and persistent immune dysfunctions are a common trait of infection with these viruses (Jacoby *et al.*, 1996). This can be exemplified with the Kilham rat virus (KRV) which can establish persistent infection in lymphoid organs such as the spleen, thymus, and lymph nodes (Brown *et al.*, 1993), and modulate the immune function of lymphocytes *in vivo* and *in vitro* (McKisic *et al.*, 1995; Chung *et al.*, 2000). Infection with KRV decreases lymphocyte viability and suppresses proliferative responses to alloantigens (Campbell *et al.*, 1977). Further studies indicated a powerful immunosuppressive function of KRV, which eventually led to the induction of autoimmune diabetes in the otherwise diabetes-resistant strain of Bio-Breeding/Worcester rats (Guberski *et al.*, 1991; Brown *et al.*, 1993; Chung *et al.*, 2000). While the impact of this virus on the host's immune system is well documented, there is only circumstantial evidence of a cellular immune response towards KRV. However, perivascular mononuclear cell infiltrates have been found in infected tissues indicative of a cellular immune response (Jacoby *et al.*, 1987; Gaertner *et al.*,

1993), although a clear analysis of the specific cells was not performed. If a cellular immune component exists, however, it could explain the finding that KRV was able to persist in rats inoculated at 6 days of age in spite of the development of high anti-RV antibody titers (Jacoby *et al.*, 2000). Furthermore, a series of studies have been undertaken, which suggest an important role of T cells in the viral clearance (Gaertner *et al.*, 1995, 1996; Jacoby *et al.*, 2000). In a recent publication, experiments were performed to identify humoral and cellular responses to the VP2 capsid protein during experimental infection of adult rats (Ball-Goodrich *et al.*, 2002). VP-2 specific proliferation, interferon- $\gamma$  production, and IgG2a humoral response were induced following KRV infection, all of which is indicative of a virus-specific Th1-mediated immune reaction. This finding is of particular interest in regard to the work being done with rats as models for autoimmune diseases. While the established immune response is virus specific, the Th1 environment, represented by high interferon- $\gamma$  levels, may affect immune responses to other antigens. This could be exemplified in the rat model for diabetes showing that KRV infection selectively activated Th1-like CD4 $^{+}$  cells (Chung *et al.*, 2000). Others (Zipris *et al.*, 2003) found that infection with KRV, but not with the closely related H-1PV, altered T-cell regulation in BBDR rats and permitted the expression of autoimmune diabetes. Although infection with either KRV or H-1PV elicited a weak CD8 $^{+}$  T cell response, only KRV infection caused a substantial decrease in splenic CD4 $^{+}$  CD25 $^{+}$  regulatory T cells and therefore a potential loss of tolerance. This would suggest a mechanism whereby a previously tightly controlled genetic predisposition may shift to declared autoimmunity in response to an environmental perturbation, namely the failure to maintain regulatory CD4 $^{+}$ CD25 $^{+}$  T-cell function. These studies clearly showed a propensity of some of these viruses to elicit autoimmune reactions. Whether the VP1-related phospholipase A2 activity common to all known members of the parvovirus family contributes to these reactions (Zadori *et al.*, 2001) remains to be investigated.

MVMp, the prototype strain of MVM, appears to be devoid of pathogenic activity in mice even if infection takes place at the neonatal stage (Brownstein *et al.*, 1992; Jacoby *et al.*, 1996). This virus is considered to have a fibrotropic host range *in vitro*. However, there is little information evaluating the *in vivo* distribution of MVMp (Kimsey *et al.*, 1986). Based on the observation that in infected mice MVMp expression has a predilection for myeloid DC and a specialized subset of B cells, the so-called B1 B cells (Raykov *et al.*, 2005), the view that this virus is devoid of lymphocytotropism must be taken with caution. These authors showed by reverse transcriptase polymerase chain reaction (RT-PCR) analysis that MVMp RNA was ubiquitously expressed in several lymphoid organs of *in vivo* treated C57/BL6 mice, such as thymus, spleen, and lymph nodes, but also lung, heart, liver and kidney after *in vivo* infection (N. Giese, personal communication). The infection of B1 B cells by MVMp

is noteworthy considering the role of these cells in autoimmune diseases (Okamoto *et al.*, 1992; Hirose *et al.*, 1993). However, MVMp has so far not been shown to exhibit any immunosuppressive properties, while eliciting antiviral immunity (Guetta *et al.*, 1986). Interestingly, the immune responses triggered by parvoviruses in *in vivo* treated mice could be distinguished from those usually seen upon virus infection. The innate side of the immune system showed weak activation of NK cells (Haag *et al.*, 2000), while on the acquired side, neutralizing antibodies (IgM, IgG) were detected in serum, however, only an insignificant virus-specific cytotoxic T lymphocyte (CTL) response was induced in C57/BL6 mice (Lang *et al.*, 2002). Like MVMp, the lymphotropic MVM variant – designated MVMi – fails to modulate T-cell functions in mice, which have been infected as adults (Kimsey *et al.*, 1986). MVMi was isolated from murine lymphoma EL-4 cells and shown to be immunosuppressive for murine lymphocytes *in vitro* (Bonnard *et al.*, 1976). Specifically, MVMi suppressed both mitogen- and antigen-induced lymphocyte proliferation, abrogated the generation of CTL in a primary mixed lymphocyte culture, and inhibited T cell-dependent B lymphocyte responses *in vitro* (Bonnard *et al.*, 1976; Engers *et al.*, 1981). The inhibition of T-cell effector functions by MVMi has been attributed to its lymphocytotropic and lytic nature. In newborn mice, MVMi infection mediates a mild reduction of hematopoietic committed precursors (Segovia *et al.*, 1995) and an involution of hematopoietic foci (Brownstein *et al.*, 1991). In adult immunodeficient SCID mice, this virus causes a persistent bone marrow infection leading to a lethal leucopenia with dysregulated erythropoiesis and megakaryopoiesis (Segovia *et al.*, 1999; Lamana *et al.*, 2001).

Recently, it was shown that MVMi suppresses short- and long-term lymphohematopoietic repopulating cells in persistently infected mice (Segovia *et al.*, 2003). The hematopoietic disorders caused by MVMi in mice may provide insight into the human B19-associated diseases in man under different immunocompetent and developmental conditions.

The mouse parvovirus-1 (MPV-1) was originally isolated from a mouse CD8 $^{+}$  T-cell clone (McKisic *et al.*, 1993). Although MPV-1 infects cloned CD4 $^{+}$ , CD8 $^{+}$ , and  $\gamma\delta$  T cells *in vitro*, so far it is not known whether the virus has a predilection for specific lymphoid subsets *in vivo*, as has been suggested for Aleutian disease virus of minks (Mori *et al.*, 1991) and MVMp (see above). MPV-1 replicates productively in cloned T cells *in vitro* and suppresses their proliferative response to IL-2 or antigen, but does not appear to inhibit the generation of cytotoxic T cells in mixed lymphocyte cultures (McKisic *et al.*, 1993). It was confirmed that MPV-1 is lymphocytotropic *in vivo* (Jacoby *et al.*, 1995) and in this context, further studies indicated that MPV-1 perturbs immune responses of Balb/c mice to ovalbumin and accelerated tumor allograft rejection (McKisic *et al.*, 1996). MPV-1 infections also exerted immunomodulatory functions in a non-neoplastic allograft rejection model (McKisic *et al.*, 1998). Therein, MPV-1 infection potentiated

the rejection of allogeneic skin grafts and even induced the rejection of syngeneic skin grafts. These data suggest that infection with MPV-1 may disrupt normal mechanisms of peripheral tolerance causing the development of autoimmune responses.

The findings summarized above show that parvovirus infections are able to elicit cellular and innate immune responses. Since some parvoviruses are endowed with oncotropic properties, this response may contribute to eliminate infected tumor cells in which the viral cytolytic program failed to go to completion. The question also arises whether parvoviruses may increase the immunogenicity of infected tumor cells through other mechanisms besides the expression of viral antigens. Ensuring that an immune response directed against infected but also non-infected tumor cells relays the initial parvoviral oncolytic effect would be of high therapeutic benefit. By inducing the lysis of neoplastic cells, parvoviruses can be expected to promote the release of tumor-associated antigens (TAA) and the subsequent loading of antigen-presenting cells (APC). The additional possibility is worth considering, that even in the absence of cell lysis, parvoviruses induce cellular phenotypic changes that stimulate innate or acquired immune reactions targeted at neoplastic cells. Further investigation of the viral cytopathic effects is clearly needed to assess the impact of parvovirus infection of tumor cells on their interactions with macrophages and DC, which is critical for the eventual induction of antitumor immunity. The principle that pathogens can provide innate immune cells with signals that subsequently direct adaptive immunity is well established (Janeway and Medzhitov, 2002). However, precisely how the system detects tissue damage and other forms of stress remains unclear. Likely candidates are the stress-inducible heat shock proteins (HSPs). HSPs are abundant soluble intracellular proteins that are present in all cells, but are still enriched in tumor cells. Members of the HSP family bind peptides, including antigenic peptides generated within cells. HSPs also interact with APC through CD91 and other receptors, eliciting a cascade of events including re-presentation of HSP-chaperoned peptides by MHC, nuclear translocation of NF- $\kappa$ B and maturation of DC. These consequences point to a key role of HSP in fundamental immunological phenomena such as activation of APC, indirect presentation (or cross-priming), and chaperoning of peptides during antigen presentation (Srivastava, 2002). Parvovirus infection appears to stimulate cells to release distress signals such as specific HSP (Moehler *et al.*, 2003), which are known to generate 'danger signals' favorable to the activation of the host immune response (Blachere *et al.*, 1997; Srivastava, 2000). Therefore, the interaction of parvoviruses with tumors may contribute not only to the destruction of some neoplastic cells, but also to the stimulation of antitumor immune responses. This possibility is supported by recent *in vitro* cross-presentation experiments indicating that H-1PV infection and resulting killing of human melanoma cells led to loading of DC with TAA, antigen cross-presentation, and

activation of tumor-specific CTL. Uninfected control melanoma cells were ineffective in this respect (Moehler *et al.*, 2005). Mechanistically, these studies suggest that parvovirus-induced tumor cell lysis may lead to the cross-presentation of tumor antigens by DC and the generation of tumor-specific CTL. Whether initiated by viral oncolysis and/or virus-activated innate immune cells, this scenario would account for the long-term protection of virus-treated animals against tumor cell challenges, in the absence of detectable viral imprints (Guetta *et al.*, 1986).

In conclusion, successful destruction of neoplastic lesions using various immunotherapeutic approaches was shown to be accompanied with autoimmune reactions, as exemplified by skin depigmentation from melanocyte destruction in the case of melanoma treatment in humans and mice (van Elsas *et al.*, 1999; Dudley *et al.*, 2002). This argues for the possibility that the competence of parvoviruses for inducing such conditions contributes to the antineoplastic activity of these agents, in particular when infection takes place prior to the induction of tumor formation. A fairly recent publication (Millar *et al.*, 2003) would support the concept that stress induction by parvoviruses via the release of inducible HSP could promote not only antigen-presenting cell functions but also conversion of T cell tolerance to autoimmunity *in vivo*. This in turn would lead to the recognition of tumor cells as foreign with their subsequent rejection.

## CONCLUSION AND PROSPECTS

A number of parvovirus-induced oncosuppressive effects were reported during the last 15 years. These studies confirmed previous observations about parvoviral anticancer properties and extended them to new parvoviruses and other experimental conditions. Though often purely phenomenological, available data convincingly demonstrate that some parvoviruses can suppress tumors in which they fail to replicate and which resist their cytopathic effects. Under such conditions, the anticancer properties of parvoviruses appear to be mediated by the immune system. Besides having an immune-modulating activity, parvoviruses are endowed with oncotropic and oncolytic properties that are also likely to contribute to tumor suppression to an extent that may vary depending on the virus and target tumor. While a few neoplastic lesions proved to be very sensitive to parvovirus-induced suppression, a number of established solid tumors were found to be rather resistant in that their growth was somewhat impaired but no curative effect (regression) was achieved upon virus infection. These limited successes account for present efforts to design mutant and recombinant parvoviruses that are more efficient than wild-type viruses in the suppression of the latter tumors.

Regarding the oncotropic properties of parvoviruses, a tight link was shown to exist between, on the one hand the

activation of certain oncogene(s) and/or the inactivation of antioncogene(s) and, on the other hand, the progress of the viral life cycle. This interrelation appears to take place on at least three levels:

- Immortalizing and transforming cellular factors have a direct or indirect impact on parvovirus gene expression and DNA replication, allowing cytotoxic NS proteins to accumulate and attain threshold levels necessary for the killing of infected cells.
- Furthermore, host cell transformation results in the functional modulation of the viral NS proteins (possibly through phosphorylation, which is known to stimulate NS replicative and cytopathic functions).
- It also results in the activation of cellular death pathways that can be mobilized by the virus.

Altogether, these effects are thought to contribute to the higher cytotoxic index of parvoviruses in transformed versus normal cells (oncolysis).

Functional studies proving the involvement of the innate as well as the adaptive branches of the immune system in tumor rejection are still outstanding. On the one hand, certain autonomous parvoviruses appear to be lymphotropic and to induce the development of a physiological milieu predisposing to autoimmune reactions, probably as a consequence of their infecting specific T cell subsets. This may result in the rejection of tissues that are sensed as alien, especially neoplastic lesions, and contribute to the parvovirus-induced suppression of tumors, even if these are resistant to the direct viral cytocidal activity. On the other hand, parvovirus oncolysis may initiate and promote a cascade of events leading to the activation of innate immune cells and, in particular, DC maturation and loading with tumor-associated antigens, a process that is necessary for the further development of a potent antitumor CTL response. Therefore, parvovirus oncosuppression is a complex phenomenon involving non-immune as well as innate and acquired immune components in proportions that depend on the type of virus, tumor, and host organism. This scenario justifies current attempts to optimize either of these interrelated facets of tumor suppression through appropriate parvovirus modifications (see also Chapter 44).

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PART **3**

# Specific Parvovirus Infections and Associated Disease

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# Human dependovirus infection

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Adeno-associated viruses (AAVs) have been studied since the early 1960s. In contrast to other human DNA viruses it has long been accepted that there is no significant correlation between the widespread infection by AAV throughout the population and any known disease entity. Although this particular characteristic has somewhat limited funded research on AAV, studies throughout the past several decades have led to an emerging view that AAV might have evolved a possibly optimal relationship with its host through a unique lifestyle that allows the virus to replicate only in cells that are infected by other viruses, which by themselves are deleterious to the host cell. Through this dependency, AAV might have overcome an apparent challenge to viral life cycles in general: on one hand viruses depend on their respective hosts for replication, on the other, most viruses hurt the hosts through their replication to various degrees. Through its dependency, AAV will only replicate in cells that are affected by the consequences of helper virus infection. Thus, if our findings from tissue culture studies can be extrapolated to the human host, infection by AAV could indeed be viewed as beneficial to the host in that cells that are infected by adenovirus, herpes viruses, and possibly papilloma viruses, will die as a result of AAV replication. In light of this aspect it is no surprise that the AAVs appear widespread throughout the vertebrate kingdom.

Nevertheless, numerous questions remain with respect to AAV infections *in vivo* (i.e. in humans). To date, AAV has been isolated only from human specimens as a contaminant of adenovirus-containing suspensions. Consequently, it has been thought that AAV infection follows the infection pattern by adenovirus, i.e. through the respiratory or gastrointestinal route (Blacklow *et al.*, 1971). In addition, and possibly consistent with herpes virus helper functions, sexual transmission has also been proposed. This hypothesis, however, does not take into account the possible latent state of AAV infection.

## BRIEF HISTORY AND EPIDEMIOLOGY OF ADENO-ASSOCIATED VIRUSES

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AAVs were first observed in the mid-1960s as 20 nm particles that contaminated adenovirus preparations (Atchison *et al.*, 1965; Hoggan *et al.*, 1966). First thought to be adenovirus breakdown products or defective adenovirus particles, AAVs were subsequently shown to be an entirely different virus that would not be able to replicate by itself (hence the terminology *Dependovirus*). These viruses could be isolated from various human tissues (Bantel-Schaal and zur Hausen, 1984; Blacklow *et al.*, 1967) and antibodies against several AAV serotypes were detectable in human serum samples (Hoggan, 1971). It is now clear that AAVs are ubiquitous in vertebrates and it has been proposed that they may occur in every species that harbours an adenovirus (Berns and Hauswirth, 1979).

In humans, antibodies to AAV serotypes 1, 2, 3, and 5 are detected in up to 80 percent (Blacklow *et al.*, 1968a, 1971; Rosenbaum *et al.*, 1971; Sprecher-Goldberger *et al.*, 1971; Mayor *et al.*, 1976; Georg-Fries *et al.*, 1984). By the age of 10 years, >60 percent of the population exhibit neutralizing antibodies to AAV serotypes 1–3 and, in many of these, antibody persists into adulthood. Interestingly, the peak ages of AAV seroconversion (i.e. 15–20 years) are very similar to those of adenovirus seroconversion.

## CLINICAL CORRELATES OF AAV INFECTION

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AAV infection of humans is accepted to be non-pathogenic. However, there may be clinical correlates of AAV infection that deserve consideration.

## AAV and cancer

Epidemiological studies suggest that AAV may be protective against certain cancers. Mayor *et al.* (1976) reported an AAV seroprevalence of 85 percent in women with normal cervical cytology and recurrent herpes infection compared with 14 percent of women who had cervical cancer. Furthermore, Georg-Fries *et al.* (1984) reported that the median AAV titer was 3-fold higher in women with a normal cervix than in those women with cervical cancer. More recently, additional evidence to support a protective effect by AAV2 infection has been provided. Smith *et al.* (2001) documented an increasing risk of invasive cervical cancer with decreasing AAV2-specific IgG antibody titer in serum, while Coker *et al.* (2001) suggested that AAV might play an inhibitory role in late-stage cervical carcinogenesis. However, although the notion of a protection through AAV infection has been put forward throughout several decades, it has not been unchallenged. For example, Strickler *et al.* (1999) reported 100 percent negative AAV detection rates in archived cervical tissue samples from normal cervical cytology, low- and high-grade cervical squamous intraepithelial lesions, and invasive cervical cancer; in addition, AAV antibodies were not associated with neoplastic grade. This observation was consistent with those by Odunsi *et al.* (2000) who could not demonstrate any correlation between cervical intraepithelial neoplasia and AAV2 infection.

As is apparent, the question of whether AAV has an anti-oncogenic effect remains unresolved. Moreover, the distinction has not yet been made between a possible direct effect of AAV on tumor viruses (likely through the Rep proteins) and the notion that, in tissue culture, transformation per se can provide limited helper factors for AAV replication. This replication, in turn, could easily account for a cytostatic effect that might be interpreted as anti-oncogenic. It is likely that answers to these questions will be provided through more detailed molecular studies. For example, Raj *et al.* (2001) have recently made the interesting discovery that AAV selectively induces apoptosis in cells that lack functional p53 and that this effect is attributable to the hairpinned ITRs of viral genome. This study has also shed some light on a similar earlier observation that AAV could inhibit oncogenicity in hamster cells (de la Maza and Carter, 1981). In addition to the inverted terminal repeat (ITR) effects, numerous cellular and viral binding partners of the AAV Rep proteins have been identified. Further studies aimed at defining the biological significance and the possible tumor protective consequences of these interactions have yet to be undertaken.

## AAV and infertility

In view of the reports that AAV can cause infection of both the female and male genital tracts, a possible correlation of AAV infection with infertility was investigated (Bantel-Schaal and zur Hausen, 1984; Tobiasch *et al.*, 1994; Han *et al.*,

1996; Malhomme *et al.*, 1997; Rohde *et al.*, 1999; Erles *et al.*, 2001; Venturoli *et al.*, 2001; Mehrle *et al.*, 2004). However, most of these findings have not yet been independently confirmed or, in some instances, have been challenged (Friedman-Einat *et al.*, 1997). Based on an epithelial raft tissue culture system, furthermore, the skin has been proposed to be permissive for AAV replication (Meyers *et al.*, 2000, 2001). Independent confirmation of these findings has yet to be provided as well.

## NATURAL AAV INFECTION

### Target tissues of AAV

Several groups have attempted to identify human target tissues for AAV infection. These efforts need to be reviewed in light of the particular lifestyle of AAV. On the one hand, as mentioned above, AAV has been found as a contaminant of adenovirus isolates obtained from respiratory and gastrointestinal tissues. Most likely, this route of infection reflects the productive, helper-assisted life cycle of AAV. It is reasonable to assume that this route is responsible for the initial infection by AAV and for the resulting seroconversion (Blacklow *et al.*, 1971). However, attempts at identifying a reservoir of latent AAV have produced results that have remained largely unconfirmed. AAV has been described to be present in a small number of hematopoietic cells (<5 percent) (Grossman *et al.*, 1992) and in the female genital tract (Friedman-Einat *et al.*, 1997). It is not clear, however, whether the latter scenario represents a true latent state because all of the AAV positive patients were suspected of being infected by herpes simplex virus (HSV). It is reasonable to hypothesize that those cases represented a 'pseudo-latency' in which AAV had, together with a helper virus, co-infected the cells but, because of HSV latency, could not undergo productive replication. This possibility is intriguing in light of the difference between herpes and adenovirus helper factors. In contrast to adenovirus, HSV does not generally induce S-phase in the infected cell and thus does not provide a milieu that is suitable for productive AAV replication. Consistent with this notion is the suggestion that in the case of herpes virus helper functions, AAV appears to utilize the HSV replication proteins (Ward *et al.*, 2001) rather than the cellular machinery used in the case of adenovirus co-infection (reviewed in Muzyczka and Berns, 2001). Consequently, during latency of HSV, AAV would not replicate and therefore would remain latent in a helper virus co-infected cell. However, more studies are required to confirm this possible form of AAV latency.

Several groups have focused on the lungs as an additional potential reservoir for AAV latency. To date, however, these attempts have not been successful. The only tissue where AAV was found at surprisingly high frequencies was muscle with 17 percent of random biopsies testing positive

(Tezak *et al.*, 2000). This observation is compelling in two ways:

- First, if the initial infection by AAV is through co-infection with helper virus, the target tissue for latency would be predicted to be infectable specifically by AAV, not the helper virus. Skeletal muscle is thought to be resistant to herpes and adenovirus infection, thereby fulfilling this hypothetic requirement.
- Second, the evolutionary selection of the specific site (AAVS1) within the human genome into which AAV integrates its genome (Kotin and Berns, 1989; Kotin *et al.*, 1990, 1991, 1992; Samulski *et al.*, 1991) in order to establish latency remains unclear.

Genetic analysis of the integration site has revealed that AAVS1 is closely linked to the slow twitch skeletal muscle gene troponin 1 (TNNT1), the cardiac troponin gene TNNI3 (Dutheil *et al.*, 2000), and more recently MBS85 (the myosin binding subunit of the cellular phosphatase PP1) (Tan *et al.*, 2001; Dutheil *et al.*, 2004). In addition, transduction experiments using recombinant AAV expressing a marker gene revealed that, when muscle is targeted, AAV preferentially transduces slow twitch fibres (Samulski, 2000, personal communication). Taken together, these observations could support the hypothesis that muscle tissue is a natural reservoir for AAV infection. However, the evidence supporting this notion is indirect and additional data demonstrating, for example, that AAV is indeed integrated in this locus in human samples is needed. In this context it would also be useful to demonstrate a link between the expression of the muscle-specific genes and AAV DNA integration frequencies.

In addition to the identification of tissues supporting helper virus mediated productive AAV replication and latency, numerous attempts have been made at identifying human tissues in which AAV can autonomously replicate.

## Host immune response to AAV

Finally, the complex lifestyle and the ubiquitous occurrence of AAV predicts a particular host immune response that would allow the establishment of latency. Since, based on its characteristics, AAV has become one of the most promising vectors for human therapeutic gene transfer (Samulski, 2003), understanding the underlying mechanisms has become particularly important. To date, however, very little is known about the immunity to AAV. Hernandez *et al.* (1999) investigated the response to wild-type (wt)AAV infection in non-human primates after intranasal, intramuscular, and intravenous infection. The authors conclude that AAV persisted at low copy numbers at the injection sites and that infection results in a 4-fold increase of neutralizing anti-AAV antibodies. However, no cell-mediated immunity was observed in the absence of adenovirus co-infection. Chirmule *et al.* (1999) went on to

characterize the immune response to naturally occurring AAV infections in a cohort of normal subjects and cystic fibrosis patients. Consistent with earlier observations the majority of patients tested positive for anti-AAV2 antibodies, although only 32 percent were characterized as neutralizing and only 5 percent of the patients showed peripheral lymphocytes that proliferated upon challenge. While a striking 'stealth' mode of AAV infection with regard to the host immune response has been observed by several laboratories, the underlying mechanisms await further investigation. It remains to be determined whether passive mechanisms play a role through the failure to infect antigen-presenting cells, and whether AAV has evolved more active mechanisms to avoid deleterious detection by the host immune system.

In summary, to date a rather complex view of the AAV life cycle is emerging that leaves open a panoply of fundamental questions with regard to some of the most basic virological aspects. It is expected that the intense efforts to develop this, to date, most promising viral vector system for therapeutic gene transfer will be paralleled by in depth attempts at further elucidating the biology of one of the smallest and genetically most simple DNA viruses.

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# Human and primate erythrovirus infections and associated disease

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## HUMAN ERYTHROVIRUS

### **Discovery and history of parvovirus B19**

Parvovirus B19 was discovered in 1975 as a cause of systemic infections of adults who were either asymptomatic or had mild non-specific symptoms such as headache, pyrexia, malaise, fatigue, and myalgia (Cossart *et al.*, 1975; Paver and Clarke, 1976; Shneerson *et al.*, 1980). In 1983, parvovirus B19 was identified as the etiological agent of erythema infectiosum, also known as fifth disease (Anderson *et al.*, 1983, 1984). The virus occurs worldwide but is restricted exclusively to human hosts. The manifestations of parvovirus B19 infection may vary with the immunologic and hematologic status of the host. In healthy individuals, parvovirus B19 infection may cause a self-limiting subclinical erythroid aplasia, that is followed by rash or arthralgia (Anderson *et al.*, 1985; Potter *et al.*, 1987). After the infection and immunological elimination from the peripheral blood, viral genomes have been shown to be present in cells of bone marrow and skin, in synovial cells, in tissues from liver, and in the endothelium of the myocardium (Söderlund *et al.*, 1997; Cassinotti *et al.*, 1998; Hokynar *et al.*, 2000; Eis-Hübingen *et al.*, 2001; Vuorinen *et al.*, 2002; Bültmann *et al.*, 2003). At present it is not clear if these cells produce viral proteins and/or infectious B19 particles and if the virus genome can be reactivated to productive replication. In patients suffering from diminished production or increased destruction of erythroid cells, parvovirus B19 infection can result in a dramatic decrease of hemoglobin leading to aplastic crisis (Anderson *et al.*, 1982). Persistent parvovirus B19 infection can occur in both immunocompetent and in

immunodeficient individuals. Persistent infection may be associated with chronic anemia and/or a variety of other symptoms, for example, myo- and pericarditis, hepatitis, and autoimmune phenomena.

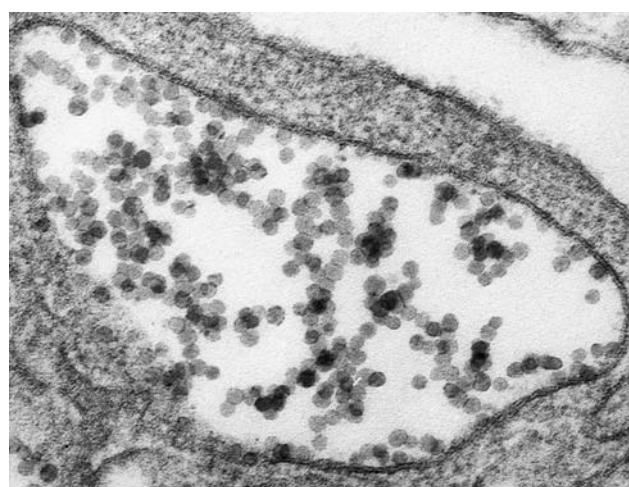
### **Classification, structure, and organization**

#### **TAXONOMY**

Parvovirus (erythrovirus) B19 occurs within the genus *Erythrovirus*, within the family *Parvoviridae*. The *Parvoviridae* comprises a family of viruses which are characterized by small (*parvus* [Latin] = small), non-enveloped particles with a diameter of 20–28 nm containing a single-stranded linear DNA molecule as genome. Members of the genus *Erythrovirus* have the ability to replicate autonomously (i.e. without requirement for helper functions as are provided to adeno-associated viruses [AAVs] by herpes or adenoviruses). Erythroviruses display a preference for infection of erythroid cells, and parvovirus (erythrovirus) B19 is the only member of the *Parvoviridae* pathogenic for humans. During the past decade closely related viruses that cause similar infections in primates and chipmunks have been identified (see later in this chapter). The genus *Parvovirus* comprises a variety of infectious agents that infect vertebrates and cause well-known infections in animals, e.g. dogs (canine parvovirus) and cats (feline panleukopenia virus).

#### **MORPHOLOGY**

The B19 virion is a small non-enveloped particle with an average diameter of 22 to 24 nm (Figure 27.1) containing a

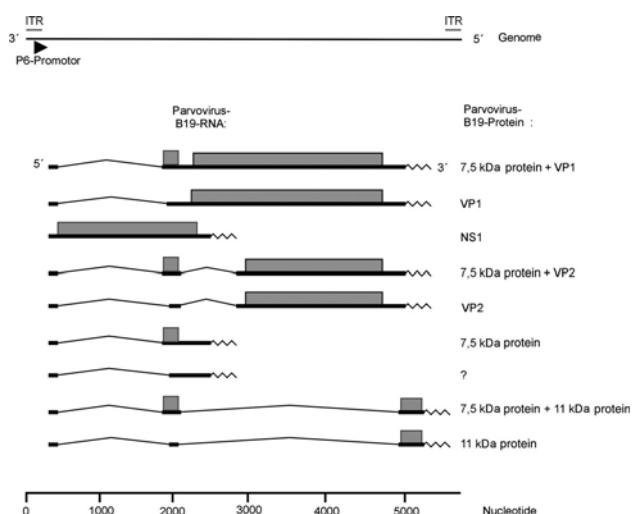


**Figure 27.1** Electron microscopy of human parvovirus B19 particles present in vacuoles of an erythroid precursor cell in a bone marrow sample of a patient with acute parvovirus B19. The picture shows an ultrathin section of the frozen cell and was kindly provided by Dr Hans Gelderblom, Robert-Koch-Institut, Berlin, Germany.

single-stranded DNA-genome of 5596 nucleotides in length. The infectious particles may contain either positive or negative strands of DNA; they show a molecular weight of  $5.6 \times 10^6$  and a buoyant density in a cesium chloride gradient of 1.41 g/ml (Berns, 1996). The icosahedral capsid does not display prominent spikes at the 3-fold axes (Agbandje-McKenna *et al.*, 1994; Kaufmann *et al.*, 2004), and consists of two structural proteins, VP1 (83 kDa) and VP2 (58 kDa), which are identical except for 227 amino acids at the amino terminal end of the VP1 protein, the so-called VP1-unique region. Each capsid consists of a total of 60 capsomers: VP2 is the major capsid protein, and comprises approximately 95 percent of the total virus particle. VP1 proteins are incorporated into the capsid structure in a non-stoichiometrical relation. The orientation of the VP1-unique region in the capsids is incompletely understood. Based on antibody-binding binding analysis, the VP1-unique region is assumed to be exposed at the surface of the virus particle (Rosenfeld *et al.*, 1992; Kawase *et al.*, 1995).

#### GENOMIC STRUCTURE AND ORGANISATION

The single-stranded DNA genome of parvovirus B19 contains 5596 nucleotides (Shade *et al.*, 1986). It is composed of an internal coding sequence of 4830 nucleotides flanked with inverted terminal repeat (ITR) elements of 383 nucleotides in length (Figure 27.2) (Deiss *et al.*, 1990). The ITR-elements are palindromic and can form double-stranded hairpin configurations, which serve as primers for the synthesis of the complementary DNA strand during genome replication (Astell, 1990). The viral genome has two large open reading frames; the left side of the genome, just downstream of the p6 promoter, encodes the



**Figure 27.2** Genome, transcription and translation map of parvovirus B19. The line at the top of the figure represents the parvovirus B19 genome with its ITR elements at the ends and the location of the p6 promoter. The lines underneath represent the various transcripts that are used for the translation of the respective viral proteins indicated by bars. Exon sequences are indicated by thick lines, the introns, which are removed by RNA splicing, by thin lines. Polyadenylation sites are represented by the jagged symbols.

non-structural protein 1 (NS1), while the capsid proteins, VP1 and VP2, are encoded by the right side (Figure 27.2). The expression of all viral genes is controlled by one promoter element, the p6-promoter, which is located upstream of the sequences encoding the NS1 gene at the extreme left side of the viral genome. The activity of the p6-promoter is enhanced by the viral NS1 protein, which acts in combination with a number of cellular transcriptional activators (Gareus *et al.*, 1998; Raab *et al.*, 2001, 2002). During productive replication a total of nine RNA species are synthesized by alternative splicing mechanisms used for the synthesis of the diverse viral proteins (Figure 27.2) (Ozawa *et al.*, 1987a; Shimomura *et al.*, 1993). Polyadenylation sites for use in termination of RNA synthesis are located in the center of the genome and at the far right side (St Amand *et al.*, 1991). Different transcription and splicing patterns have been observed in cells with restricted cell tropism of parvovirus B19 (Leruez *et al.*, 1994; Brunstein *et al.*, 2000).

#### SEQUENCE VARIATION

The original nucleotide sequence of the parvovirus B19 genome was obtained from a virus isolate present in the serum of a child suffering from homozygous sickle cell anemia (Shade *et al.*, 1986). This virus isolate has been designated pbaua and is today known as the prototype of parvovirus B19, genotype 1. A large number of isolates have been entirely or partly sequenced resulting in the identification of three distinct genotypes of parvovirus B19

(Nguyen *et al.*, 1999; Hokynar *et al.*, 2002; Servant *et al.*, 2002). Between the different genotypes, nucleotide variations of up to 11 percent are observed, which are preferentially localized in genomic regions encoding the carboxyterminal part of the NS1 protein and the VP1-unique region, respectively (Erdman *et al.*, 1996; Hemauer *et al.*, 1996; Dorsch *et al.*, 2001; Servant *et al.*, 2002). Despite the fact that nucleotide variations of up to 2 or 3 percent may be observed when comparing members of the same genotype, the viral genomes show a rather high degree of amino acid sequence conservation. The limited number of point mutations resulting in amino acid variations in the VP1-unique region of genotype 1 are not associated with differences in immunological recognition (Dorsch *et al.*, 2001). Furthermore 100 percent serologic cross-reactivity was observed between the VP2 proteins of genotypes 1 and 3 (Heegaard *et al.*, 2002a). The question as to whether serological differences that result in the formation of variant serotypes may be associated with the amino acid variations in the immunodominant VP1-unique regions of all three genotypes has not yet been answered.

Genotype 1 seems to be the most common genotype worldwide, whereas genotype 2 has been reported preferentially in skin biopsies in Scandinavia (Hokynar *et al.*, 2002), in the bone marrow of an anemic HIV-patient in Denmark (Nguyen *et al.*, 2002) and in few liver explants in Germany (Eis-Hübiner, personal communication). Recently a persistent productive parvovirus B19 genotype 2 infection with recurrent high viremia was observed in a renal transplant patient in Germany (Liefeldt *et al.*, 2005). Parvovirus B19 genotype 3, also known as V9 isolate, has been occasionally observed in patients with transient aplastic anemia in France (Nguyen *et al.*, 1999; Heegaard *et al.*, 2002b). Parvovirus B19 genotype 3 may be the predominant genotype in West Africa (Candotti *et al.*, 2004). To date, specific disease symptoms have been correlated with neither members of the various genotype groups nor with individual sequence variations (Umene and Nunoue, 1990; 1991; Kerr *et al.*, 1995a; Hemauer *et al.*, 1996; Lehmann *et al.*, 2003; Liefeldt *et al.*, 2005).

## CAPSID PROTEINS

The capsid of parvovirus B19 is composed of two capsomer proteins, VP1 and VP2, which are encoded by overlapping reading frames (Figure 27.2). VP2 possesses the functional activities of the virion for binding to the cellular receptor, globoside (Brown *et al.*, 1993a), and for particle production. VP2 proteins that are produced via gene technology methods in mammalian, insect, or yeast cells can assemble to non-infectious virus-like particles (VLPs), that are physically and antigenically similar to native virions (Kajigaya *et al.*, 1989; 1991; Brown *et al.*, 1991; Lowin *et al.*, submitted). The minor capsid protein VP1 is identical to VP2 with the addition of 227 amino acids at the amino terminal end. Neutralizing antibodies are directed against both

capsid proteins. The long-lasting neutralizing antibody response is mainly directed against the VP1-unique region, which is thought to be exposed at the particle surface. Recently a phospholipase A2-like activity has been linked to the carboxyterminal half (amino acids 130–195) of the VP1-unique region of human parvovirus B19 (Zadori *et al.*, 2001; Dorsch *et al.*, 2002). This enzyme activity may contribute to the inflammatory processes induced by the production of leukotrienes and prostaglandins, but may also lead to the generation of unnatural cleavage products from cellular phospholipid compounds that may induce antiphospholipid antibodies in combination with a distinct genetic background (von Landenberg *et al.*, 2003).

## NON-STRUCTURAL PROTEINS

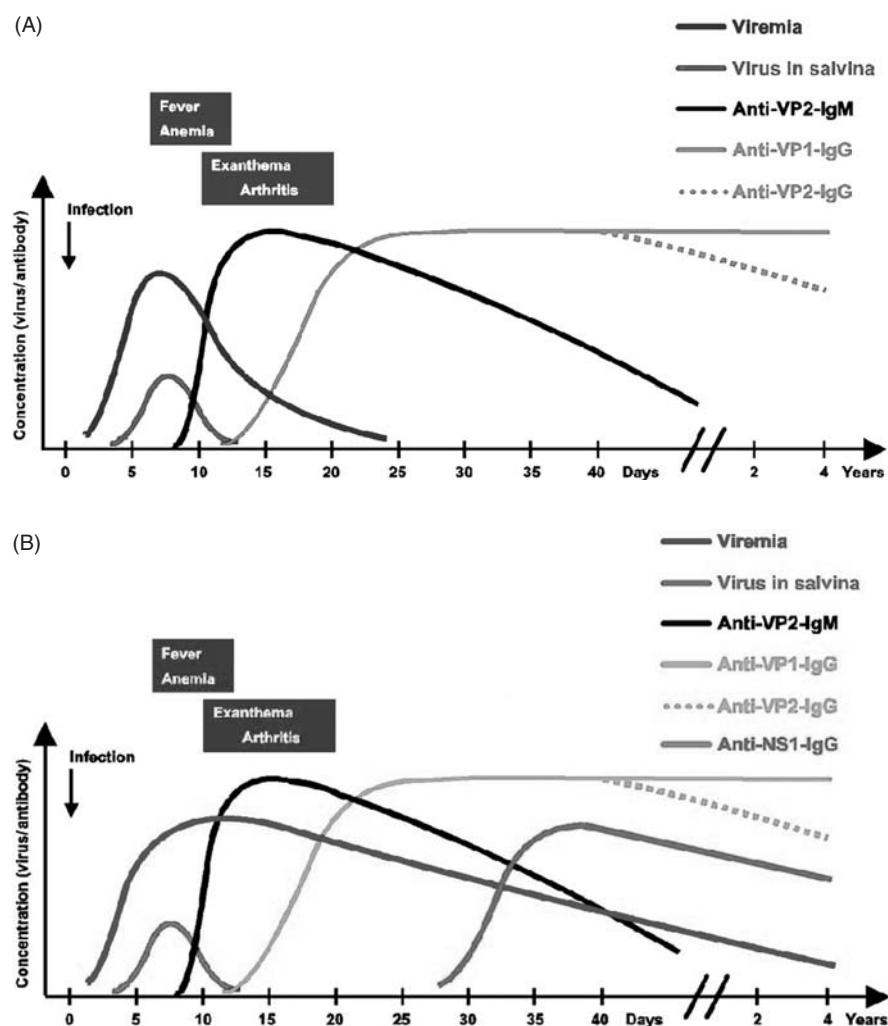
In addition to the structural proteins the viral genome encodes a number of non-structural proteins (Figure 27.2): the NS1 protein and two small polypeptides of 11 kDa and 7.5 kDa. The major non-structural protein, NS1 (77 kDa), is a multifunctional protein. It has been shown to possess site-specific DNA-binding and helicase activities and is functionally active as a transactivator of the viral p6 and various cellular promoters (Gareus *et al.*, 1998; Vassias *et al.*, 1998; Raab *et al.*, 2001, 2002), for example the cellular promotors for the expression of tumor necrosis factor (TNF)- $\alpha$  and interleukin 6 (Moffatt *et al.*, 1996; Fu *et al.*, 2002; Mitchell, 2002). Additionally the NS1 protein contains a well-conserved nucleoside triphosphate-binding motif, which is essential for a variety of biological functions, such as the ATPase activity and the pronounced cytotoxicity of the protein. The cytotoxic NS1 activity was demonstrated to be closely related to cell-cycle arrest (Morita *et al.*, 2003) and apoptosis by a pathway involving caspase 3, whose activation may be a key event during NS1-induced cell death (Moffatt *et al.*, 1996, 1998; Sol *et al.*, 1999).

Knowledge is limited regarding the functions of the 11 kDa non-structural proteins, a family of three variants using different start codons for translation (St Amand and Astell, 1993). The sequence contains three proline-rich regions and *in vitro* the interaction with SH3-domain protein Grb2 (growth factor receptor-binding protein 2) has been reported (Fan *et al.*, 2001). No functional activities have been reported for the 7.5 kDa protein (Luo and Astell, 1993).

## Virus-host interactions

### IMMUNE RESPONSE

During the course of parvovirus B19 infection, a specific humoral immune response occurs against the viral proteins (Figure 27.3). Viremia has its onset 6 days after infection with levels up to  $10^{13}$  virus particles per milliliter blood. A few days later, mostly in combination with the appearance of IgM antibodies, viremia begins to decline. The initial IgM antibody response is almost entirely directed against



**Figure 27.3** (A) Serological parameters found in acute and past parvovirus B19 infections associated with virus elimination from the peripheral blood. (B) Serological parameters found in persistent parvovirus B19 infections. See also Color Plate 27.3.

linear and conformational VP2-specific epitopes, which are common for both the structural proteins VP1 and VP2. IgM antibodies binding specifically to the VP1-unique region are found occasionally. In general, IgM antibodies persist up to 10 weeks after infection but may be found for several months in some patients. They are replaced by IgG against both VP1 and VP2 capsid proteins. Specific IgG becomes detectable at about 2–3 weeks after infection. During the first weeks the majority of IgG-antibodies are directed against both conformational and linear epitopes present in the VP2 protein and the VP2-specific part of the VP1 protein and against linear epitopes of the VP1-unique region (Söderlund *et al.*, 1995; Kerr *et al.*, 1996; Manaresi *et al.*, 1999; Modrow and Dorsch, 2002). About 4–6 months after infection, antibodies against linear VP2-specific epitopes begin to decline whereas IgG against conformational epitopes persists together with the VP1-specific antibodies. Immunoglobulin specific for the VP1-unique region, particularly those against epitopes in the amino-terminal half of this protein domain, offer life-long protection against

re-infection (Saikawa *et al.*, 1993; Anderson *et al.*, 1995; Gigler *et al.*, 1999; Zuffi *et al.*, 2001).

In individuals who develop persistent B19 infection, viremia may continue for several months and years after acute infection with levels of  $10^3$  to  $10^6$  genome copies per milliliter blood or synovial fluid. In immunocompetent patients with persistent parvovirus B19 infections, VP1- and VP2-specific IgG antibodies can be detected preferentially in combination with NS1-specific antibodies (von Poblotzki *et al.*, 1995a,b; Hemauer *et al.*, 1999a, 2000; Kerr and Cuniffe, 2000; Zakrzewska *et al.*, 2001; Lehmann *et al.*, 2002). Usually, the synthesis of these antibodies follows anti-VP1/VP2-IgG and starts at about 3–4 weeks after infection (Hemauer *et al.*, 2000; von Poblotzki *et al.*, 1995a).

In immunodeficient patients, for example, HIV-infected individuals, therapeutically immunosuppressed transplant recipients, or tumor patients and persons with congenital immune defects, persistent parvovirus B19 infections are frequently observed (Kurtzman *et al.*, 1988; Frickhofen and Young, 1989; Kurtzman *et al.*, 1989a,b;

Frickhofen *et al.*, 1990; Flunker *et al.*, 1998; Koduri *et al.*, 1999; Cavallo *et al.*, 2003). In these cases antibodies against both the structural and non-structural proteins may be totally lacking or may be detectable whether or not at reduced levels.

In healthy individuals infected with parvovirus B19 the predominant immune response is humoral and based on the production of antibodies (Kurtzman *et al.*, 1989a). Various studies using immunoglobulin therapy for treatment of persistent parvoviremia in immunodeficient patients show that the application of immunoglobulins is followed by a clear reduction of the virus load in the peripheral blood indicating that specific IgG is the mainstay in the control of infection and virus elimination (see section on *Immunoglobulin treatment*). B-cell memory seems to be mainly directed against conformational epitopes of the viral capsid proteins (Corcoran *et al.*, 2004). Cellular immunity based on either Th1- or Th2-cells is more difficult to analyze and only limited data are available. T-helper cell proliferation against epitopes present in viral capsid proteins has been shown in individuals with past B19 infection and in volunteers immunized with non-infectious virus-like particles (von Poblotzki *et al.*, 1996; Fransilla *et al.*, 2001). In *ex vivo* experiments elevated secretion of interferon- $\gamma$  and interleukin (IL)-2 was observed following VP1 and VP2 antigen stimulation in previously infected individuals indicating a Th1-mediated cellular immune response (Corcoran *et al.*, 2000, 2003). In addition there are few studies on epitopes in the amino acid sequence of the viral proteins that are presented in a major histocompatibility complex (MHC) class I-dependent manner (Klenerman *et al.*, 2002).

## PATHOGENESIS

The pathogenesis of a parvovirus B19 infection, or the process of causation of disease, is dependent on both viral and host factors, the interplay of which will determine the particular outcome resulting from a particular virus-host interaction.

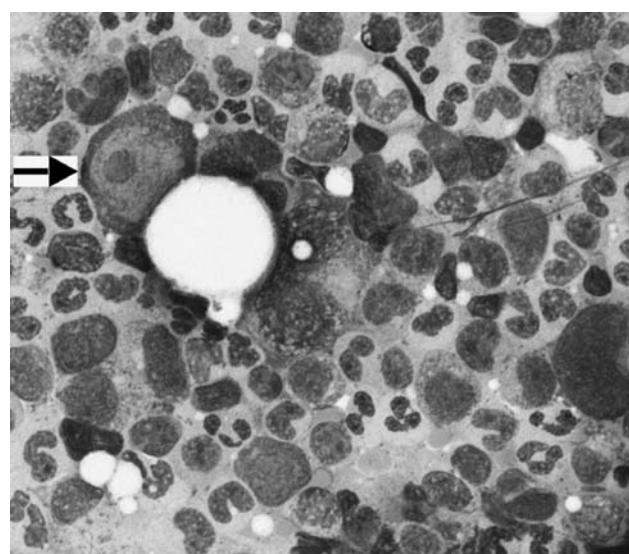
### Effects induced by virus factors

#### *Virus acquisition*

The pathogenesis of B19 infection begins with acquisition of virus usually via the respiratory tract, following which replication is presumed to occur in the nasopharyngeal lymphoid tissue leading to viremia on day 6 (Anderson *et al.*, 1985) with subsequent infection of erythroblasts in the bone marrow.

#### *Lytic infection*

Various studies document a direct pathogenic or 'lytic' effect on erythroid cell precursors (Takahashi *et al.*, 1990; Koduri, 1998), and the giant pronormoblast is virtually pathognomonic of, but not invariable in, B19 infection (Figure 27.4) (Morey *et al.*, 1992a; Caper *et al.*, 1996). This cell is also referred to as the 'lantern cell'. These are early



**Figure 27.4** Bone marrow smear of a renal transplant patient with pure red cell aplasia (PRCA) from persistent parvovirus B19 infection, genotype 2. The bone marrow is depleted of all erythroid elements except for a few giant pro-erythroblasts with intranuclear inclusions (indicated by the arrow). The granulopoiesis is not affected and the morphology of megakaryocytes remains unchanged (kindly provided by L. Liefeld, Charité, Humboldt University, Berlin Germany). See also Color Plate 27.4.

erythroid cells (25–32  $\mu\text{m}$  diameter), which contain large eosinophilic nuclear inclusion bodies, cytoplasmic vacuolization and occasionally 'dog-ear' projections (Caul *et al.*, 1988; Brown *et al.*, 1991). Ultrastructural changes are revealed by electron microscopy including marginated chromatin, presence of nuclear virions, and pseudopod formation (Young *et al.*, 1984). In one study of patients with B19-associated pure red cell aplasia (PRCA) (Koduri, 1998), the appearance of giant pronormoblasts correlated with the course of parvovirus B19 infection in terms of detection of serum B19 DNA and specific IgM. One study shows that giant pronormoblasts stained negative for p21, activated caspase-3, and TUNEL, but stained positive for p53, Ki-67 or Bax (Sadahira *et al.*, 2001).

#### *Apoptosis and cytotoxicity*

In contrast to the destruction of erythroblasts during parvovirus B19 infection, which follows viral replication, the pathogenesis of thrombocytopenia associated with acute B19 infection is thought to be explained by expression of the left side of the viral genome encoding the NS1 protein in the absence of replication (Pallier *et al.*, 1997). The cytotoxicity of the NS1 protein was first demonstrated by Ozawa and colleagues (1987b) using cells transfected with an NS1 expression plasmid. The cytotoxicity of the NS1 protein has also been proposed as a possible mechanism, which may account for certain clinical other manifestations of B19 infection including leucopenia and arthralgia (Brown *et al.*, 1994a).

The gradual cytocidal effect mediated by the NS1 protein (Ozawa *et al.*, 1986) that is observed during parvovirus B19 infection of erythroid lineage cells is combined with features of apoptosis including margination chromatins, cytoplasmic vacuolization, and nuclear blebbing (Morey *et al.*, 1993). An apoptotic function for the NS1 protein has been revealed using stable cell lines and inducible expression of the NS1 gene (Moffatt *et al.*, 1998; Sol *et al.*, 1999). Activation of caspases 3, 6, and 8 was induced by NS1 protein synthesis in UT7/Epo-cells (Moffatt *et al.*, 1998). NS1 expression results in an increase in sensitivity to apoptosis induced by tumor necrosis factor-alpha (TNF- $\alpha$ ) (Sol *et al.*, 1999).

#### *Transactivation of host cellular genes*

The viral NS1 protein has been described as a transactivator of both the viral p6-promoter as well as a variety of cellular promoters. These include the promoter region controlling the expression of TNF- $\alpha$  (Fu *et al.*, 2002) and IL-6 genes (Moffatt *et al.*, 1996). Elevated levels of TNF- $\alpha$  have been shown to be present in patients during the acute and convalescent phases of B19 infection (Kerr *et al.*, 2001). The prolonged or continuous presence of these proinflammatory cytokines during acute-convalescent and persistent B19 infection respectively may result in the induction of long-lasting clinical symptoms and autoimmune reactions.

#### *Persistence and latency*

Infectious parvovirus B19 has been demonstrated during the acute phase at various sites in the human body, including nasopharynx, peripheral blood, bone marrow, liver, skin, and synovium. Cell types infected by B19 virus include erythroblasts, megakaryoblasts, granulocytes, macrophages, follicular dendritic cells, T and B lymphocytes, hepatocytes, and endothelial cells (Saal *et al.*, 1992; Moffatt *et al.*, 1996; Takahashi *et al.*, 1998). The viral DNA has been shown to persist in various sites including bone marrow (Kerr *et al.*, 1997), synovium (Kerr *et al.*, 1995b; Söderlund *et al.*, 1997), testis (Diss *et al.*, 1999), myocardium (Bültmann *et al.*, 2003; Pankweit *et al.*, 2003), and skin (Nikkari *et al.*, 1999; Hokynar *et al.*, 2002). In one study, B19 DNA positivity in peripheral blood was shown to persist for 3–5 years from the time of acute infection in 7 of 53 persons (Kerr *et al.*, 1995c). However, the mechanism that facilitates this persistence is unclear. It has been suggested that parvovirus B19 DNA may persist by integration into the human genome (Reed *et al.*, 2000) as is the case with minute virus of mice (MVM) (Corsini *et al.*, 1997) and the dependoviruses (Kotin *et al.*, 1990a,b). However, experimental data that unequivocally demonstrate integration of the B19 genome into human chromosomal DNA are not available.

#### **Effects exerted by host factors**

##### *Cellular receptors*

B19 virus enters the cell by first binding the B19 cell receptor, P blood group antigen (globoside, Gb4) (Brown *et al.*, 1993a), and then entering the cell by binding a co-receptor,  $\alpha 5\beta 1$  integrin, in high affinity conformation (Weigel-Kelley *et al.*,

2003). Since *in vitro* binding to membrane-associated pure globoside could not be demonstrated, the interaction with globoside appears to be dependent on additional factors (Kaufmann *et al.*, 2005). It is thought that the particular distribution of P antigen may in part determine the particular clinical manifestations of B19 infection (Cooling *et al.*, 1995).

##### *Immune complex deposition*

Experimental infections of human volunteers have enabled elucidation of the pathogenesis of infection (Anderson *et al.*, 1985; Potter *et al.*, 1987). These studies revealed that production of specific IgG following infection was coincident with appearance of the B19-related skin rash, arthralgia and peripheral neuropathy. On this basis, immune complex deposition was proposed to account for these clinical manifestations (Anderson *et al.*, 1985). This hypothesis is further supported by the fact that rash and arthralgia are known to occur in chronically infected patients following administration of immunoglobulin (Pattison *et al.*, 2000).

##### *Autoantibody production*

Some clinical features of both acute and chronic B19 infection are very similar to those of autoimmune connective tissue diseases, a fact that led to investigations into the possibility of autoimmunity. Various autoantibodies have been documented during and following acute B19 infection. Detection of antinuclear antibody (ANA) (both homogeneous and speckled pattern) and rheumatoid factor (RF) appears to be common (Kerr *et al.*, 2002a; Scroggie *et al.*, 2002) and one study found an association between development of chronic arthralgia and presence of ANA during the acute phase (Kerr *et al.*, 2002a).

Recently an association between persistent parvovirus B19 infection and the production of antiphospholipid antibodies in pediatric and adult patients with rheumatic disease has been described (von Landenberg *et al.*, 2003). The antiphospholipid syndrome (APS) (Cervera *et al.*, 2002) is, like some parvovirus B19 infections, characterized by a wide variety of hemocytopenic and vaso-occlusive manifestations. Additionally recurrent fetal loss and the association with autoantibodies directed against negatively charged phospholipids and protein cofactors, mainly  $\beta 2$ -glycoprotein-I are important features of the APS. The hypothesis regarding a common pathogenicity of the antiphospholipid syndrome and the autoimmunity features observed in B19 infection is supported not only by the close association between the presence of antiphospholipid antibodies and parvovirus B19 infection, but also by the similarity in the presenting clinical symptoms in patients with parvovirus B19 infection and in those with APS. Furthermore, Loizou *et al.* demonstrated a close similarity in the specificity of antibodies against different phospholipids induced by parvovirus B19 infection as compared with antibodies found in systemic lupus erythematosus (SLE) patients (Loizou *et al.*, 1997).

It has been shown that VP1-specific IgG that has been affinity-purified using the immunodominant synthetic peptide, reacts specifically with human keratin, collagen type II,

single-stranded DNA and cardiolipin (Lunardi *et al.*, 1998). The main reactivity was against keratin and collagen type II, and there was a correlation between the clinical features and the main autoantigen specificity; immunoglobulin from patients with arthritis reacted preferentially with collagen II, while immunoglobulin eluted from patients with skin rashes reacted preferentially with keratin. As type II collagen is a target antigen for autoantibodies and clonally-expanded T cells in the rheumatoid arthritis (RA) synovium (Sekine *et al.*, 1999), this finding may have considerable significance for the link between B19 infection and rheumatic disease.

One of the pathogenic mechanisms involved in triggering the production of antiphospholipid antibodies may be the phospholipase A2-like activity observed in the VP1-unique region of the structural protein VP1 of parvovirus B19 (Dorsch *et al.*, 2002). This enzyme activity may contribute to the inflammatory processes induced by the production of leukotrienes and prostaglandins, but may also lead to the generation of unnatural cleavage products from cellular phospholipid compounds that may induce aPL antibodies in combination with a distinct genetic background. These effects may be combined with mechanisms based on molecular mimicry that have been discussed as a major cause for the formation of antiphospholipid antibodies (Serra *et al.*, 1999; Reitbalt *et al.*, 2000). These effects have been demonstrated by Blank and coworkers in an experimental study, using peptides from different bacteria for the induction of the antiphospholipid syndrome in a mouse model (Blank *et al.*, 2002).

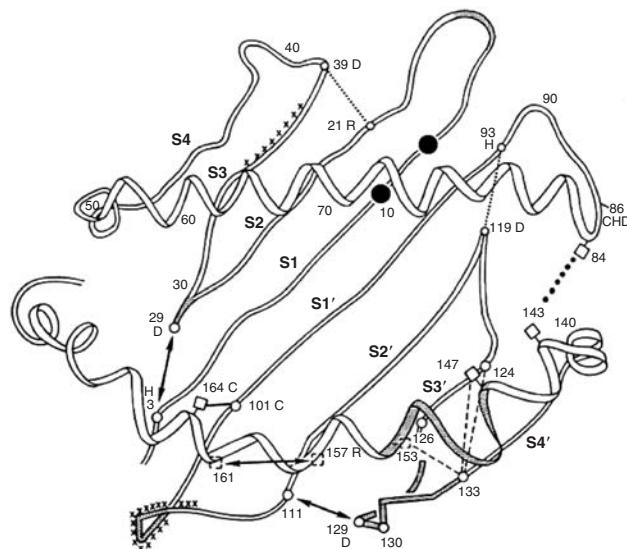
#### *Genetic determinants of immune response*

As certain symptoms of B19 infection are similar to those of various autoimmune connective tissue diseases, several groups have studied the HLA types of patients suffering from B19-associated arthritis. Several studies have examined the role of HLA-DR in the pathogenesis of parvovirus B19 arthritis. The first of these examined a small number of patients ( $n = 18$ ) and found a positive association between HLA-DR4 antigen and acute parvovirus B19 arthritis (Klouda *et al.*, 1986), but this was not borne out in further studies (Dyckmans *et al.*, 1986; Woolf *et al.*, 1989; Gendi *et al.*, 1996).

Gendi *et al.* (1996) used molecular typing in cases of acute parvovirus B19 infection (i.e. positive for parvovirus B19-specific IgM in serum) associated with the occurrence of arthritis. They typed HLA-DRB1 alleles in 34 patients and 297 control subjects. Results, reported only in terms of antigen frequency, revealed no associations between HLA-DR and parvovirus B19 arthritis, although they did find that symptoms of the joints persisted for more than one week in all HLA-DR4-positive patients. The role of the HLA-B locus was examined by Woolf *et al.* (1989), who found no association when comparing patients with control subjects; however, these authors used serologic methods in only 26 patients and 318 controls. Persistent parvovirus B19-associated arthritis has been linked with HLA-B27 (Jawad *et al.*, 1993), which is strongly associated

with spondyloarthropathy, but this was not confirmed in other studies (Kerr *et al.*, 2002b; Lehmann *et al.*, 2002).

Kerr *et al.* (2002b) compared HLA class I and II alleles in 36 patients with acute symptomatic parvovirus B19 infection (positive for parvovirus B19-specific IgM) with those observed in more than 900 control subjects from the North West of England. In 32 out of 36 of the patients symptoms of B19 infection consisted of rash, arthralgia, fatigue, myalgia, and lymphadenopathy. One patient presented with intrauterine death without preceding maternal rash, two patients developed thrombocytopenia, and one transient aplastic crisis. The frequency of each of HLA-DRB1\*01 ( $P = 0.016$ ), DRB1\*04 ( $P = 0.007$ ), and DRB1\*07 ( $P < 0.0001$ ) alleles was significantly higher in patients with symptomatic parvovirus B19 infection than in control subjects. In the parvovirus group, 63.9 percent carried the rheumatoid arthritis-associated shared epitope sequence, compared with 45 percent of control subjects (odds ratio [OR], 2.2; 95 percent CI 0.97–4.8;  $P = 0.04$ ) and carriage was associated with fatigue during the acute phase (OR 4.2; 5 percent CI 0.8–23.9;  $P = 0.047$ ). HLA-DRB1\*01, \*04, and \*07 carry a neutrally charged glutamine at position 10 and a positively charged lysine at position 12 of the first hypervariable region, resulting in an overall positive charge in this region compared with other molecules (Figure 27.5). Other HLA-DRB1 alleles with charge similarity to these alleles in the first hypervariable region include HLA-DRB1\*09



(rare allele), \*15, and \*16. In addition it could be shown that DRB1\*15 and \*16 are also associated with clinical manifestations of B19 infection in the absence of DRB1\*01, \*04, and \*07 alleles in the setting of B19-associated meningocephalitis (Kerr *et al.*, 2002c) and B19-associated new onset of acute leukemia (Kerr *et al.*, 2003a).

The significance of this finding is not clear as these residues do not contribute directly to contact of the binding groove with bound peptide (Brown *et al.*, 1993b; Stern *et al.*, 1994). However, they may influence the binding ability of adjacent residues such as the tryptophan at position 9. Polymorphism at position 9 has been associated with development of dermatomyositis-specific Mi-2 autoantibodies (Mierau *et al.*, 1996) and susceptibility to demyelinating polyneuropathy in plasma cell dyscrasia (Vrethem *et al.*, 1993). HLA-B49 was also associated with symptomatic parvovirus B19 infection independently HLA-DRB1\*01, \*04, and \*07, while HLA-DQB1 and DPB1 showed no associations (Kerr *et al.*, 2002b).

There are several possible explanations for these conflicting findings on the role of HLA polymorphisms in B19 infection. First, early studies were done using serologic typing, and results were reported in terms of HLA-antigen frequency. However, methods using oligonucleotide probes that determine specific HLA-DR alleles are more accurate and provide information on the particular HLA genotype of each patient. Second the different ethnic background of the parvovirus B19-infected patients. Although this is not well documented, it appears that in most studies the subjects were from southern England, which is known to have greater ethnic heterogeneity than north-western England (Kerr *et al.*, 2002b).

These patients, along with normal controls, were also tested for the following cytokine gene polymorphisms using a polymerase chain reaction (PCR)-based method: tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) -308; interferon- $\gamma$  +874; interleukin-6 (IL-6) -174; IL-10 -592, -819, and -1082; and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) +869 (codon 10) and +915 (codon 25). Polymorphism at each of these positions had previously been reported to have a bearing on the level of transcription. The TNF- $\gamma$  -308\*A allele occurred in 13.9 percent of the parvovirus group compared with 27 percent of controls ( $P = 0.02$ ). The TGF- $\beta$ 1 CG/GC haplotype was more frequent in the parvovirus group than in the controls ( $P = 0.02$ ). Within the group of parvovirus B19-infected patients, the TGF- $\beta$ 1 +869T allele was associated with development of skin rash at acute infection ( $P = 0.005$ ) (Kerr *et al.*, 2003b), supporting the hypothesis that the exanthema observed during B19 infection has an immune pathogenesis. We have subsequently shown that the TGF- $\beta$ 1 + 869T allele is associated with increased inflammatory activity, poor functional outcome and increased mortality in patients with rheumatoid arthritis (Mattey *et al.*, 2005).

These results suggest that inherited variability in antigen presentation and cytokine responses may affect the likelihood of developing symptoms during acute parvovirus infection. However, the role of these polymorphisms remains

to be confirmed and this will require testing of more cases of symptomatic and also, importantly, cases of asymptomatic parvovirus B19 infection.

#### *Induction of an invasive cellular phenotype*

Ray *et al.* (2001) established an experimental *in vitro* system in which normal primary human synovial fibroblasts were tested for their ability to degrade reconstituted cartilage matrix. They showed that treatment of fibroblasts with parvovirus B19-containing human sera resulted in an increase in invasion of up to 248 percent compared with untreated fibroblasts. In addition, pre-incubation of viremic serum with neutralizing antibodies abrogated the effect. This work provides a significant biological link between exposure to B19 and phenotypic changes in human synovial fibroblasts, and adds further support to the proposed link between B19 and rheumatoid arthritis.

#### *Modification of host gene expression*

Kerr and colleagues (2005) investigated the possibility that human gene expression in peripheral blood mononuclear cells (PBMC) is altered long after acute infection in seropositive versus seronegative persons. They tested total RNA extracted from peripheral blood of 57 seropositive and 13 seronegative normal healthy persons using a microarray representing 9522 human genes and found that 92 genes were differentially expressed at a fold-change cut-off of 1.5. For 6 of 42 of genes tested by taqman real-time PCR in a second group of normal healthy persons (21 seropositive and 17 seronegative), differential expression was confirmed in the same direction as in the arrays. Although these differences were small (fold-changes of 0.8 – 1.2), they were statistically significant and reproducible among the 108 subjects tested. All samples were negative in real-time PCR testing for the B19 NS1 and VP1/2 genes. Functional roles for these six genes included the cytoskeleton (SKIP, MACF1, SPAG7, FLOT1), integrin signaling (FLOT1, RASSF5), HLA class III (c6orf48), and tumor suppression (RASSF5). Involvement of the integrin signaling pathway is particularly interesting due to the role of beta-integrins as a co-receptor for B19 virus entry (Weigel-Kelley *et al.*, 2003). Possible mechanisms that may account for these findings include a prolonged immune response, supported by studies showing prolonged, detectable cytokine levels even in the absence of symptoms (Kerr *et al.*, 2001), and the possible integration of the viral genome, as has been previously suggested (Reed *et al.*, 2000). B19 infection leads to a prolonged viremia during which the virus has access to hemopoietic stem cells. Infection of and integration into these cells represents a plausible event which could result in such long-term effects on human gene regulation (Kerr *et al.*, 2005).

## Epidemiology

Parvovirus B19 occurs worldwide but is restricted exclusively to human hosts. The majority of infections occur

during childhood and adolescence. Seroprevalence (specific IgG against the capsid proteins) is approximately 2–15 percent in children at an age of 1–5 years, 30–40 percent in adolescents (15 years of age) and 40–60 percent in young adults (20 years of age) (Cohen and Buckley, 1988; Tsujimura *et al.*, 1995; Kelly *et al.*, 2000). The prevalence of IgG-antibodies reaches maximal levels in the elderly in which more than 90 percent are positive. Although the infection is endemic, regional epidemics also occur during late winter and spring. Every 3–4 years the rates of infection may again rise to epidemic levels. During outbreaks the spread of the virus to seronegative individuals is very common.

Since the virus binds via the VP2 proteins to erythrocyte precursor cells using blood group P antigen (globoside) as the cellular receptor for adsorption (Brown *et al.*, 1993a), P antigen-negative individuals have been shown to be genetically resistant to infection (Brown *et al.*, 1994b).

## Transmission

In general parvovirus B19 is transmitted by respiratory aerosol spread from individuals with acute infection (Chorba *et al.*, 1986). Due to the massive productive replication of parvovirus B19 that takes place in erythroid progenitor cells (Ozawa and Young, 1987), the virus load is extremely high in acutely infected individuals before the onset of a detectable immune response. Up to  $10^{13}$  particles and/or virus genomes may be present per milliliter of peripheral blood. At the time of high viremia, viral DNA may also be detected in respiratory secretions and in urine, tears, and other body fluids. In acutely infected pregnant women parvovirus B19 may also be transmitted vertically, from mother to fetus (Brown *et al.*, 1984; Kinney *et al.*, 1988; Yaegashi *et al.*, 1998) (see below).

B19 virus may also be transmitted parenterally via transfusion of blood and blood products (Blümel *et al.*, 2002; Hayakawa *et al.*, 2002a; Prowse *et al.*, 1997). About 1000 genome equivalents of viral DNA present in factor VIII products have been shown to be sufficient to induce an acute parvovirus B19 infection in previously seronegative hemophilic children (Blümel *et al.*, 2002). With sensitive PCR, approximately 1 in 1000 blood donations has been shown to contain viral DNA at concentrations of  $1 \times 10^3$  to  $1 \times 10^7$  geq/ml (Yoto *et al.*, 1995; Prowse *et al.*, 1997; Jordan *et al.*, 1998; Aubin *et al.*, 2000). High virus loads in blood donations obtained from acutely infected individuals are detected with a prevalence of 0.003–0.017 percent (Tsujimura *et al.*, 1995; Wakamatsu *et al.*, 1999; Heegaard *et al.*, 2001b), which may, however rise to 0.03 percent during seasonal epidemics (McOmish *et al.*, 1993). Owing to the lack of a lipid envelope, the virus is very stable. Consequently, many standard methods are insufficient to abolish the infectivity of parvovirus B19; these include ethanol, isopropanol, detergents, treatment by dry heat (72 hours at 80°C or 30 minutes at 100°C) and pasteurization

(Prowse *et al.*, 1997; Santagostini *et al.*, 1997). Parvovirus B19 has been detected not only in blood plasma, bone marrow, stem cells, erythrocyte, and thrombocyte concentrates, but also in clotting factor products (factor VIII, factor IX, PPSP), immunoglobulin, serum albumin, and other products produced from human blood. Both symptomatic and asymptomatic infections have been observed in patients as a consequence of treatment with parvovirus B19-contaminated products (Erdman *et al.*, 1997; Heegaard and Laub, 2000; Blümel *et al.*, 2002; Hayakawa *et al.*, 2002a). In hemophiliac children, a seroprevalence of >90 percent has been observed, depending on the total amount of administered clotting factor products (Rollag *et al.*, 1991; Azzi *et al.*, 1999).

## Diagnosis

Acute infections are routinely diagnosed by the detection of VP2-specific IgM antibodies in enzyme-linked immunosorbent assay (ELISA) or Western blot assays in combination with the presence of high levels of viral DNA. The beginning of convalescence is characterized by the simultaneous presence of IgM and IgG antibodies and declining levels of detectable B19 genome. Past B19 infections are characterized by VP1/VP2-specific IgG without the presence of viral DNA in peripheral blood samples. In patients with persistent infections viral DNA is present in combination with VP1/VP2- and NS1-specific IgG and occasionally also VP2-specific IgM. Expressing the VP2 gene by recombinant baculovirus (Brown *et al.*, 1991a; Kajigaya *et al.*, 1991), eukaryotic (Kajigaya *et al.*, 1989), or yeast vector systems (Lowin *et al.*, submitted), empty VP2 capsids can be obtained that do not contain viral genome but show antigenic characteristics similar to infectious B19 virus. It is important that these empty VP2 particles are included as antigen in ELISA or Western blot test systems since varying amounts of the antibodies may be directed against conformational epitopes that are not part of monomeric VP2 proteins (Söderlund *et al.*, 1995).

Detection of viral DNA is routinely performed by either nested or quantitative polymerase chain reaction assays (Hemauer *et al.*, 1996; Aberham *et al.*, 2001; Harder *et al.*, 2001; Knöll *et al.*, 2002; Manaresi *et al.*, 2002). It is important that the primers and probes used for DNA amplification are suitable for amplification of all three genotypes of parvovirus B19 (Heegaard *et al.*, 2001b; Servant *et al.*, 2002; Liefeldt *et al.*, 2005). B19 viral antigen may also be detected in the circulation during the acute phase using immunoblotting; however, this is much less sensitive than PCR detection of B19 DNA, and is therefore not useful in routine diagnosis. Using electron microscopy (EM), characteristic particles of parvovirus B19 may be observed, particularly in cases where the viral load is high, such as in the circulation during the acute phase and in fetal tissues in cases of hydrops fetalis. However, because of the technically demanding

nature of this method and also the ready availability of tests to detect virus-specific IgM and viral nucleic acid, EM is no longer applied routinely.

## Clinical aspects

### ASYMPTOMATIC INFECTION

B19 infection may be asymptomatic in up to 50 percent of children and 25 percent of adults (Woolf *et al.*, 1989). Analyzing a cohort of IgM-positive acutely infected women <50 percent showed signs of rash or arthralgia (Cartter *et al.*, 1991). However, reticulocytopenia occurs with both symptomatic and asymptomatic acute parvovirus B19 infection. During this phase reticulocyte numbers drop to undetectable levels for about 7 days, followed by a mild and transient depression of hemoglobin lasting for 3–7 days in normal persons (Young, 1988).

### SYMPOTOMATIC INFECTION

Clinical syndromes associated with parvovirus B19 infection are listed in Table 27.1.

#### Clinical syndromes commonly associated with parvovirus B19 infection

##### *Non-specific illness*

Commonly, parvovirus B19 infection may result in an influenza-like illness, which may consist of pyrexia, chills, myalgia, headache, and malaise (Anderson *et al.*, 1982). This generally coincides with the viremic stage of infection and precedes development of the typical skin rash of B19 infection by 1 week.

##### *Erythema infectiosum*

Erythema infectiosum (EI, fifth disease, slapped cheek syndrome), the major manifestation of B19 infection, is a self-limiting rash illness (Anderson *et al.*, 1983; Cherry, 1999). ‘Fifth disease’ refers to the five known infectious diseases of childhood associated with rash: measles, rubella, varicella-zoster (chicken pox), scarlet fever, and erythema infectiosum. The disease was well known as ‘rubeola sine katarrhys’ prior to the discovery of parvovirus B19. Prodromal symptoms may include fever, coryza, headache, nausea, and diarrhea. In classic cases of erythema infectiosum, the rash evolves in three stages. The first stage begins as a rash of medium intensity symmetrically involving the cheeks, but with relative circumoral pallor and beginning approximately 12–14 days after onset (Figure 27.6). Together with the appearance of the erythema, immunoglobulins against the viral capsid proteins VP1 and VP2 become detectable for the first time. During the following days, the second stage of rash develops with evolution into an erythematous maculopapular pattern involving the limbs and the trunk (Figure 27.6). The rash may be transient or recurrent and usually exhibits a central fading in the pattern of ringlets or garlands. It is frequently lacy or reticular. The third stage is

highly variable in duration, lasting 1 to 3 or more weeks, with fluctuations of intensity that may be influenced by environmental factors such as UV light and heat (Naides, 1999). Itching, vesicles, and scaly dermatitis have also been reported (Woolf *et al.*, 1989; Zerbini *et al.*, 1992).

##### *Transient aplastic crisis (TAC)*

The first clinical symptom to be associated with B19 infection was TAC in patients with sickle cell disease (Pattison *et al.*, 1981). Previous to this, TAC had been well recognized as a precipitous drop in hemoglobin associated with cessation of reticulocyte production against a background of chronic hemolytic anemia. B19 infection is now recognized to cause TAC in association with shortened red cell survival caused by a variety of underlying conditions. These include sickle cell anemia (Pattison *et al.*, 1981; Kelleher *et al.*, 1984), α- and β-thalassemia (Lefrere *et al.*, 1986a; 1986b), autoimmune hemolytic anemia (Bertrand *et al.*, 1985; Saarinen *et al.*, 1986), glucose-6-phosphate dehydrogenase deficiency (Goldman *et al.*, 1990), hereditary spherocytosis (Tsukada *et al.*, 1985; Lefrere *et al.*, 1986c), hereditary stomatocytosis (Mabin and Chowdhury, 1990), iron deficiency anemia (Lefrere and Bourgweis, 1986), pyruvate kinase deficiency (Duncan *et al.*, 1983), sideroblastic anemia (Mehta *et al.*, 1992), congenital dyserythropoietic anemia (West *et al.*, 1986; Carpenter *et al.*, 2004), pyrimidine 5'-nucleotidase deficiency (Rechavi *et al.*, 1989), malaria (Jones *et al.*, 1990), and paroxysmal nocturnal hemoglobinuria (Lakhani *et al.*, 1987). Blood loss may also represent a predisposition to TAC upon B19 infection (Harris, 1992).

##### *Transient arthropathy*

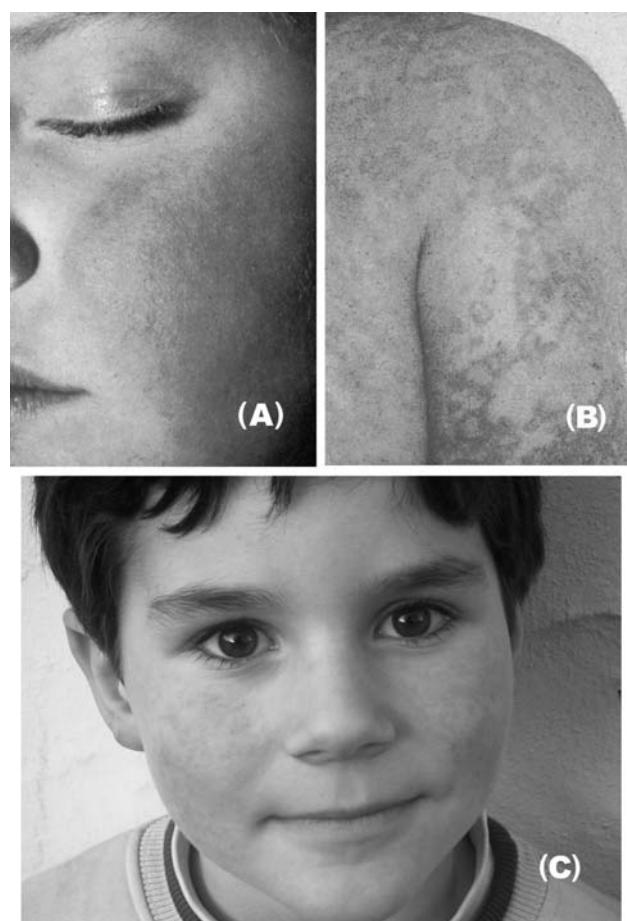
Soon after the discovery of parvovirus B19 as the infectious agent causing erythema infectiosum an association between acute infection and arthralgia was reported (Reid *et al.*, 1985; White *et al.*, 1985). Arthralgias and arthritis are commonly observed in association with parvovirus B19 infection in adults whereas erythema infectiosum is the main symptom observed in children (Figure 27.7) (Cassinotti *et al.*, 1995; Hoebe *et al.*, 2002). In a similar fashion to erythema infectiosum, the occurrence of arthralgia coincides with the initial detection of circulating IgM and IgG antibodies against the viral structural proteins VP1 and VP2. Therefore it is assumed that immune complexes deposited in the synovial fluid of the affected joints are involved in the pathogenesis of B19-associated arthralgias. B19 infection may produce a symmetrical peripheral polyarthropathy or polyarthritis in 40–80 percent of infected adults; this occurs more frequently in females (70 percent) than in males (30 percent) (Anderson *et al.*, 1985; Joseph, 1986; Woolf *et al.*, 1989; Murai *et al.*, 1999; Scroggie *et al.*, 2000; Moore, 2000; Kerr *et al.*, 2002a). Arthralgia is usually self-limited but may persist for several months or recur over months to years (Guillaume *et al.*, 2002). Seishima and co-workers (2003) describe 42 adult patients who were studied for >2 years after primary parvovirus B19 infection; 34 patients (81 percent) had complained of polyarthralgia

**Table 27.1** Disease manifestations associated with parvovirus B19 infection

Frequency of association	System	Disease
Common		Non-specific illness Erythema infectiosum (EI) Transient aplastic crisis (TAC) Transient arthropathy Fetal death Chronic PRCA in immunocompromised persons
Less common	Cutaneous	Henoch-Schönein Purpura Papular-purpuric gloves and socks syndrome (PPGSS) Gianotti-Crosti syndrome Desquamation Erythema multiforme Livedo reticularis Erythema nodosum
	Haematological	Aplastic anemia Thrombocytopenia (including ITP) Neutropenia Transient erythroblastopenia of childhood (TEC) Virus-associated hemophagocytic syndrome (VAHS) Acute leukemia and myelodysplasia (MDS) Lymphadenopathy Kikuchi disease (with SLE) Hypersplenism Congenital red cell aplasia
	Hepatobiliary	Hepatitis Acute liver failure
	Cardiovascular	Myocarditis Pericarditis Vasculitis Acute heart failure
	Neurological	Meningitis Encephalitis Guillain-Barré syndrome Cerebellar ataxia Transverse myelitis Peripheral neuropathy Carpal tunnel syndrome Congenital neurological disease
	Rheumatological	Arthritides (including adult, juvenile, RA, JIA) Vasculitides (including SLE) Chronic fatigue syndrome (CFS) Systemic sclerosis (SS) Myositis Uveitis
	Renal	Glomerulonephritis

during the acute phase of parvovirus B19 infection. In three female patients arthralgia persisted for up to 6 months; one woman developed symptoms persisting over >2 years. In another study none of 54 adult patients with arthralgia from acute parvovirus B19 infection reported persistence of joint swelling after 5 years (Speyer *et al.*, 1998). However, a further study found that 11 of 39 (28 percent) patients had persistent arthralgia 1–3 years following acute B19 infection (Kerr *et al.*, 2002a). Transient arthropathy is also reported

to occur in children in association with acute parvovirus B19 infection; however, this seems to occur more rarely than in adults (Barash *et al.*, 2002; Oguz *et al.*, 2002). There appears to be a correlation between recent or persistent parvovirus B19 infection and the development of juvenile rheumatoid arthritis. In the peripheral blood and in the synovial fluid of children suffering from various forms of rheumatic disease, both parvovirus B19 genomes and immune complexes have been detected frequently indicating that the virus infection



**Figure 27.6** (A, C) Facial rash of two different children acutely infected with parvovirus B19 as symptoms associated with erythema infectiosum. (B) Rash symptoms with an erythematous maculopapular pattern in the form of ringlets in the arm and shoulders of patient A with acute parvovirus B19 infection. See also Color Plate 27.6.

may be involved both in causing and/or triggering the autoimmune and inflammatory process (Nocton *et al.*, 1993; Lehmann *et al.*, 2002; Oguz *et al.*, 2002; Lehmann *et al.*, 2003).

#### Fetal death

The link between parvovirus B19 infection occurring during pregnancy and hydrops fetalis and fetal death was first reported in 1984 (Brown *et al.*, 1984). Normal fetal erythrocytes have a life span of 60–80 days (Pearson, 1966), but this figure decreases in preterm infants of low birth weight (Kaplan and Hsu, 1961). In addition, during the second trimester, fetal red cell mass increases 30-fold (Gray *et al.*, 1987). Therefore the fetus is extremely dependent on this increased rate of erythropoiesis. During fetal parvovirus B19 infection, erythropoiesis is arrested and the affected fetus develops an aplastic crisis with high output cardiac failure and edema (Kinney *et al.*, 1988). Viral myocarditis has also been reported (Naides and Weinen, 1989; Morey *et al.*, 1992a) and may further contribute to the edema. However,



**Figure 27.7** Swelling of the knee joint in a patient with acute B19 infection and arthritis (kindly provided by Dr H. W. Lehmann, Clinic for Pediatrics, University of Giessen). See also Color Plate 27.7.

non-hydropic fetal death has also been reported and may be under-recognized (Skjoldebrand-Sparre *et al.*, 2000). Congenital syndromes have been reported following intrauterine infection (Weiland *et al.*, 1987); however, studies have shown that this is rare (Public Health Laboratory Service, 1990; Guidozzi *et al.*, 1994).

Primary parvovirus B19 infection during pregnancy occurs with an incidence of 1–5 percent (Porter *et al.*, 1988; Bruu and Flugsrud, 1994; Kerr *et al.*, 1994; Harger *et al.*, 1998) and has been estimated to result in fetal infection in approximately 30 percent of maternal infection cases (Public Health Laboratory Service, 1990). Early reports of the incidence of fetal death after acute parvovirus B19 infection in pregnancy were sometimes in excess of 30 percent (Mortimer *et al.*, 1985a); however, the actual incidence is probably of the order of 1.6 percent (Gratacos *et al.*, 1995; Harger *et al.*, 1998; Miller *et al.*, 1998), and the likelihood of a favorable outcome is therefore very high (Public Health Laboratory Service, 1990; Gratacos *et al.*, 1995). However, in pregnant women with a confirmed acute infection, the risk of an abnormal outcome is 5–10 percent (Public Health Laboratory Service, 1990; Rodis *et al.*, 1998). It has been estimated by meta-analysis that parvovirus B19 infection accounts for 15–20 percent of cases of non-immune hydrops fetalis (NIHF) (Yaegashi *et al.*, 1999). The likelihood of hydrops fetalis from B19 infection appears to be greatest between 11 and 23 weeks gestation, which coincides with the hepatic period of hemopoietic activity (Yaegashi *et al.*, 1998, 1999). The case fatality rate in B19-associated hydrops fetalis is of the order of 50 percent (Smolonicie and Pillai, 1994; Miller *et al.*, 1998).

#### Pure red cell aplasia (PRCA)

Patients with immunosuppression may not be able to control B19 viremia, which may result in persistently low titer B19 viremia accompanied by PRCA and chronic anemia. Conditions predisposing to persistent B19 viremia and PRCA include AIDS (Naides *et al.*, 1993), chemotherapy for acute lymphoblastic leukemia (Kurtzman *et al.*, 1988; Rao

*et al.*, 1990), acute myeloblastic leukemia (Weiland *et al.*, 1989), chronic myelomonocytic leukemia (Gilsanz *et al.*, 1995), chronic myeloid leukemia (Frickhofen *et al.*, 1992), myelodysplastic syndrome (Baurmann *et al.*, 1992; Hasle *et al.*, 1996), chemotherapy for cancer (Graeve *et al.*, 1989), transplantation of bone marrow (Niitsu *et al.*, 1990), heart (Amiot *et al.*, 1998), and liver (Ndimbie *et al.*, 1996), steroid treatment of systemic lupus erythematosus (SLE) (Koch *et al.*, 1990), and congenital immunodeficiency including Nezelof syndrome (Kurtzman *et al.*, 1987), common variable immunodeficiency (Davidson *et al.*, 1989), and severe combined immunodeficiency (Gahr *et al.*, 1991). Persistent B19 infection is thought to result from a defect in the humoral immune response to B19 structural proteins such that the virus is not neutralized (Kurtzman *et al.*, 1989a). Normal human immunoglobulin, which provides these neutralizing antibodies, is the only specific treatment. However, there is accumulating evidence that persistent B19 viremia does not always result in the development of anemia or PRCA. LaMonte and co-workers (2004) recently confirmed that low level viremia may exist both in immunosuppressed and in immunocompetent children without the development of symptomatic anemia. In addition we have observed that immunosuppressed adult renal and bone marrow transplantation patients may be asymptomatic during phases of low level viremia ( $10^3$ – $10^5$  genome equivalents per ml). Reactivation to high level viremia may result in recurrent phases of severe anemia and reticulocytopenia (Liefeldt *et al.*, 2005; Plentz *et al.*, 2004). It is not known whether the development of anemia is dependent on the virus load or is influenced by individual host factors.

### Clinical syndromes less commonly associated with parvovirus B19 infection

#### Cutaneous manifestations

Various cutaneous eruptions have been reported in addition to the classic picture of erythema infectiosum (Table 27.1). Petechial and purpuric rashes, including Henoch-Schönlein purpura, have been reported in association with acute B19 infection, both with and without thrombocytopenia (Lefrère *et al.*, 1985, 1986; Mortimer *et al.*, 1985b; Shiraishi *et al.*, 1989; Heegaard *et al.*, 1999b). Papular eruptions have also been described, including papular purpuric 'gloves and socks' syndrome (Figure 27.8) (Bagot and Revuz, 1991; Halasz *et al.*, 1992) and Gianotti-Crosti syndrome (Lacaze *et al.*, 1987; Borreda *et al.*, 1991). Other cutaneous manifestations, reported rarely, include desquamation (Dinerman and Corman, 1990), vesiculobullous and vesiculopustular eruptions, including erythema multiforme (Naides *et al.*, 1988; Lobkowicz *et al.*, 1989; Garcia-Tapia *et al.*, 1993), livedo reticularis (Dereure *et al.*, 1995), and erythema nodosum (Imbert *et al.*, 1989).

#### Hematological manifestations

B19 infection has been associated with aplastic anemia in patients who do not have a recognized immune deficiency state (Bertoni *et al.*, 1997; Osaki *et al.*, 1999) and has been



**Figure 27.8** Papular purpuric 'gloves and socks' syndrome (PPGSS) associated with acute parvovirus B19 infection (kindly provided by Dr Fölster-Holst, Clinic for Dermatology, Venereology und Allergology, Universitätsklinik Schleswig-Holstein, Campus Kiel). See also Color Plate 27.8.

associated with a variety of other hematological manifestations. Thrombocytopenia and idiopathic thrombocytopenic purpura have been described in association with B19 infection in children and adults and this may or may not be clinically apparent (Anderson *et al.*, 1985; Potter *et al.*, 1987; Foreman *et al.*, 1988; Yoto *et al.*, 1993; Murray *et al.*, 1994; Oeda *et al.*, 1994; van Elsacker-Niele *et al.*, 1996; Heegaard *et al.*, 1999a,b) and appear to respond to intravenous immunoglobulin in some cases (Heegaard *et al.*, 1999a). The pathogenesis of B19-associated thrombocytopenia may be mediated either centrally or peripherally (Kaplan *et al.*, 1992) where the central type results from bone marrow suppression and NS1-induced cytotoxicity to megakaryocytes (Ozawa *et al.*, 1988) and the peripheral type is associated with antiplatelet autoantibodies and enhanced clearance by the reticuloendothelial system (Foreman *et al.*, 1988; Oeda *et al.*, 1994).

Neutropenia occurring during the acute phase of B19 infection has been reported (Anderson *et al.*, 1985; Potter *et al.*, 1987; Doran and Teall, 1988; Hanada *et al.*, 1989; Naides and Weiner, 1989; Wodzinski and Lilleyman, 1989; Koch *et al.*, 1990; Pont *et al.*, 1992; Gautier *et al.*, 1997), and has been shown to correlate with bone marrow positivity for B19 virus (McClain *et al.*, 1993).

B19 infection has been demonstrated in transient erythroblastopenia of childhood (TEC) (Wodzinski and Lilleyman, 1989; Muir and Fitzsimmons, 1992; Nagai *et al.*, 1992; Nikkari *et al.*, 1993; Miyata *et al.*, 1994), which is a disorder of young children characterized by anemia and reticulocytopenia. However, the particular role that B19 virus plays in the pathogenesis of TEC, if any, remains unclear (Young and Alter, 1993; Rogers *et al.*, 1996).

Acute B19 infection has been associated with virus-associated hemophagocytic syndrome (VAHS) in both children and adults (Uiike *et al.*, 1993; Shirono and Tsuda,

1995; Wakamoto and Miyazaki, 1996; Hoang *et al.*, 1998; Matsumoto *et al.*, 1998; Sadahira *et al.*, 1998; Toyoshige and Takahashi, 1998; Sano *et al.*, 1999; Barah *et al.*, 2001). VAHS is a virus-induced disease characterized by macrophage proliferation with marked hemophagocytosis and cytopenia, and may be precipitated by infection with a variety of viral and other agents. B19-associated VAHS typically occurs in previously immune competent persons, but may also occur in those with underlying immune suppression. It is typically benign, self-limiting, and reversible, although it may also be fatal (Barah *et al.*, 2001).

As mentioned previously, persistent B19 infection has frequently been reported in cases of acute leukemia, many of whom are undergoing chemotherapy. These cases typically present with anemia in the absence of rash and arthralgia. B19 infection has also been associated with cases of acute myeloblastic and lymphoblastic leukemia at onset of disease (Kerr *et al.*, 2003a) and also preceding leukemic disease onset (Heegaard *et al.*, 2001a; Fisgin *et al.*, 2002). B19 infection has also been associated with myelodysplasia (Hasle *et al.*, 1994; 1996; Rinn *et al.*, 1995; Yarali *et al.*, 2000; Tezuka *et al.*, 2001). One published study examined serum from 65 patients with ALL for B19 DNA and specific IgM (Heegaard *et al.*, 1999a). The results showed that, although there was one positive case, which was also published separately (Heegaard *et al.*, 2001a), serum anti-B19 IgG was positive in 30 percent of patients, which is consistent with the population prevalence adjusted for age. Another study (Kerr *et al.*, 2003a) examined cerebrospinal fluid (CSF) samples from cases of acute lymphoblastic leukemia (ALL) ( $n = 14$ ), two of which were of T-cell origin, and acute myeloblastic leukemia (AML) ( $n = 2$ ) along with CSF samples from a comparison group consisting of benign intracranial hypertension (BIH) ( $n = 10$ ) and hydrocephalus with a ventriculoperitoneal shunt ( $n = 13$ ) presenting during an outbreak of parvovirus B19 in the UK (1997–98), for B19 DNA using nested PCR for the NS1 and VP1 genes. Four leukemia cases were positive – two patients with common ALL, one with null cell ALL, and one with M7-AML – whereas all controls were negative. All four patients were significantly anemic, but none was encephalitic or had evidence of central nervous system leukemia. In three of these patients, serum TNF- $\alpha$ , interferon- $\gamma$ , interleukin 6, granulocyte-macrophage colony stimulating factor (range, 34.93–3800.06 pg/ml), and macrophage chemoattractant protein 1 were detectable (Kerr *et al.*, 2003a). All of these four patients carried at least one of the HLA-DRB1 alleles, which have been associated with symptomatic parvovirus B19 infection (Kerr *et al.*, 2002b). Erythroid suppression, immune cell proliferation, and HLA class II alleles are associated with B19 infection and are also thought to be important in the pathogenesis of acute leukemia. This potential association requires further investigation.

Acute B19 infection has been associated with lymphadenopathy (Plummer *et al.*, 1985; Kerr *et al.*, 1996, 2002a), necrotizing lymphadenitis (Johnson *et al.*, 2003),

and chronic necrotizing lymphadenitis (Kikuchi's disease) in association with SLE (Meyer *et al.*, 1991). B19-associated hypersplenism has also been reported (Currie *et al.*, 1992).

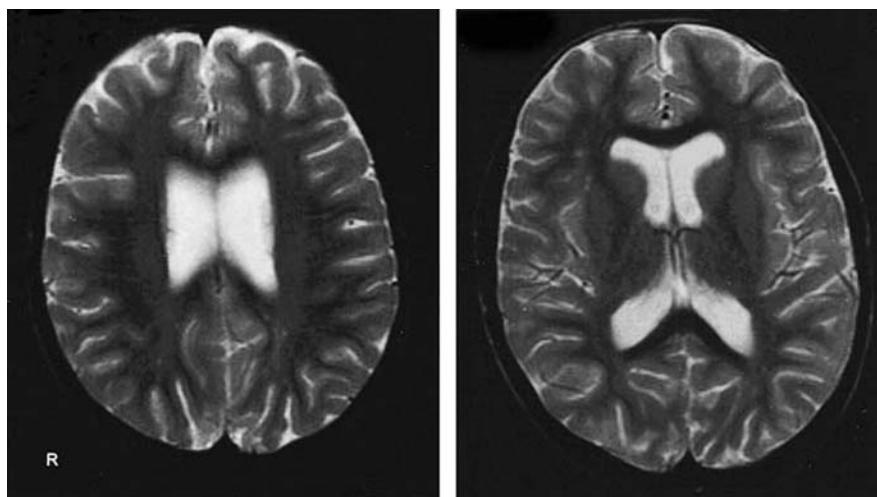
In addition to the anemia observed in B19-associated fetal hydrops, B19 infection has also been associated with congenital anemia albeit at low incidence (Brown *et al.*, 1994c; Heegaard *et al.*, 1996; Rugolotto *et al.*, 1999). One study describes three infants with B19-associated congenital anemia following transplacental infection in which the bone marrow was positive for B19 DNA while the sera were negative (Brown *et al.*, 1994c). Another study reported B19 DNA positivity in bone marrow from 3 of 11 cases (Heegaard *et al.*, 1996).

#### *Hepatobiliary disease*

Parvovirus B19 infection has been associated with mildly raised liver enzymes in adults and neonates (Naides, 1987; Tsuda, 1993), and acute childhood hepatitis (Yoto *et al.*, 1994; Sokal *et al.*, 1998; Hillingsø *et al.*, 1998; Drago *et al.*, 1999). Although the virus has been proposed as a causative agent of non-A, non-B, non-C hepatitis, and fulminant liver failure with or without aplastic anemia requiring liver transplantation (Langnas *et al.*, 1995; Karetnyi *et al.*, 1999), studies are conflicting (Safadi *et al.*, 2001; Wong *et al.*, 2003).

#### *Cardiovascular disease*

In fetal infection associated with hydrops fetalis, parvovirus B19 DNA could be shown to be present in the nuclei of myocytes indicating a cardiac tropism of the infection (Porter *et al.*, 1988; Naides and Weiner, 1989; Berry *et al.*, 1992; Morey *et al.*, 1992a,b). The infection of myocardial cells is thought to contribute to the development of hydrops fetalis (see section above on *Clinical syndromes commonly associated with parvovirus B19 infection*). During the past years parvoviral genome sequences have been shown to be present in a high number of myocardial biopsies as well of adult and pediatric patients with myocarditis, dilated cardiomyopathy and perimyocarditis as in healthy transplant donors (Bültmann *et al.*, 2003; Lotze *et al.*, 2003; Pankuweit *et al.*, 2003; Donoso Manke *et al.*, in press). This may be used as an indication that parvovirus B19 may persist in a stage of latency in myocardial cells. The question as to whether viral proteins are produced in this setting has not yet been answered. However, in pediatric cardiac transplant patients parvovirus B19 infection has been reported to cause generalized disease as well as possible myocarditis (Nour *et al.*, 1993; Janner *et al.*, 1994; Enders *et al.*, 1998; Murry *et al.*, 2001; Dettmeyer *et al.*, 2003). In addition several groups have reported that acute parvovirus B19 infection may be associated with myocarditis both in children and in adults (Papadogiannakis *et al.*, 2002; Lamparter *et al.*, 2003; Munro *et al.*, 2003). It is clear that as parvovirus B19 infection is common and the development of myocarditis is rare, important, but as yet unknown factors are involved in the causation of parvovirus B19-associated heart disease.



**Figure 27.9** *T2 weighted magnetic resonance imaging of the head of a 9-year-old boy who suffered B19-associated meningoencephalitis during the acute phase, showing enlarged ventricles and high signal intensity within the white matter. This patient recovered from the acute illness but had severe and irreversible neurological sequelae following the infection.*

#### *Neurological disease*

Neurological complications of erythema infectiosum were recognized prior to the discovery of B19 virus (Balfour *et al.*, 1970; Breese and Homer, 1977). It has subsequently been shown that several neurological syndromes, which may complicate erythema infectiosum, are associated with positivity for virus-specific IgM and/or B19 DNA in serum and/or cerebrospinal fluid. These syndromes include encephalitis/encephalopathy (Conry *et al.*, 1993; Watanabe *et al.*, 1994; Heegaard *et al.*, 1995; Haseyama *et al.*, 1997; Isumi *et al.*, 1999; Barah *et al.*, 2001), aseptic meningitis (Tsuiji *et al.*, 1989; Cassinotti *et al.*, 1993; Okumura and Ichikawa, 1993; Koduri and Naides, 1995; Suzuki *et al.*, 1995; Tabak *et al.*, 1999; Sinclair *et al.*, 1999; Druschky *et al.*, 2000), Guillain-Barré syndrome (Winer *et al.*, 1988; Minohara *et al.*, 1998; Yamaoka *et al.*, 2000), cerebellar ataxia (Barah *et al.*, 2001; Torok, 1995), transverse myelitis (Nagahama *et al.*, 1992), neuralgic amyotrophy (Denning *et al.*, 1986; Walsh *et al.*, 1988; Pellas *et al.*, 1993; Staud *et al.*, 1995; Maas *et al.*, 1996; Puechal *et al.*, 1998; Kirchoff-Moradpour *et al.*, 2001), peripheral neuropathy (Faden *et al.*, 1990, 1992; Wilhelm *et al.*, 1998), and carpal tunnel syndrome (Samii *et al.*, 1996; Kerr *et al.*, 2002a).

The incidence of parvovirus B19 meningoencephalitis in the UK was studied during an outbreak period by testing cerebrospinal fluid samples from 162 patients suffering from undiagnosed meningoencephalitis for the presence of parvovirus B19 DNA. Viral genomes were detected in 7 (4.3 percent) cerebrospinal fluid samples. This study also reported that parvovirus B19-infected patients who exhibit meningoencephalitis may also suffer other symptoms, such as pyrexia, skin rash, arthralgia, lymphadenopathy, raised liver enzymes, and anemia, which might provide a clue to the parvoviral etiology (Barah *et al.*, 2001). Two of these

cases of B19 meningoencephalitis were diagnosed one day following birth, and presumably followed an intrauterine infection with parvovirus B19. After several years follow-up, three patients had neurological sequelae, all of whom had abnormal brain scans (enlarged ventricles, high signal intensity from white matter) at the time of presentation of acute neurological illness (Figure 27.9). One patient's symptoms had lasted 40 days when they were abrogated by administration of oral corticosteroids, but returned after cessation of therapy; subsequently a course of intravenous immunoglobulin led to a complete cure in this patient (Barah *et al.*, 2001). Success with intravenous immunoglobulin (IVIG) therapy for B19-associated neurological disease has previously been reported (Nigro *et al.*, 1994a).

The mechanism by which parvovirus B19 causes CNS damage is not clear. Although viral DNA has been detected by several groups in cerebrospinal fluid samples of these cases (Cassinotti *et al.*, 1993; Okumura and Ichikawa, 1993; Watanabe *et al.*, 1994; Yoto *et al.*, 1994), detection in brain tissue is rare (Isumi *et al.*, 1999). Where such tissue is available, histological examination suggests an immune-mediated pathogenesis rather than direct viral cytotoxicity. The neuronal cell loss in those who suffer neurological sequelae (Barah *et al.*, 2001; Kerr *et al.*, 2002c) is likely the result of demyelination, which is a recognized complication of viral infection of the central nervous system and believed to be caused by an inappropriate immune response (Stohlman and Hinton, 2001). In addition, markedly raised levels of inflammatory cytokines (IL-6, TNF- $\alpha$ , IFN- $\gamma$ , GM-CSF, and MCP-1) were detected in serum ( $n = 4$ ) and CSF ( $n = 1$ ) from cases of parvovirus B19-associated meningoencephalitis during the acute phase as compared with normal controls who had minimal levels in serum (Kerr *et al.*, 2002c).

**Table 27.2** Autoimmune diseases that are reported in association with parvovirus B19 infection

Involved organs	Disease category	Disease
Joints	Arthralgias Arthritis	Monoarthritis Oligoarthritis Polyarthritis Rheumatoid arthritis (?) Juvenile idiopathic arthritis
Connective tissue/vessels	Systemic lupus erythematosus (SLE) Vasculitis	Leukoclastic vasculitis Purpura Henoch–Schönlein Papular purpuric gloves and socks syndrome (PPGSS) Kawasaki disease? Giant cell arteritis (GCA) Polyarteritis nodosa Wegener's granulomatosis
Blood cells	Dermatomyositis Autoimmune neutropenia Autoimmune thrombocytopenia Idiopathic thrombocytopenic purpura (ITP) Autoimmune hemolytic anemia Virus-associated hemophagocytic syndrome (VAHS)	

#### Rheumatological and autoimmune disease

Parvovirus B19 infection may induce a broad spectrum of autoimmune phenomena. The clinical spectrum ranges from mild arthralgias to severe necrotizing vasculitis (Table 27.2).

**Arthritis.** Parvovirus B19 infection may have an impact on the development of arthritis via several different pathogenic mechanisms. In persons with a genetically determined susceptibility for the development of rheumatic diseases an acute B19 infection is directly followed by long-lasting arthritis. In these cases the infection is the initial trigger for the establishment of the rheumatic disease. In B19-negative patients with pre-existing arthritis the clinical status worsens coincidentally with onset of B19 infection. This phenomenon has not only been observed in patients with idiopathic rheumatic disease but also in arthritis caused by other infectious agents, e.g. *Borrelia* spirochetes. Occasionally, the presence of parvovirus B19 viremia combined with IgM/IgG antibodies against structural proteins VP1 and/or VP2 has been shown in patients with long-lasting polyarthralgia/polyarthritides. Some of these cases meet criteria for diagnosis of rheumatoid arthritis (Naides *et al.*, 1990; Murai *et al.*, 1999; Stahl *et al.*, 2000a). However, development of rheumatoid arthritis after acute parvovirus infection appears to be rare (Nikkari *et al.*, 1994; Moore, 2000).

The fact that parvovirus B19 infections generally occur during childhood or adolescence may explain the finding that B19-induced and/or triggered arthritis is more frequently observed in children than in adults (Lehmann *et al.*, 2002, 2003). Therefore factors affecting the investigation of the role of B19 infection in children with rheumatic

diseases are different from those in adult rheumatic patients. Among 73 adults with different forms of arthritis, only one synovial fluid sample (1.4 percent) contained parvovirus B19 DNA (Cassinotti *et al.*, 1998). In children with rheumatic disease the relation of the B19 infection with arthritis-arthralgias is well recognized. Some children with acute B19 infection may develop chronic arthritis indistinguishable from juvenile idiopathic arthritis (Lehmann *et al.*, 2002; Nocton *et al.*, 1993; Mimori *et al.*, 1994; Oguz *et al.*, 2002). The clinical spectrum encompasses mono-, oligo-, and polyarthritis. Furthermore persisting viremia is frequently observed in children with systemic-onset juvenile idiopathic arthritis. In a group consisting of 74 children with various forms of juvenile arthritis, antibodies against B19 were detected in 62 percent (Lehmann *et al.*, 2003). In 57 percent of these antibody-positive patients, B19-DNA was detected in serum and/or synovial fluid. In many patients the continuous presence of viral particles and immune complexes could be demonstrated in the peripheral blood as well as in synovial fluid. Despite the development and presence of B19-specific antibody, the children showed a prolonged state of viremia or viral persistence in synovial fluid. They were clearly incapable of eliminating the virus. It has been proposed that this may be due to an inadequate immune reaction against the viral capsid proteins (Kurtzman *et al.*, 1989a,b). Both VP1- and VP2-proteins have been shown to contain epitopes that induce the production of virus neutralizing antibodies. However, no defects in production of either VP1- or VP2-specific IgG were observed in pediatric arthritis patients with persistent

B19 infection (Lehmann *et al.*, 2003). The inability of the patients to eliminate B19-virus might be associated with reduced antibody affinity. None of the patients had symptoms of impaired T-cell or general B-cell dysfunctions. The presence of viral DNA in synovial tissue has been shown up to different degrees in patients with arthritis as well as in healthy controls. This indicates that parvovirus B19 infects cells of the synovial membrane and may persist in a latent state (Nikkari *et al.*, 1995; Söderlund *et al.*, 1997; Hokynar *et al.*, 2000; Stahl *et al.*, 2000b,c). It is possible that reactivation may occur sporadically or in association with inflammation owing to rheumatic disease and/or infection with other infectious agents such as *Borrelia* or streptococci.

**Vasculitis.** Parvovirus B19 infection has been associated with various forms of collagenosis and may mimic systemic lupus erythematosus (SLE) in children and adults (Nikkari *et al.*, 1995; Moore, 2000; Narvaez Garcia *et al.*, 2001; Negro *et al.*, 2001; Tovari *et al.*, 2002). Seishima and co-workers (2003) describe 42 adult patients who were studied for more than 2 years after primary parvovirus B19 infection. Three of these patients developed transient arthralgia lasting for up to 6 months. One patient developed persistent arthralgia in combination with antinuclear antibodies and hypocomplementemia. These symptoms were similar to SLE but did not fulfill the criteria for SLE and rheumatoid arthritis.

Similar to the situation in arthritis patients, parvovirus B19 infection has been described as an agent both causing and triggering SLE (Cope *et al.*, 1992; Kalish *et al.*, 1992; Trapani *et al.*, 1999; Hemauer *et al.*, 1999b; Hsu and Tsay, 2001; Diaz *et al.*, 2002). Hsu and Tsay (2001) found that 24 percent of 72 SLE patients contained parvovirus B19 DNA in the peripheral blood. The majority of the viremic SLE patients did not produce antibodies against the B19 structural proteins, an observation that may be explained by the immunosuppressive treatment. In another study VP1/VP2-specific IgG could be demonstrated in 86 percent of adult SLE patients, and 36 percent were shown to be viremic (von Landenberg *et al.*, 2003). Although none of these SLE patients displayed typical signs of acute B19 infection, all had developed antiphospholipid antibodies.

Patients with recent B19 infection may develop leukocytoclastic vasculitis (Chakravarty and Merry, 1999). Vasculitis-like syndromes in association with a B19 infection are also found in patients with Henoch-Schönlein purpura, papular-purpuric gloves and socks syndrome (PPGSS, see above) and Kawasaki disease (Nigro *et al.*, 1994b; Smith *et al.*, 1998; Grilli *et al.*, 1999; Cioc *et al.*, 2002). However, causality of these and other manifestations of autoimmune disease is often difficult to infer. One report of children with Kawasaki disease found B19 DNA in 67 percent; however, other studies have been negative (Cohen, 1994; Yoto *et al.*, 1994; Chua *et al.*, 2000). In patients with giant cell arteritis (GCA) a statistically significant association between histologic evidence of GCA and the presence of B19 DNA in temporal artery biopsies has been described (Gabriel *et al.*, 1999).

Detection of parvoviral RNA by *in situ* reverse transcription polymerase chain reaction (RT-PCR) demonstrated that the endothelial cells and surrounding mononuclear cells were the viral targets in various connective tissue diseases. Using immunohistochemistry, viral protein was shown to coincide with endothelial cell distribution in these tissue specimens (Magro *et al.*, 2002). Polyarteritis nodosa has been linked with acute or persistent B19 infection and may be cured by immunoglobulin treatment (Corman and Dolson, 1992; Viguier *et al.*, 2001; Durst *et al.*, 2002). Lewkonia *et al.* (1995) and Chevrel *et al.* (2000) reported the detection of viral genomes in muscle biopsies of a child and a 48-year-old woman with dermatomyositis respectively. However this appears to be rare. In a follow-up study all seven new patients were B19-DNA negative in muscle biopsies (Chevrel *et al.*, 2003). In addition parvovirus B19 has been implicated in Wegener's granulomatosis (Nikkari *et al.*, 1994; Finkel *et al.*, 1994).

#### Chronic fatigue syndrome (CFS)

Several groups have documented an association between acute parvovirus B19 infection and the subsequent development of fatigue (Adlakha and Schultz, 1994; Hillingsø *et al.*, 1998; Hayakawa *et al.*, 2002b; Kerr *et al.*, 2002a; Keonigbauer *et al.*, 2000; Manato *et al.*, 2003), CFS (Jobanputra *et al.*, 1995; Kerr *et al.*, 1996; Jabobsen *et al.*, 1997; Kerr *et al.*, 2002a, Chia and Chia, 2003) and the related disorder, fibromyalgia (Leventhal *et al.*, 1991). Regarding the diagnosis of parvovirus B19-associated CFS (in all of the cases except one) (Jobanputra *et al.*, 1995), the CDC criteria for the diagnosis (Fukuda *et al.*, 1994) were fulfilled. Four studies have reported the proportion of CFS patients associated with active parvovirus B19 infection: 0 percent (0/22) (Koelle *et al.*, 2002), 1.5 percent (3/200) (Chia and Chia, 2003), 14.3 percent (1/7 with hematologic abnormalities) (Ilaria *et al.*, 1995) to 7.7 percent (4/52) (Kerr *et al.*, unpublished data). Because several different infections may precipitate CFS (Chia and Chia, 2003), the proportion resulting from any one agent, such as parvovirus B19, is likely to vary according to the sampling strategy, time and place, and its relation to the prevalence of each infection (Kerr and Tyrrell, 2003).

One study reported that 13 of 39 cases of acute symptomatic B19 infection had prolonged fatigue that was associated with raised circulating levels of interferon- $\gamma$  (IFN- $\gamma$ ) ( $\geq 7$  pg/ml) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) ( $\geq 40$  pg/ml;  $P = 0.0275$ ) as compared with B19 convalescence without fatigue and normal cases (Kerr *et al.*, 2001, 2002a). In 5 of these 13 cases with fatigue, their symptoms fulfilled the CDC criteria for a diagnosis of CFS. Mean circulating IFN- $\gamma$  and TNF- $\alpha$  in these five CFS cases were 8.37 and 65.71 pg/ml, respectively (Kerr *et al.*, 2001). The above abnormalities have also been documented among the multiple immune defects that have been reported in cases of idiopathic CFS (Kerr and Tyrrell, 2003). Four of the five were viremic at follow-up, suggesting the use of a serum test for B19 DNA in the investigation of CFS. Among cases

of acute symptomatic parvovirus B19 infection, the symptom of fatigue was associated with carriage of the shared epitope sequence at position 70–74 of the third hypervariable region of the DRB1 chain ( $P = 0.047$ ) (Kerr *et al.*, 2002b). This forms part of the peptide-binding groove, which is critical in determining which antigenic peptides are presented to T cells and which is a risk factor for the development and severity of RA (Brown *et al.*, 1993b; Stern *et al.*, 1994).

A recent study on the safety and immunogenicity of a recombinant parvovirus B19 vaccine revealed short-lived fatigue occurring in eight of 24 healthy volunteers after vaccination (Ballou *et al.*, 2003). Since these recombinant virus-like particles lack DNA and are replication-defective, this observation may suggest that B19-associated fatigue may occur in the absence of virus replication.

**Systemic sclerosis.** Several reports have described an association between parvovirus B19 infection and the development of systemic sclerosis (SSc) and Raynaud phenomenon (Harel *et al.*, 2000). Since these are rather rare disorders, the data are not clear and rather difficult to interpret. Initially Ferri and coworkers (1999) described the presence of parvovirus B19 DNA in the bone marrow in 12 of 21 patients with SSc in comparison with none of the control group. None of these patients displayed viremia, but NS1-specific IgG as a marker of persistent infection was frequently observed. A similar association between persistent parvovirus B19 infection and the development of SSc was reported by other groups (Hamamdzic *et al.*, 2002; Lehmann *et al.*, 2002). Recently Magro and co-workers (2004) studied a group of 12 SSc patients: nine showed B19-specific IgM and/or IgG antibodies and in four of these patients viral DNA was detected in the skin and/or bone marrow. These patients also displayed enhanced expression of TNF- $\alpha$  in the tissue together with prominent deposition of C5b-9 in the cutaneous vasculature. The data indicate that parvovirus B19 may be involved in the development of systemic sclerosis, however not exclusively.

**Uveitis.** Two cases of B19 associated uveitis have been reported: one in an adult with EI, tonic pupils, and ophthalmoplegia (Corridan *et al.*, 1991) and one in a young girl associated with transient antinuclear antibody and rheumatoid factor production (Maini and Edelstein, 1999). Anti-B19 IgG was found in vitreous fluid from three of six IgG seropositive cases of chronic intermediate uveitis, but none had evidence of local anti-B19 IgG production (De Boer *et al.*, 1993).

#### *Renal disease*

Renal disorders have been reported to be occasionally associated with acute parvovirus B19 infections. They present as proteinuria, hypocomplementemia, and erythrocyte casts in urine sediment (Bleumink *et al.*, 2000; Taylor *et al.*, 2001). Immunofluorescence microscopy of renal biopsies revealed C3c deposits with immunoglobulins along the glomerular capillary walls and in the walls of small arteries and arterioles. Electron microscopy showed swelling of the

endothelial cells and small electron dense deposits in mesangium consistent with immunocomplex-mediated nephritis (Takeda *et al.*, 2001). In addition, B19 infection has been linked to collapsing glomerulopathy, a distinct variant of focal and segmental glomerulosclerosis (Moudgil *et al.*, 2001).

## Treatment

For uncomplicated cases of erythema infectiosum, usually no therapy is required; however, paracetamol may be useful to reduce temperature. For TAC and TEC, hospital admission and supportive blood transfusion are mandatory until recovery of erythropoiesis.

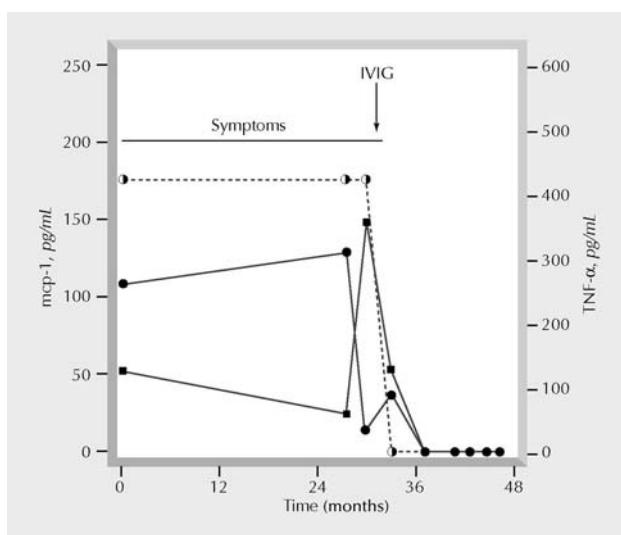
For parvovirus B19-associated transient arthralgia, rest and non-steroidal anti-inflammatory drugs have been recommended.

#### IMMUNOGLOBULIN TREATMENT

During the last decade the treatment of various sequelae of chronic parvovirus B19 infection with high dose intravenous immunoglobulin (IVIG, 0.4 g/kg/day body weight over 5 days) has emerged as a therapeutic option that may frequently be beneficial. However, clinical studies reporting on IVIG therapy as a general tool to improve the various symptoms associated with B19 infections are not available. Therefore until today there is no general therapeutic regimen for the treatment of parvovirus B19-associated rheumatic disease.

Most experience of the use of IVIG to treat B19 infection has been gained in HIV-infected and bone marrow and organ transplant patients. Solid organ transplant patients (e.g. kidney) have also been shown to become infected by parvovirus B19 and respond to IVIG treatment (Yango *et al.*, 2002; Liefeldt *et al.*, 2005). Furthermore, successful treatment of idiopathic thrombocytopenic purpura and polyarteritis nodosa associated with parvovirus B19 infection has been reported (Heegaard *et al.*, 1999a, 1999b; Wehmaier *et al.*, 2000; Magro *et al.*, 2002). For PRCA, IVIG has been found to be curative, although with profound immunosuppression, more than one course may be required (Heegaard and Brown, 2002). The clinical status of B19-triggered oligo- and polyarthritic children has been improved with high dose intravenous immunoglobulins shown to be rich in virus-specific antibodies (Stahl *et al.*, 2000d; Lehmann *et al.*, 2004). However, the question as to whether this treatment is able to induce a long-lasting symptom-free period or a final cure has yet to be answered. Besides antiviral treatment with immunoglobulins, all patients with autoimmune disease require an anti-inflammatory and immunosuppressive treatment according to the treatment guidelines for the particular disease.

IVIG also improves B19-associated meningoencephalitis and CFS (Nigro *et al.*, 1994a; Jacobsen *et al.*, 1997; Barah



**Figure 27.10** Time course of a patient with parvovirus B19-associated chronic fatigue syndrome (CFS) showing duration of clinical symptoms, serum viral DNA (half circle), serum cytokines (TNF- $\alpha$ ; full circle), and MCP-1 (square), before and after the administration of intravenous immunoglobulin (IVIG). (Reproduced from Kerr and Tyrrell, 2003 with permission.)

et al., 2001; Kerr et al., 2003b). Kerr and co-workers have treated three cases of B19-associated CFS, which followed acute parvovirus infection, with a 5-day course of IVIG (0.4 g/kg/day). During the pretreatment phase of the illness, each patient was viremic and had consistently elevated levels of MCP-1, TNF- $\alpha$ , IFN- $\gamma$  and IL-6. IVIG therapy led to clearance of parvovirus B19 viremia, resolution of symptoms with improvement in physical and functional ability in all patients, and resolution of cytokine dysregulation (Figure 27.10). In one of these patients who was negative for B19-specific IgG, these antibodies were detected for the first time after IVIG treatment and its appearance was associated with an isolated increase in IL-2 and IL-4, cytokines that are known to be important in immunoglobulin class switching (Spellberg and Edwards, 2001; Fehmiger et al., 2002).

#### INTRAUTERINE BLOOD TRANSFUSION

In pregnant women, there are several indications for investigation of suspected B19 infection: rash illness, unexplained arthralgia, maternal contact with B19 infection, non-immune fetal hydrops, unexplained maternal polyhydramnios, and elevation of serum alphafetoprotein (AFP). If a maternal serum IgM test is positive, the diagnosis of maternal B19 infection is confirmed. However, if the test is negative, it should be remembered that maternal parvovirus B19-specific IgM may become undetectable some weeks after infection and that fetal death can occur from 1 to  $>20$  weeks after a maternal B19 infection and therefore in the absence of detectable B19-specific IgM-antibodies in

the serum samples of the pregnant women (Nyman et al., 2005; Lehmann and Woodrow, unpublished). Following contact with an B19-infected individual, confirmation of the status of infection by PCR is obligatory. In addition a susceptible mother should be tested 4 weeks post contact; it should be remembered that a subsequent maternal B19 infection may not be symptomatic. A detailed ultrasound is indicated on the finding of fetal hydrops or polyhydramnios. Fetal blood should then be tested for the presence of B19 DNA and hemoglobin, white cell, and platelet counts. Fetal blood transfusion should be considered for severe fetal anemia (hemoglobin  $<8$  to 10 mg/dl) (Knöll et al., 2002). In general, further management aims to support the fetus with blood transfusion.

#### Prevention

Until now a vaccine to prevent parvovirus B19 infection was not available. Purified recombinant empty capsids consisting of VP1/VP2 proteins expressed by baculovirus vectors have been used in a phase I trial and showed the successful induction of B19-neutralizing antibodies in volunteers (Ballou et al., 2003).

### SIMIAN ERYTHROVIRUSES

#### Discovery and history

Although parvovirus B19 is the only officially recognized member of the erythroviruses, there are several other proposed members of this genus. Simian parvovirus (SPV) was first identified in 1992 in a group of anemic cynomolgus monkeys (O'Sullivan et al., 1994); cloning and sequencing of viral DNA confirmed the isolation of a novel SPV (Brown et al., 1995). A second outbreak of anemia in pig-tailed macaques used for experimental SHIV-2 infection was shown to be due to a new parvovirus with limited sequence homology to both B19 and SPV, and was named pig-tailed macaque parvovirus (PmPV) (Green et al., 2000). A third outbreak of anemia in rhesus macaques at Kansas University Medical Center was shown to be due to a novel virus with limited sequence homology to parvovirus B19, SPV, and PmPV, and was named Rhesus parvovirus (RmPV) (Brown and Young, 1997). These animals were again involved in infection studies using SHIV-2.

#### Epidemiology

In both the published outbreaks of SPV infection (O'Sullivan et al., 1994; 1996), monkeys with severe anemia also had concurrent active infection with type D simian retrovirus (SRV), which is known to be immunosuppressive. In the second of these outbreaks, monkeys were involved in

preclinical drug trials for an experimental antidiabetic drug (O'Sullivan *et al.*, 1996). In both outbreaks, there was evidence of asymptomatic infection, a phenomenon which was confirmed in experimental infection of cynomolgus monkeys (O'Sullivan *et al.*, 1997). Therefore, it is likely that most SPV infections are subclinical.

The seroprevalence of SPV in primates has been studied using a recombinant Western blot assay. Results showed that approximately 50 percent positivity in captive cynomolgus monkeys, and approximately 35 percent in captive rhesus macaques (which may represent detection of antibodies to RmPV, which may cross-react with SPV proteins) (Brown and Young, 1997). Seroprevalence in wild monkeys remains undetermined.

It is thought that transmission of SPV occurs via the respiratory route or via fomites, as during the second outbreak (O'Sullivan *et al.*, 1996); the animals were separated in cages, but shared the same ventilation system (Brown and Young, 1997).

## Clinical manifestations of infection

In the first outbreak of SPV infections, the monkeys were initially healthy when they became ill with diarrhea and dehydration. A moderate anemia also occurred with a hemoglobin of 7.4 g/dL that decreased over a week to a level of 3.8 with a reticulocyte count of zero. The animal deteriorated despite receiving a blood transfusion and was killed (Brown *et al.*, 1997). Other monkeys developed severe normocytic normochromic anemia and reticulocytopenia. Tissues showed widespread infection with type D SRV, which causes immunosuppression and is common in captive monkeys. In addition to signs of SRV infection (splenomegaly and lymphadenopathy), the most pronounced post-mortem finding was within the bone marrow. Microscopic bone marrow examination showed reduced mature erythroid and myeloid cells, the presence of many medium to large undifferentiated cells, and increased numbers of megakaryocytes. Intranuclear inclusions were also found with degenerate giant pronormoblasts. Ultrastructural examination of inclusions revealed ~24 nm virus particles. All of these features correspond to the morphology of human bone marrow infected with parvovirus B19 (Brown and Young, 1997).

## Experimental infection

O'Sullivan and colleagues (1997) experimentally inoculated cynomolgus monkeys with SPV to investigate the potential of this system as an animal model of human parvovirus B19 infection. Six adult animals received SPV via either the intranasal or intravenous route and were then studied for clinical manifestations and hematological and virological profiles. A low grade viremia was universal, the

peak of which coincided with appearance of specific antibodies with consequent virus clearance. The symptoms at this stage were mild, some suffering with anorexia and weight loss, unlike human volunteers who suffered the characteristic rash of erythema infectiosum and arthropathy on appearance of specific antibody (Anderson *et al.*, 1985). Red cell destruction was manifested by a mild decrease in hemoglobin. Monkeys with prior presence of SPV antibody were immune to re-infection, as previously found with parvovirus B19 studies in humans (Anderson *et al.*, 1985). Therefore, this animal system revealed that SPV infection of monkeys was highly similar to infection of normal humans with B19 virus, in that the infection was mild and self-limited with transient cessation of erythropoiesis. Therefore, study of SPV infection in cynomolgus macaques may be useful as an animal model in order to inform our understanding of the various aspects of the pathogenesis of human parvovirus B19 infection, which presently remains unclear.

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# Rodent parvovirus infection and associated disease

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Rodent parvoviruses provide important models for studying virus – host interactions *in vitro* and *in vivo*. The small genome is easily manipulated *in vitro* to elucidate the roles of viral proteins in infection and cell tropism. Experimental infection of mice and rats facilitates *in vivo* analysis of parvoviral pathogenesis and identification of host factors involved in viral clearance, viral persistence, and prevention of infection.

Parvoviruses are a major cause of adventitious infections in laboratory rodents and they can distort biological responses that depend on cell proliferation. Natural infections tend to be tenacious and often require laborious and costly countermeasures such as intensive serologic monitoring and rederivation or replacement of animals, all of which disrupt research. Therefore, understanding the pathogenesis and epidemiology of rodent parvoviruses is essential for detection, containment, elimination, and prevention of infection.

Most parvovirus infections are clinically silent, although weanling and immunocompromised rodents are susceptible to persistent infection with prolonged excretion. Pathogenic and/or lethal infections typically occur in fetal or neonatal rodents, which have numerous mitotically active cells and an immature immune system. This chapter will describe *in vivo* infections for each laboratory rodent species, and compare infections for species infected by multiple parvoviral strains.

## **PARVOVIRUSES OF MICE**

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Crawford discovered the first parvovirus of mice, minute virus of mice (MVM), as a contaminant of a mouse adenovirus stock (Crawford, 1966). After culture adaptation and plaque purification in fibroblast cells, Tattersall and Bratton

designated this prototype strain MVMp (Tattersall and Bratton, 1983). An allotropic variant, designated MVMi, was isolated from a transplantable mouse lymphoma by Bonnard and co-workers (Bonnard *et al.*, 1976) and was shown to inhibit lymphocyte functions *in vitro* (Engers *et al.*, 1981; McMaster *et al.*, 1981). Molecular and *in vitro* differences between MVMi and MVMp are described in more detail elsewhere in this book.

During the mid-1980s, diagnostic testing of mouse sera for MVM antibodies detected samples that did not uniformly react with MVM antigens, implying the presence of an antigenically distinct parvovirus in mouse colonies (Smith *et al.*, 1993). McKisic and colleagues subsequently isolated a lymphocytotropic parvovirus from maintenance cultures of established murine T-cell clones (McKisic *et al.*, 1993). This new 'orphan' parvovirus had adverse effects on *in vitro* immune responses: it grew lytically in a CD8<sup>+</sup> T-cell clone designated L3 and inhibited the proliferation of cloned T cells stimulated with antigen or interleukin-2 (IL-2). In contrast to MVMi, it did not inhibit the generation of cytotoxic T cells in mixed lymphocyte cultures. Shortly thereafter, two additional 'orphan' parvoviruses with similar characteristics were isolated from the spleens of naturally infected mice (Smith *et al.*, 1993; Besselsen *et al.*, 1996), and serologic studies confirmed that all three isolates were antigenically distinct from MVM. They were subsequently renamed mouse parvovirus (MPV) after sequence analysis confirmed that isolates in this new serogroup had numerous nucleotide and coding differences from MVM (Ball-Goodrich and Johnson 1994).

## **Minute virus of mice (MVM)**

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The mouse is the natural host of MVM, although experimental infections have been induced in rats and hamsters

by parenteral or prenatal inoculation of virus (Kilham and Margolis, 1970; Toolan, 1983). MVMi appears to be more pathogenic for mice than MVMp. Kimsey *et al.* found that oronasal inoculation of MVMi caused disseminated, lethal infection in most neonatal mice (Kimsey *et al.*, 1986) and growth-retardation in survivors. MVMp, by contrast, did not induce clinical morbidity or growth retardation but rather localized, asymptomatic infection. Mice infected with either strain developed an antibody response to MVM, but neither infection affected host T-cell function. Brownstein and co-workers (Brownstein *et al.*, 1991) showed that mouse genotype determined susceptibility to lethal MVMi infection. After oronasal inoculation, resistant and susceptible strains of mice developed widely disseminated infection. However, C57BL/6 neonates sustained asymptomatic infection, whereas BALB/c, SWR, SJL, CBA, and C3H mice developed lethal infection, which featured renal papillary hemorrhage. Lethal infection of DBA/2 neonates also included extensive intestinal hemorrhage and occasional intussusception. Equivalent levels of infectious MVMi were detected in resistant (C57BL/6) and susceptible (C3H) mice, although *in situ* hybridization (ISH) and immunohistochemistry (IHC) demonstrated that C57BL/6 mice had fewer infected cells. Primary cell types infected in C3H mice were endothelium, lymphocytes, and hepatic erythropoietic precursors. The authors suggested that accumulation of non-infectious viral products and infection of more cells may explain susceptibility to lethal infection. All strains were resistant to lethal infection by 24 hours after birth.

To delineate MVM genomic regions responsible for pathogenicity, Brownstein *et al.* compared infections of neonatal mice with MVMi, MVM(1035) (a recombinant MVMp genome with the MVMi allotropic determinant; nucleotides 3522–4342), MVMi (NS2–1990) (MVMi that does not express NS2 [non-structural protein]), and MVMp (Brownstein *et al.*, 1992). MVM (1035) and MVMi infected the same target cells (endothelium, lymphoid cells, and hematopoietic cells) and caused lethal infection with renal papillary infarcts. Therefore, MVM sequence from nucleotides 3522 to 4342 conferred *in vivo* tropisms for endothelium and hematopoietic cells in addition to the expected lymphocyte tropism. The cell tropism of MVM (1035) also extended to the external germinal layer of cerebellum and smooth muscle cells of the stomach and colon, none of which was a target of MVMi infection. MVMi (NS2–1990) infection was asymptomatic, although limited viral replication occurred in some MVMi target organs (heart, liver, spleen, and intestine).

In some studies, MVMi infected neonates developed motor disabilities and intention tremors (Segovia *et al.*, 1991; Ramirez *et al.*, 1996). Although Brownstein *et al.* (1991) noted viral infection in brain endothelium, Ramirez *et al.* (1996) also demonstrated replication of MVMi in brain parenchyma. Immunohistochemistry detected viral capsid proteins at post-inoculation day (PID) 2 in the laterodorsal thalamic and the pontine nuclei and at PID 6 in three main germinal

centers of the cerebrum: the subventricular zone, the subependymal zone of the olfactory bulb, and the dentate gyrus of the hippocampus. In the cerebellum, viral capsid proteins were confined to the internal granular layer rather than the proliferative external granular layer. Therefore, MVMi infection appeared to impair postmitotic neuronal migration which occurs during the first week of life.

Hematopoiesis was examined in MVMi infected neonatal BALB/c mice to determine whether *in vitro* tropisms of MVMi for primary myeloid cell cultures (Segovia *et al.*, 1991) occurred *in vivo* and resulted in myelosuppression. Segovia *et al.* (1995) determined that peak infectious virus titers were reached in lung, spleen, thymus, and bone marrow from PID 4 to PID 8. Accumulation of MVM DNA replicative intermediates in tissues was highest at PID 4 and 6 but decreased sharply by PID 8. Humoral immunity to MVM correlated with the decrease in viral replication and titers of infectious virus in tissues, although it did not protect mice from lesions or mortality. By PID 10, most infected mice had significantly decreased cellularity in femoral bone marrow and spleen as well as decreased granulocyte-macrophage progenitors and erythroid progenitors. Maximal mortality (85 percent) at PID 12 correlated with decreased splenic and bone marrow cellularity and hemopoietic precursors.

Segovia *et al.* (1999) extended their analysis to MVMi infection of adult severe combined immunodeficient (SCID) mice. By PID 30, hematological analysis of infected mice revealed acute, severe leukopenia but no significant change in the number of platelets or erythrocytes. However, infection was lethal by PID 100. In the bone marrow, granulocyte-macrophage and erythrocyte clonogenic progenitors were deeply suppressed during maximum MVMi production. Viral infection in the bone marrow was limited to a small subset of primitive cells, which declined in number with disease progression. Infection resulted in a lasting unbalance of marrow hemopoiesis as granulomacrophagic cells were depleted and erythroid cell numbers increased to maintain effective erythropoiesis. Thus, parvovirus infection of SCID mice caused novel dysregulation of murine hematopoiesis. Subsequent research (Lamana *et al.*, 2001) found that intranasal MVMi inoculation of adult SCID mice resulted in a progressive decrease in bone marrow megakaryocyte and myeloid cells to below 1 percent of those in mock-infected animals by PID 35. However, the SCID mice compensated for the depletion of megakaryocyte cells as platelet counts in peripheral blood were close to normal values. An additional study showed that MVMi-infected SCID mice efficiently engrafted immunocompetent lymphohematopoietic precursors (Segovia *et al.*, 2003). The graft rescued the mice from lethal leukopenia, and the progressive recovery of circulating white blood cell numbers correlated with graft size.

Studies of oronasal MVMi infection in immunocompetent adult mice are fragmentary. In unpublished work, we found that infection of adult mice was asymptomatic, and

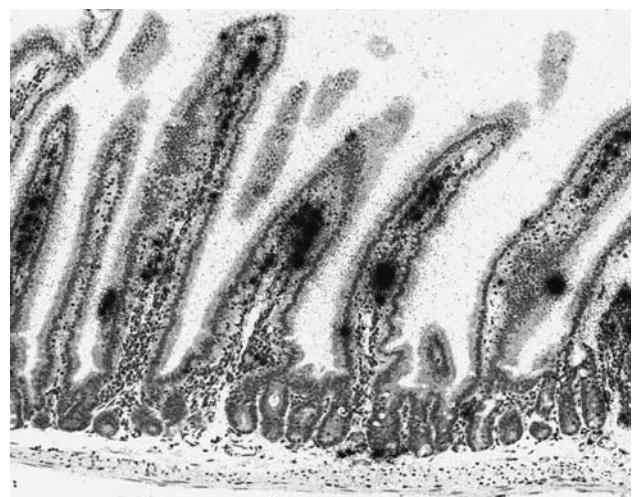
mice seroconverted to MVM from PID 7 to PID 10. Viral replication and dissemination were limited (Ball-Goodrich, unpublished), with moderate to high levels of MVM DNA detected in small intestine and mesenteric lymph nodes (MLN) at PID 6. Trace amounts of viral DNA were present at PID 14, and none was detected by PID 21.

## Mouse parvovirus (MPV)

Current serologic evidence indicates that MPV causes natural infection only in mice and that it is widely distributed in laboratory mouse colonies. A national survey in 1997 (Jacoby and Lindsey, 1997) found that MPV infection was present at about 32 percent of responding institutions, all of which were among the top 100 recipients of NIH funding. The prevalence of murine parvoviruses is attributable to their persistence in infected animals, resistance to environmental inactivation (Harris *et al.*, 1974; Saknimit *et al.*, 1988; Boschetti *et al.*, 2003), and contamination of biologicals used for animal inoculation (Parker *et al.*, 1970; Nicklas *et al.*, 1993).

MPV infection, whether naturally occurring or experimentally induced (Smith *et al.*, 1993; Jacoby *et al.*, 1995; Shek *et al.*, 1998), is clinically silent in neonatal and adult mice. The absence of substantive lesions indicates that MPV is minimally cytopathic, although infection persists after inoculation of infant or adult mice. Persistence was first suspected after mice inoculated with MPV as neonates transmitted virus to cagemates for up to 6 weeks, and mice inoculated with MPV as young adults transmitted virus for up to 4 weeks (Smith *et al.*, 1993). Pathogenesis studies (Jacoby *et al.*, 1995) confirmed that infection of adult, immunocompetent mice with the prototypic MPV-1 strain is persistent and lymphocytotropic: infected cells are detected in lymphoid tissues at 9 weeks post inoculation. Both age groups seroconvert to MPV at 7–10 days after inoculation, indicating that antiviral immunity does not terminate virus excretion or result in viral clearance.

The absence of obvious lesions and low sensitivity of immunostaining led to the use of ISH for assessment of MPV pathogenesis. Using ISH with  $^{32}\text{P}$ -labeled random-primed probes to detect sites of viral replication after oronasal inoculation of MPV, we showed that acute infection appears to begin in the small intestine with subsequent dissemination to lymphoid tissues, liver, lung, and kidney (Jacoby *et al.*, 1995). Virus-positive cells in the small intestine at PID 7 included enterocytes in the crypts and villus walls and mononuclear cells and capillary endothelium of underlying lamina propria (Figure 28.1). The random pattern of enterocytic MPV infection is unusual because enterotropic parvoviruses of other species often target mitotically active crypt cells (Robinson *et al.*, 1980; Carlson *et al.*, 1987; Uttenhal *et al.*, 1990). Infected cells rapidly become more prominent in capillary or lymphatic endothelium and among mononuclear cells in the lamina propria,

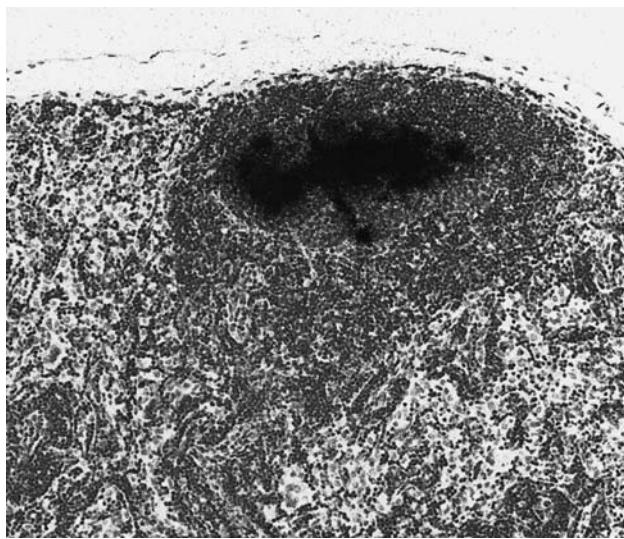


**Figure 28.1** Small intestine was collected from an adult mouse PID 7 after oronasal inoculation with MPV and analyzed by *in situ* hybridization with a random-primed  $^{32}\text{P}$  probe. Intestinal epithelium contains several positive cells (black cells), but most positive cells are in the lamina propria. See Color Plate 28.1.

and virus infection extends to Peyer's patches, MLN, thymus, spleen, peripheral lymph nodes, lung, kidney, and liver. This distribution demonstrates that lymphocytotropic occurs early in infection and may contribute to viral dissemination. It also suggests that some virus-positive cells in intestinal mucosa could include intra-epithelial lymphocytes, a supposition compatible with the *in vitro* tropism of MPV for cloned gamma-delta T cells (McKisic *et al.*, 1993).

The intestine also appears to be a pathway for viral excretion and transmission. Polymerase chain reaction (PCR) analysis of DNA extracted from feces identifies MPV DNA in feces collected from infected mice (Compton *et al.*, 2004), and alimentary-based infection could result after ingestion of contaminated feces, bedding, or food. Excretion through the respiratory or urinary tracts, attributes of other rodent parvoviruses, has not been adequately assessed, although rare infected cells in these tissues indicate that excretion from these sites may be possible secondary modes of transmission.

In adult mice, seroconversion to MPV occurs by PID 10 and subsequent time points show a gradual decrease in virus-positive cells. However, infected cells are detectable in lymph nodes and splenic white pulp through at least 9 weeks post inoculation. They are localized to lymphoid follicles and germinal centres (Figure 28.2), in a pattern similar to lymphocytotropic parvovirus infection of rats, mink and other species (Alexandersen *et al.*, 1987; Carlson *et al.*, 1987; Uttenhal *et al.*, 1990). Because MPV packages primarily minus-sense ssDNA and transcribes plus-sense mRNA (Ball-Goodrich and Johnson, 1994), ISH with strand-specific molecular probes can aid in distinguishing sites of virus replication from sites of virus sequestration (Alexandersen and Bloom, 1987; Bloom *et al.*, 1989): plus-sense probes detect genomic



**Figure 28.2** Mesenteric lymph node was collected from an adult mouse 9 weeks after oronasal inoculation with MPV and analyzed by *in situ* hybridization with a random-primed  $^{32}\text{P}$  probe. Signal is localized to a germinal center. See also Color Plate 28.2.

single-stranded (ss)DNA and double-stranded replicating DNA (RF DNA) and minus-sense probes detect RF DNA and mRNA. Persistently infected cells in lymphoid tissues label with plus-sense riboprobes but not minus-sense riboprobes, suggesting that persistent virus is quiescent or that levels of replication intermediates are decreased and undetectable by ISH. Although it is unclear whether MPV infects specific lymphoid cell subsets, infection of multiple lymphoid cell types is suggested by the random distribution of infected cells during acute infection, presence of virus in the MLN during persistent infection, and immune dysfunction associated with MPV infection (see below). Even without targeted infection of specific cell types, infection of lymphoid cells could facilitate MPV persistence in lymphoid tissues by interfering with the host's antiviral immune response. Unlike MVM infection of neonatal mice, hematopoietic cells are not conspicuous targets for MPV as only a few virus-positive cells occur in splenic red pulp during acute infection.

A major complication of MPV infection in laboratory mouse colonies is the potential for interference with biomedical research. Distortion of biological responses dependent on cell proliferation is inferred by the predilection of parvoviruses for actively dividing cells. Association of rodent parvoviruses with transplantable neoplasms is well known, but proliferation-dependent immune functions may be adversely impacted by MPV infection owing to its lymphocytotropism. As previously noted, MPV was initially detected because it interfered with the ability of cloned T cells to thrive and proliferate (McKisic *et al.*, 1993). Subsequent investigation has confirmed the disruptive effects of MPV on T-cell responses *in vivo* (McKisic *et al.*, 1996, 1998). MPV-1 infection provoked abnormal CD8 $^+$  T cell-mediated

rejection of tumor and skin allografts for at least 3 weeks following inoculation (McKisic *et al.*, 1996, 1998). MPV-1 infection also diminished *in vitro* cytolytic and proliferative responses of alloantigen-reactive lymphocytes from tumor and skin graft sensitized mice. In addition, syngeneic grafts on MPV-infected mice were rejected at the same rate as allogeneic grafts, and depletion of T lymphocytes or passive immunization with anti-MPV immune serum prevented syngeneic graft rejection. Neither tumor cells nor transplanted skin were sites of viral replication *in vivo*. Thus, MPV infection may disrupt normal mechanisms of peripheral tolerance.

Established CD4 $^+$  T cell-mediated responses also were altered by MPV-1 infection, and the effect differed depending on the source of T cells (McKisic *et al.*, 1996). Proliferative responses of cells from splenic and popliteal lymph node cells were suppressed, while those of cells from MLN were augmented. Normal proliferation patterns re-emerged within 2 to 3 weeks. While the mechanism(s) of MPV-associated immunological modulation remains unclear, these experiments highlight the impact of MPV infection on mouse-based research.

## Differences between MPV and MVM

Although MPV and MVM are both lymphocytotropic, they differ in significant ways:

- MVMi can cause acute hemorrhagic disease and death in infant mice of several strains (Kimsey *et al.*, 1986; Brownstein *et al.*, 1991), whereas MPV is non-pathogenic in mice of all ages (Smith *et al.*, 1993).
- MVM appears to modulate immune responses only *in vitro* (Engers *et al.*, 1981; McMaster *et al.*, 1981), whereas MPV can modulate immune responses *in vitro* and *in vivo* (McKisic *et al.*, 1993, 1996, 1998).
- MVM infection appears to be short-lived regardless of host age at inoculation (Smith and Paturzo, 1988), whereas MPV inoculation of neonatal or adult mice results in persistent infection (Smith *et al.*, 1993; Jacoby *et al.*, 1995). Given the high prevalence of MPV in contemporary mouse colonies, persistent infection of lymphoid tissues, and modulation of host immunity, MPV infection is of greater concern among mouse users in the biomedical community.

## PARVOVIRUS OF HAMSTERS

### Hamster parvovirus (HaPV)

HaPV was isolated from a naturally occurring outbreak in Syrian hamsters (Besselsen *et al.*, 1999). Morbidity and mortality were appreciable for neonatal and weanling hamsters, and surviving 2–4 week-old hamsters had dome-shaped

crania, a potbellied appearance, small testes, and discolored, malformed, and lost incisor teeth. The virus was isolated by serial passage of infected hamster tissues in neonatal hamsters followed by multiple passages in baby hamster kidney (BHK) cells. Neonatal hamsters infected with a high dose of virus developed clinical signs, including stunted growth and discoloration of incisors, prior to death at 7–8 days after infection (Besselsen *et al.*, 1999). Lesions included pale spleen and hemorrhagic syndrome involving the gastrointestinal tract, kidneys, testes, uterus, and brain. Histologically, hamsters had evidence of necrosis and/or hemorrhage within the tooth pulp, subcapsular renal hemorrhage, and random and multifocal hemorrhage in the gastrointestinal tract, testes, and uterus. In addition, there was hemorrhage adjacent to the lateral ventricles in the cerebrum and hippocampus as well as adjacent to the granular layer of the cerebellum. By *in situ* PCR, viral DNA was evident in capillary endothelial cells, neuroglia, and neurons in the brain and capillary endothelial cells in the renal cortex. Death was the result of severe, multisystemic hemorrhagic disease.

Neonatal hamsters inoculated with a lower dose of HaPV developed clinical disease with no lethality (Besselsen *et al.*, 1999). At 6 weeks after infection, hamsters had discolored or missing incisors and abnormally small testes, gross lesions similar to those observed during the initial outbreak. Histologically, hamsters had enamel hypoplasia with mineralization and fibrosis of remaining incisor tissues, suppurative periodontitis, atrophy of the seminiferous tubular epithelium, and multifocal myelomalacia in the cerebrum. Inoculation of HaPV in weanling or adult hamsters resulted in seroconversion to the virus but no clinical disease or histologic lesions.

When compared at the nucleotide level, tissue culture-adapted HaPV and MPV are approximately 95 percent identical, a lower identity than among MPV strains (98 percent) but higher identity than between MPV or HaPV and MVM (88 percent). Serologically, HaPV antisera inhibits hemagglutination by MPV antigen to a limited extent, approximately 10-fold lower than the HAI titer using HaPV antigen, and the converse cross-reactivity was evident for MPV antisera and HaPV antigen. Although passage of the clinical isolate in hamster cells *in vivo* and *in vitro* may have increased the tropism of HaPV for hamsters, the nucleotide and antigenic similarities with MPV suggest that HaPV should be tested for infectivity in mice.

## PARVOVIRUSES OF RATS

Kilham and Olivier isolated the first parvovirus, Kilham rat virus (KRV), as a contaminant of a transplantable neoplasm and found it to be apathogenic for newborn and weanling rats (Kilham and Olivier, 1959). Subsequent research, however, showed that parenteral inoculation of

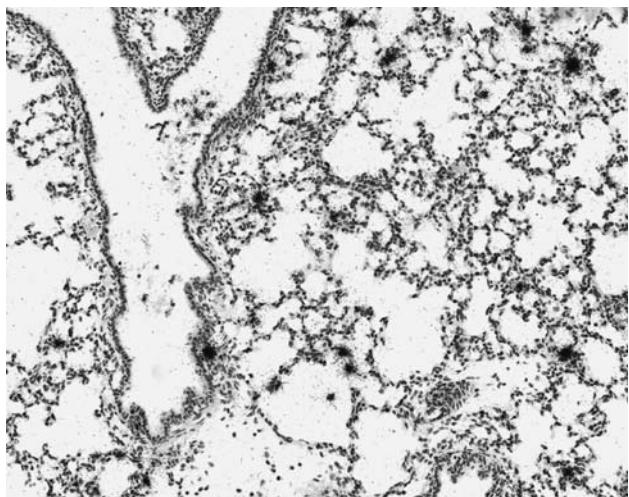
concentrated virus into newborn hamsters resulted in distinctive malformations including dwarfism, flattened foreface, abnormal or absent teeth and bone fragility (Kilham, 1961). In 1960, Toolan isolated a second parvovirus, H-1 virus, from rat-passaged transplantable human tumors and it caused similar malformations when neonatal hamsters were infected (Toolan, 1960). Both agents traversed the placenta of pregnant hamsters and rats, which resulted in deformity or death of embryos (Toolan *et al.*, 1960; Kilham and Margolis, 1969).

While the rat is the natural host for KRV, the natural host for H-1 virus has not been determined. H-1 virus was isolated from a human tumor (Toolan, 1960) and from aborted human fetuses (Toolan, 1968). Antibodies against H-1 virus are present in humans (Guglielmino *et al.*, 1978), but they also have been detected in rat sera (Moore and Nicastri, 1965; Kilham and Margolis, 1969). These and other pioneering studies described the predilection of rodent parvoviruses for mitotically active cells and explained their pathogenicity for fetal and infant rats (Toolan, 1960; Kilham and Maloney, 1964; Kilham and Margolis, 1966; Cole *et al.*, 1970; Margolis and Kilham, 1970). More recently, the prototype strain of a third rat serotype, rat parvovirus (RPV), was isolated from naturally infected rats and characterized *in vivo* and *in vitro* (Ball-Goodrich *et al.*, 1998). A similar agent, 'rat orphan parvovirus', was identified by Japanese workers (Ueno *et al.*, 1996). Inoculation with RPV results in a non-pathogenic, persistent infection in infant and adult rats (Ueno *et al.*, 1997; Ball-Goodrich *et al.*, 1998).

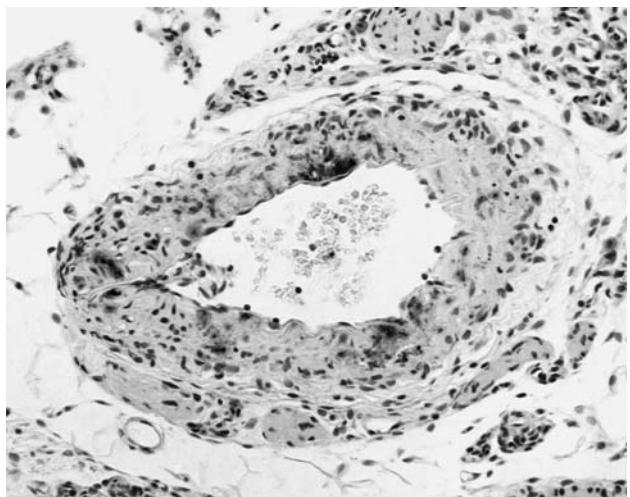
## Rat virus (RV)

Early RV studies, prompted by the potential for virus-induced models of congenital malformations, examined lethal infection in fetal or neonatal rats. Lesions were characterized by necrosis and hemorrhage primarily in liver, brain, and lymphoid tissues (Kilham and Margolis, 1966; Coleman *et al.*, 1983; Jacoby *et al.*, 1987). Infection of the cerebellum can include destruction of the external germinal layer, which leads to granuloprival cerebellar hypoplasia and clinical ataxia. In addition, necrosis and hemorrhage in the brain or spinal cord can lead to infarction and sudden death in infants and occasionally weanlings (Coleman *et al.*, 1983). Correlative clinical signs in rats involve varying combinations of icterus, diarrhea, hemorrhage, ataxia, sudden acute death, or late death due to chronic hepatic disease. The clinical presentation and lesions depend on virus strain and dose, host age, and route of exposure.

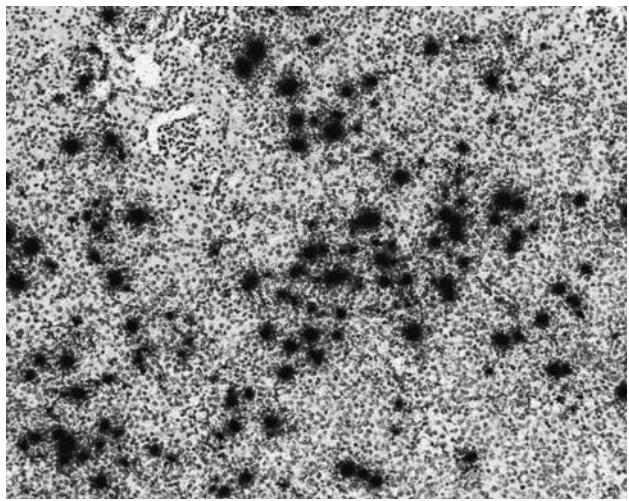
More contemporary studies used oronasal inoculation to mimic infection during natural outbreaks (Kilham and Margolis, 1966; Coleman *et al.*, 1983; Jacoby *et al.*, 1987; Gaertner *et al.*, 1996). Oronasal inoculation of pathogenic RV strains in rats 6 days of age or less results in primary infection of the respiratory tract, followed by viremic dissemination (Jacoby *et al.*, 1987; Gaertner *et al.*, 1989, 1993).



**Figure 28.3** At PID 8 after oronasal inoculation of RV in 6-day-old euthymic rats, lung was collected and analyzed by in situ hybridization with a  $^{35}\text{S}$ -labeled riboprobe to detect viral genomic and replicative DNA. Positive cells (black cells) are visualized among many alveolar septae. See also Color Plate 28.3.



**Figure 28.5** In situ hybridization, with a random-primed biotinylated probe, of a branch of a mesenteric artery collected at PID 28 after oronasal RV inoculation of 6-day-old athymic rats. Signal (brown nuclei and cells) is seen primarily in smooth muscle cells of the arterial wall. See also Color Plate 28.5.



**Figure 28.4** At PID 8 after oronasal inoculation of RV in 6-day-old euthymic rats, kidney was collected and analyzed by in situ hybridization with a  $^{35}\text{S}$ -labeled riboprobe to detect viral genomic and replicative DNA. Tubular epithelium in the renal cortex contains numerous positive cells (black cells). See also Color Plate 28.4.

High viral titers are attained in blood approximately 4 days after infection and are maintained until seroconversion occurs at PID 10. Anemia and thrombocytopenia may develop in some rats. Acute lesions often occur in liver and include necrosis, hemorrhage, and transient formation of intranuclear inclusions, especially in hepatocytes and vascular endothelium. Prolonged infection of liver can cause chronic active hepatitis and fibrosis. Lymphoreticular lesions in thymus, lymph nodes, and spleen also are typified by necrosis, and infection of lung (Figure 28.3), kidney

(Figure 28.4), and urinary bladder are likely sources of virus excretion (Gaertner *et al.*, 1993; Jacoby *et al.*, 2000).

Hemorrhagic lesions and viral inclusions in endothelium illustrate the importance of vasculotropism during RV infection. Virus has been demonstrated in endothelium (Margolis and Kilham, 1970; Jacoby *et al.*, 1987; Gaertner *et al.*, 1993), where it causes cells to swell or lyse. This may result in disruption of vascular integrity and contribute to infarction and hemorrhage (Cole *et al.*, 1970). Endothelial infection also may exacerbate viremia and facilitate fetal infection through involvement of placental and fetal vasculature. In addition, platelet-fibrin aggregates were found to attach preferentially to RV-infected cells, suggesting that endothelial infection activates clotting (Baringer and Nathanson, 1972).

Viral DNA also has been detected in smooth muscle myofibres (SMC) during both acute and persistent infection, with SMC providing a major site of persistent virus infection. Sites include vascular SMC in many tissues (Figure 28.5) and SMC in muscle tunics of the small intestines (Jacoby *et al.*, 2000). Inflammation is not a prominent component of RV infection, although mononuclear cell infiltrates can occasionally be found at sites of persistent infection in liver, kidney, and perivascular (Gaertner *et al.*, 1993). As noted above, resistance to lethal infection develops by 6 days of age (Gaertner *et al.*, 1996). Typically, immunocompetent juvenile and adult rats develop asymptomatic infection that is cleared within 4 weeks, although fatal RV infection in juvenile and adult rats has occasionally occurred as a result of hemorrhagic infarcts (El Dadah *et al.*, 1967; Cole *et al.*, 1970; Coleman *et al.*, 1983).

Circumstantial evidence for persistent RV infection appeared in the early literature when Robey detected

infectious virus in seropositive wild rats (Robey *et al.*, 1968) and Lipton reported similar findings for experimentally infected laboratory rats (Lipton *et al.*, 1973). RV persistence was not pursued in depth until Jacoby showed that inoculation of 2-day-old infant rats with the RV-Yale (Y) strain, a field strain isolated during a natural outbreak, resulted in infection lasting at least 6 months (Jacoby *et al.*, 1991) and in transmission to cagemates for up to 3 months (Jacoby *et al.*, 1988). Subsequent studies with RV-UMass, a more pathogenic strain of RV isolated at the University of Massachusetts, confirmed that persistent virus is detectable by both ISH and explant culture (Gaertner *et al.*, 1996; Jacoby *et al.*, 2000). These results also confirmed that establishment of persistent RV infection depends on virus inoculation during the first week after birth of immunocompetent rats or inoculation of immunodeficient rats as infants or adults (Gaertner *et al.*, 1989, 1996; Jacoby *et al.*, 1991, 2000).

The mechanism of RV persistence is unknown, but Jacoby *et al.* (2000) used several criteria to confirm that it includes active viral replication. Southern analysis of DNA from persistently infected tissues detected viral replicative forms and single-stranded virion DNA. Immunohistochemical evaluation of SMC at 4 and 8 weeks post inoculation detected both RV NS and VP proteins. ISH with strand-specific riboprobes detected viral DNA and mRNA, although the prevalence of viral DNA-positive cells was consistently higher than for viral mRNA-positive cells. ISH on synchronized RV infections *in vitro* showed that mRNA concentrations peak early during RV replication (Ball-Goodrich *et al.*, 2001). Thus, the low prevalence of mRNA-positive cells *in vivo* may indicate concentrations below the threshold for ISH detection because the virus has neared the end of its replicative cycle. Alternatively, it may reflect the accumulation of non-replicating virus (Mori *et al.*, 1990) or the lability of mRNA in tissue sections.

Autonomous rodent parvoviruses require factors present during the S-phase of the cell cycle to replicate, including cellular DNA polymerase. Thus, during persistent infection, RV mRNA should be detected solely in cycling SMC, a cell type with a low turnover rate (Clowes *et al.*, 1983). To test this possibility (Jacoby *et al.*, 2000), tissues from persistently infected euthymic rats were co-labeled for KRV mRNA (minus-sense riboprobe) and proliferating cell nuclear antigen (PCNA), a cell-cycle protein expressed during late G1- and S-phase but not during G0 (Takasaki *et al.*, 1981). Approximately half of 113 mRNA-positive SMC were PCNA positive and half were negative. These results suggest that RV replication may continue in SMC past S-phase of the cell cycle, a possibility consistent with an *in vitro* study of feline parvovirus replication (Lenghaus *et al.*, 1985). Possible mechanisms include continuing viral transcription in cells beyond S-phase, viral transcription beginning at more than one stage of the cell cycle if double-stranded DNA templates were formed prior to exit of S-phase, or maintenance of viral transcripts if replication does not peak prior to the end of S-phase. In all these instances, transcripts

would be present when the cell returned to G0, an interval when PCNA is not expressed. Thus, cell death may not be the sole outcome of RV infection during a single cell cycle, and viral replication may extend through more than one phase of the cell cycle (Jacoby *et al.*, 2000).

While RV infection of neonatal rats results in morbidity or viral persistence, RV infection of adult immunocompetent rats induces both humoral and cellular immune responses, which limit infection and result in viral clearance within 4 weeks. Infected rats develop IgM and IgG antibodies against RV VP2 by PID 7, anti-VP2 IgG titers increase through PID 35, and the predominant IgG isotype is IgG2a (Ball-Goodrich *et al.*, 2002). Perivascular accumulations of mononuclear cells at sites of infection indicate activation of a cellular response to RV (Jacoby *et al.*, 1987, 1991; Gaertner *et al.*, 1993). Cellular responses to viral antigens were confirmed by examining cytokine secretion and splenocyte (SC) proliferation weekly through PID 35 (Ball-Goodrich *et al.*, 2002). While no viral specific response was detected at PID 7, SC harvested at PID 14 proliferated and secreted high levels of interferon (IFN)- $\gamma$  after incubation with RV VP2. Proliferation plateaued at PID 21 and remained high through PID 35, and IFN- $\gamma$  secretion was maintained through PID 35. These results indicate that Th1-mediated humoral and cellular immunity contribute to RV clearance in adult rats. Additionally, studies in T-cell deficient (athymic) rats indicate a primary role for cell-mediated immunity in viral clearance. Neither neonatal nor juvenile athymic rats mount an effective immune response after oronasal RV inoculation, and the resulting infection is persistent for >8 weeks (Gaertner *et al.*, 1989, 1991, 1995, 1996; Jacoby *et al.*, 2000). This result also implies that natural killer cells are not effective in clearing infection as they are functional in athymic rats.

Several studies have indicated a functional role for humoral immunity in preventing RV infection. Most athymic adult rats given immune serum at the time of virus inoculation did not become persistently infected, whereas the majority of those given immune serum at PID 7, the natural time of seroconversion, were infected with RV at PID 28 (Gaertner *et al.*, 1995). Infant rats administered immune serum before or at the time of virus inoculation were protected from infection. However, the protective effects were diminished if immune serum was administered one day after virus (Gaertner *et al.*, 1991).

As was shown for murine parvoviruses, RV infection modulates the host immune response. Early studies showed that RV infection can suppress lymphocyte proliferation stimulated by mitogens and alloantigens, including tumor cell alloantigens (Campbell *et al.*, 1977). More recently, McKisic *et al.* demonstrated that RV replicated in lymphocytes and suppressed effector functions in adult rats (McKisic *et al.*, 1995). All lymphoid cell types (B cells, CD4 $^{+}$ , and CD8 $^{+}$  T cells) were infected 7 days after oronasal inoculation of RV, and the number of infected cells increased after *in vitro* stimulation of lymphoid cells with mitogen (Con A)

for 4 days. In lymphocytes isolated at 7, 14, and 28 days after RV infection, there was diminished T lymphocyte proliferation in response to alloantigen and decreased cytolysis of cells expressing alloantigen. This down-regulation of T-lymphocyte responsiveness could provide a mechanism by which parvovirus-infected cells escape cytolytic destruction. Workers at the University of Massachusetts found that RV infection of lymphoid tissue provoked diabetes in diabetes-resistant BB/Wor rats (Guberski *et al.*, 1991; Brown *et al.*, 1993). In this model, RV infection of BB rats activated autoreactive CD8<sup>+</sup> T cells that were responsible for the diabetic phenotype and decreased responsiveness to extraneous antigens (Chung *et al.*, 2000).

RV can cross the placenta after oronasal or intraperitoneal inoculation of pregnant rats, and prenatal infection can lead to fetal death and resorption. The prevalence and severity of prenatal disease depends on virus strain, dose, and timing of infection (Gaertner *et al.*, 1996; Jacoby *et al.*, 2001). For example, fetuses of dams inoculated with high doses of RV at day 9 in gestation sustained severe multisystemic necrosis and mortality. In contrast, fetuses of dams inoculated with high doses of RV at gestation day 12 had primarily asymptomatic infections that persisted after birth (Gaertner *et al.*, 1996). Further studies showed that dams inoculated with a lower dose of RV on gestation day 9 delivered live litters, and 75 percent of progeny were viral DNA positive by ISH at 3 weeks after birth (Jacoby *et al.*, 2001). Analysis was extended to determine whether and when pups infected *in utero* transmitted RV. These studies showed that 70 percent of prenatally-infected progeny, from dams inoculated with RV-UMass, transmitted virus at 9 weeks of age (Jacoby *et al.*, 2001). In addition, persistently infected female progeny were held for breeding, and 64 percent of their breeding partners seroconverted to RV. These results indicate that RV persistence included virus transmission for an extensive period to cagemates. In contrast, fetuses and resultant pups were protected from RV infection, an outcome attributed to maternally acquired humoral immunity.

The foregoing discussion demonstrates that RV infection has the potential to distort or disrupt biological responses of rats that require or result in cell proliferation. Risks to research can include immune dysfunction, clinical illness and death, poor reproductive performance, and contamination of cell lines and transplantable tumors (Jacoby *et al.*, 1979; Tattersall and Cotmore, 1986). In addition, RV stability and resistance to inactivation (Yang *et al.*, 1995) increase the risk of transmission and prolong the impact of infection on research laboratories and vivariums. These features, along with persistent infection of the host, contribute to make RV one of the most common infectious agents of laboratory rats.

## H-1 virus

Besides Toolan's classic studies of H-1 (described at the beginning of this section), little research has focused on the

pathogenesis of H-1 in rats. Toolan extended analysis of H-1 inoculation of pregnant hamsters and found that the peak of fetal mortality was mid-gestation, although fetal infection occurred after inoculation at later times in pregnancy (Toolan, 1978). Li and Rhode compared *in vivo* infection of newborn rats and hamsters using H-1 virus and an NS2 null mutant H-1SA (Li and Rhode, 1991). While both viruses caused lethal infection of hamsters, albeit slower kinetics for the mutant, only H-1 virus infection resulted in lethal infection of newborn rats. In addition, tissues from rats infected with H-1SA had no detectable virus whereas those from H-1 virus infected rats contained high titers of infectious virus. They concluded that NS2 is required for productive infection of newborn rats but not newborn hamsters. Ohshima *et al.* (1998) inoculated H-1 virus into newborn rats, and infected pups suffered emaciation, jaundice, and ataxia. Seven days after infection, ISH on brain tissue detected viral DNA in granular layer cells of the cerebellum. By TUNEL assay, a higher frequency of apoptosis-specific signal was present in infected tissues and co-localized to the same areas as virus-infected cells. These results *in vivo* extend and confirm the authors' *in vitro* observations, which show induction of apoptosis in H-1 infected C6 rat glioblastoma cells.

## Rat parvovirus (RPV)

Recently, a new rat parvovirus, originally called rat orphan parvovirus (ROPV), was identified in rat colonies worldwide. Ueno *et al.* found that rat sera from a colony in Japan was parvovirus antibody positive by IFA test but not by HAI (Ueno *et al.*, 1996). An epidemiology study determined the duration of viral infection and excretion after ROPV infection of juvenile rats (Ueno *et al.*, 1997). PCR analysis detected ROPV DNA in feces and urine of infected rats at 1–4 weeks post inoculation. ROPV was detected through 8 weeks in DNA from blood, bone marrow, thymus, MLN, spleen, liver, kidney, lung, pancreas, and intestine. At 24 weeks, ROPV was detected in DNA from MLN and spleen, indicating persistent infection of lymphoid tissue. Rats appear to be the host species for ROPV as infection of mice and hamsters did not result in seroconversion or production of infectious virus.

In the United States, analysis of a different ROPV isolate distinguished it from the RV serogroup based on serology, genomic sequence, and pathogenicity (Ball-Goodrich *et al.*, 1998). Therefore, it was classified as the prototype of a new serogroup, RPV-1. Sera from RPV-infected rats was negative by HAI using RV or H-1 virus as antigens, and it neutralized RPV infectivity but not that of RV or H-1 virus. The divergence between RPV and RV at the nucleotide and protein levels is much greater than that between RV and H-1 virus, and the divergence is high in both the NS and VP coding regions.

RPV infection of neonatal rats, at a dose similar to the LD<sub>50</sub> of RV-UMass, caused only asymptomatic infection

(Ball-Goodrich *et al.*, 1998). Viral replication, as assessed by ISH, was first evident in small intestine, kidney, and lymphoid tissues at PID 5. The prevalence of infected cells increased in these tissues through PID 10, by which time rats had seroconverted to RPV. Endothelial infection was prominent in small intestine, lung, brain, liver, and kidney, but there was no evidence of hemorrhage or necrosis. By PID 20, the number of affected tissues remained high, although the prevalence of positive cells had decreased in all tissues except lymph node and kidney. Oronasal inoculation of juvenile rats resulted in a similar, non-pathogenic infection. As in neonates, small intestine and lymphoid tissues were primary sites of replication at PID 5, although capillary endothelium in lung, brain, liver, and kidney also were infected. The peak prevalence of infected cells in small intestine and lymphoid tissues was attained by PID 7, by which time rats had seroconverted to RPV. The prevalence of infected cells decreased progressively through PID 10 and 20, although MLN remained positive through PID 20.

## Comparison of RPV and RV pathogenicity

Although both agents become widely disseminated in rat tissues and can persist after development of immunity, small intestine appears to be the primary site of RPV infection whereas RV initially infects the respiratory tract with little or no infection of the intestine. More significantly, RPV is non-pathogenic in infant rats where, despite high levels of infected endothelia in many tissues, there was no evidence of hemorrhage or necrosis. Significant coding differences in the NS proteins of RPV from RV may explain the lack of pathogenicity in infant rats.

## Other rat parvoviruses

More recently, a number of new parvoviruses have been detected in laboratory animal colonies. DNA for three such agents, named rat minute virus (RMV)-1a, -1b, and -1c, was PCR amplified from infected tissues and sequenced (Wan *et al.*, 2002). All three are closely related to RV and H-1 virus but significantly different from RPV-1a. However, the viruses have not been isolated, and therefore, no information is available about their pathogenesis or mechanisms and duration of transmission.

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# Pathogenesis of feline panleukopenia virus and canine parvovirus

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Feline panleukopenia virus (FPV) and canine parvovirus (CPV) and a number of closely related parvoviruses are widespread in nature, and cause disease in many different carnivores. The viruses are all closely related to each other, being over 97 percent identical in genomic sequence, and they have historically often been named after the host from which they are isolated – hence CPV, FPV, raccoon parvovirus, mink enteritis virus (MEV), and blue fox parvovirus (BFPV) from Arctic foxes. The virus is very stable in the environment and can remain infectious in nature for days or weeks allowing persistence and making the virus very difficult to control or eliminate, and disease is most often prevented by vaccination with effective modified live vaccines, or in the case of MEV, with inactivated vaccines.

FPV has been known as the cause of diseases in large and small cats, raccoons and some related carnivores for many years (Hindle and Findlay, 1932; Hammon and Enders, 1939b), but CPV is a new virus, probably derived from FPV or a close relative during the early 1970s (reviewed by Parrish, 1990). Studies of the emergence of CPV show that it spread around the world during early 1978; and that the original strain of virus (called CPV type-2) was replaced between 1979 and 1981 by a genetically and antigenically distinct virus (CPV type-2a), which has itself subsequently also been largely replaced by further variants, including the CPV-26 antigenic variant (Parrish *et al.*, 1988; Truyen *et al.*, 1995).

The host range differences between the viruses are complex, and the 1978 strain of CPV (CPV type-2) clearly replicates in both canine and feline cells in culture, as well as in dogs, but it did not infect cats (Truyen and Parrish, 1992), while the later strains (CPV type-2a and type-2b) can infect

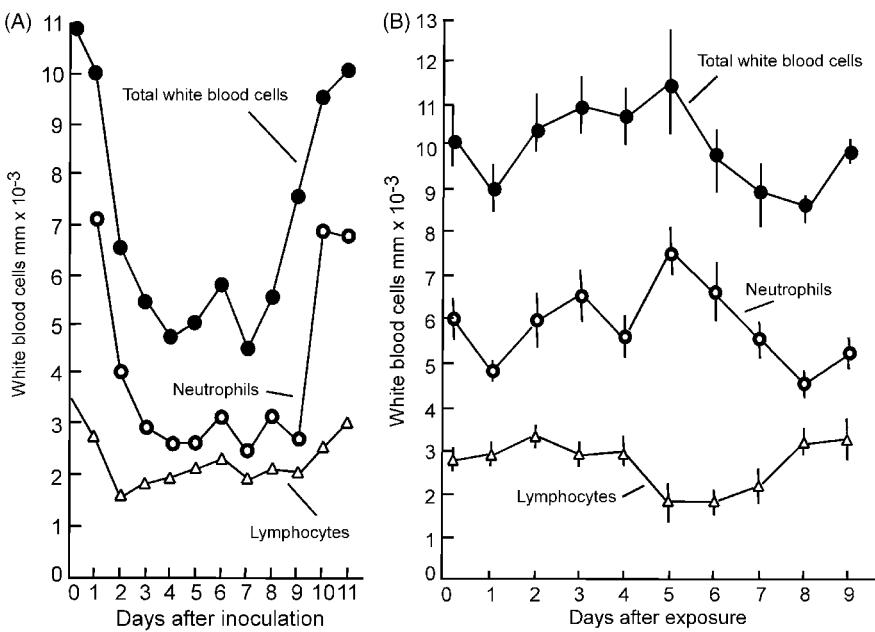
cats as well as dogs. In contrast, FPV replicates in feline but not canine cells in culture and in cats, but it can replicate in certain canine tissues after inoculation of animals, including thymus and bone marrow cells (Truyen and Parrish, 1992). The natural animal host range of CPV includes dogs and close relatives such as wolves, coyotes, South American dogs, and Asiatic raccoon dogs. FPV and the FPV-like viruses infect both large and small cats, as well as mink, raccoons, and possibly foxes (reviewed by Parrish, 1990).

This review considers the pathogenesis of FPV in cats, the very similar MEV in mink, and CPV in dogs. The pathogeneses of these infections are very similar, although small differences in different hosts and the age of the animal also affect the outcome of the infection.

## DISEASES AND PATHOGENESIS

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The pathogenesis of parvovirus infections is influenced primarily by the requirement for viral DNA replication for mitotic cells, which determines many of the differences in the outcomes of infections in fetal, neonatal, or older animals. However, not all the dividing cells in an animal are permissive for virus replication and, while the dividing lymphoid and intestinal epithelial cells are primary targets for virus replication by FPV, MEV, and CPV, developmentally regulated properties of some differentiated dividing cell populations may restrict parvovirus replication at the cellular level and determine the specific outcome of infection. The precise relationship between the presence of dividing cells



**Figure 29.1** The changes in circulating leukocyte numbers after infection of cats or dogs with FPV or CPV, respectively. (A) The mean circulating, total leukocyte, lymphocyte, and neutrophils in 8 FPV infected cats at various days after infection. (From Larsen *et al.*, 1940.) (B) The mean counts and standard deviations of total circulating leukocytes, neutrophils, and lymphocytes of dogs after infection with CPV. (From Carman and Povey, 1985b.)

in tissues and their susceptibility to parvovirus infection in dogs, mink or cats has not been defined.

## Older animals

The pathogeneses of infections by FPV in cats or CPV in dogs are very similar. Both viruses are considered together, with differences between the infections being noted where those have been defined. The site of entry and initial virus infection is most likely through cells of the nasopharynx, the tonsils or other lymphoid tissues (Reynolds, 1970; Csiza *et al.*, 1971b; Carman and Povey, 1982; Pollock, 1982; Macartney *et al.*, 1984a). Animals also can be infected by most parenteral routes. Virus is isolated between 1 and 3 days later from the tonsil, retropharyngeal lymph nodes, thymus, and mesenteric lymph nodes; after approximately 3 days virus is also recovered from the intestinal-associated lymphoid tissues and Peyer's patches (Csiza *et al.*, 1971b; Carlson *et al.*, 1977; Macartney *et al.*, 1984a; Meunier *et al.*, 1985b). Virus spreads systemically through a plasma viremia, resulting in widespread infection of the lymphoid tissues including the thymus and lymph nodes, and there is also widespread circulation of infected lymphocytes, which could carry the virus to many tissues.

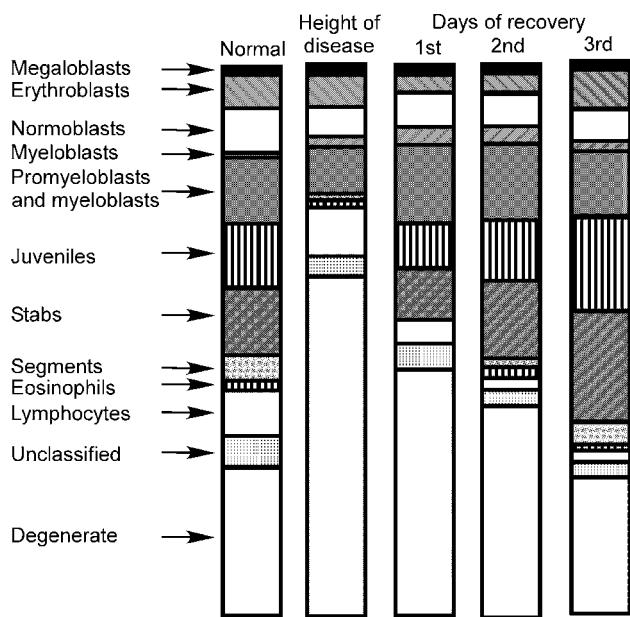
## HEMATOPOESIS

The incidence of leukopenia or lymphopenia varies between the different viruses. Effects on erythrocyte levels are not

seen after infection, possibly because of the long life span of the erythrocytes compared with the course of the disease. Panleukopenia is a common feature of many FPV infections of cats (Figure 29.1A), where the total white cell counts may fall to 2000 per mm<sup>3</sup> or less, and neutrophil counts decrease to <1200 per mm<sup>3</sup>. Lymphocyte numbers decline, although to a lesser degree, but there is little effect on eosinophil, basophil, monocyte, or red cell numbers (Lawrence and Syverton, 1938; Hammon and Enders, 1939a; Lawrence *et al.*, 1940; Rohovsky and Griesemer, 1967; Reynolds, 1969; Carlson *et al.*, 1977). Panleukopenia is very uncommon in CPV infections, although a relative lymphopenia is often observed (Figure 29.1B), and some animals develop neutropenia, but total leukocyte counts are generally not markedly affected (Robinson *et al.*, 1980b; Carmichael *et al.*, 1981; Pollock, 1982; Macartney *et al.*, 1984a; Carman and Povey, 1985b).

## Bone marrow

In FPV and CPV infections of cats and dogs the bone marrow may be severely affected, with a marked decrease in cellularity. Most animals show decreased numbers of myeloid, erythroid, and megakaryocytic cells (Figure 29.2) (Hammon and Enders, 1939a; Lawrence *et al.*, 1940; Robinson *et al.*, 1980b; Boosinger *et al.*, 1982). Individual animals differ in both the extent of the depletion and the effects on individual cell types. Many cells in feline bone marrow cell cultures were susceptible to infection by FPV. On average about 10–20 percent of the cells showed virus antigen or DNA by



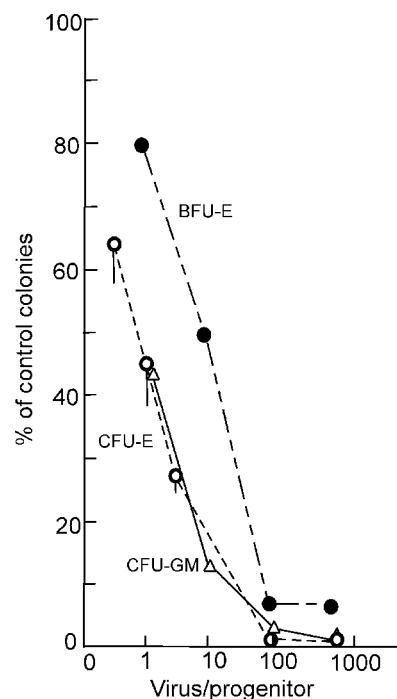
**Figure 29.2** Differential bone marrow cell counts of cats at various stages of infection with FPV. The data represents 13 normal marrows, and 31 marrows from infected cats. (From Lawrence et al., 1940.)

fluorescent antibody staining or by *in situ* hybridization (Kurtzman et al., 1989). At high doses of FPV there were reductions in both erythroid and myeloid colony formation, but at lower virus doses there was a greater suppression of the myeloid colony forming units-granulocyte macrophage (CFU-GM) colony formation compared with the erythroid burst-forming units erythroid (BFU-E- and [colony-forming units-erythroid] CFU-E-derived) colonies (Figure 29.3). The precise differentiated stages of the FPV-susceptible cell populations were presumed to be early progenitors. They proposed that virus infection of the myeloid precursors would rapidly lead to reduced circulating neutrophil levels owing to the rapid turnover of those cells (Kurtzman et al., 1989).

No effect of CPV on the regeneration of erythrocytes was observed when hemolytic anemia was induced in dogs with phenylhydrazine before CPV infection, indicating that, at least for CPV in dogs, the virus does not greatly depress erythroid cell production (Brock et al., 1989). Although CPV infects canine bone marrow cells (Macartney et al., 1984b; Meunier et al., 1985b; Truyen and Parrish, 1992), this does not result in a panleukopenia, suggesting that CPV and FPV infect different target cells in the bone marrow and probably other tissues of their respective hosts.

## Lymphoid tissues

The infection of the lymphoid tissues results in lymphocytolysis, cellular depletion, and, subsequently, tissue regeneration in surviving animals. Virus replication and cell

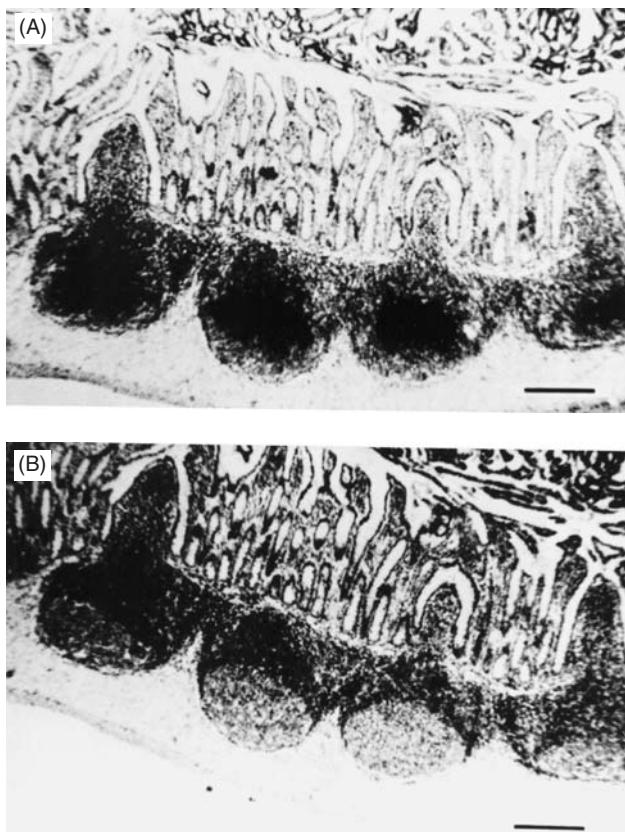


**Figure 29.3** Inhibition of hematopoietic colonies in cultures of feline bone marrows inoculated with various multiplicities of infection of FPV. The numbers of colonies of the three types are given compared to uninfected cultures. (From Kurtzman et al., 1989.)

destruction in lymphoid tissues occurs mostly in areas of dividing cells, including germinal centres of lymph nodes and in the thymus cortex (Figure 29.4) (Hammon and Enders, 1939a, 1939b; Reynolds, 1970; Carlson et al., 1978; Macartney et al., 1984b; Uttenthal et al., 1990). Although some cells must be lysed due to virus infection, at least some of the reduction in cell numbers in the lymphoid tissues is likely due to indirect effects, such as virus binding to the cells, and to the actions of lymphotoxic cytokines such as tumor necrosis factor (TNF)- $\alpha$  (McNally and Welsh, 2002; McNally et al., 2001).

## INTESTINAL INFECTION

Intestinal infections appear very similar for all the carnivore parvoviruses. FPV or CPV first infect the rapidly dividing cells in the crypts of the intestinal villi of the ileum and jejunum 3–5 days after inoculation, and virus is found throughout the epithelium between 4–8 days after infection (Figure 29.4) (Carlson and Scott, 1977; Carman and Povey, 1985a; Meunier et al., 1985b; Uttenthal et al., 1990). The virus infection and loss of epithelial cells results in a flattened and attenuated epithelium with shortened intestinal villi leading to loss of osmotic regulation, with a resulting diarrhoea often containing blood and mucus. Animals may become dehydrated and pyretic, in part because of endotoxin uptake from the gut. The degree



**Figure 29.4** Ileal sections of mink 8 days after infection with MEV, probed with (A) a plus-sense RNA probe that detects viral DNA present in virions and replicative form DNA, or (B) a minus-sense probe that would detect primarily replicative DNA forms. Hybridization is seen over both the gut-associated lymphoid tissues, as well as the intestinal epithelial cells. Bar – 230  $\mu\text{m}$ . (From Uttenhal et al., 1990.)

and the severity of the infection are in part determined by the rate of turnover of the intestinal epithelial cells (Rohovsky and Griesemer, 1967; Carlson and Scott, 1977), and coinfection with intestinal parasites, pathogenic bacteria, or viruses such as rotavirus or coronavirus are likely to increase the severity of the disease. Germ-free cats showed reduced FPV replication in the small intestine, while treating cats with mild hydrochloric acid (HCl) enemas resulted in increased cell replication and virus infection of the colonic epithelium. During the intestinal phase of the infection virus is excreted in large amounts in the feces, with up to  $10^7$ – $10^9$  infectious units being shed per gram.

## DISEASE SEVERITY

The clinical disease probably results from the damage by virus infecting the small intestine. There is variation in the response of individual animals to virus infection, and serological studies indicate that infections by CPV in dogs

(and probably by FPV in cats) are often mild or subclinical (Meunier et al., 1980; Parrish et al., 1982). A correlation was observed between the viral titers in serum and feces and the severity of the disease observed in dogs inoculated with CPV (Meunier et al., 1985a). A number of attenuated strains of CPV and FPV have been isolated by repeated passage of the viruses in cells in tissue culture (Carmichael et al., 1981). Although the attenuating mutations in those viruses are not completely defined, the viruses are shed at lower titers in the feces, suggesting that decreased replication in the intestine resulted in decreased enteritis.

## FETAL OR NEONATAL INFECTIONS

Infection of neonates results in different diseases from those seen in older animals, and is characterized by infection of the developing cerebellum in kittens or ferrets, or of the heart in puppies. Enteritis is not observed in very young animals. Infection of kittens either *in utero* or shortly after birth can result in viral replication in the cells of the external germinal epithelium of the cerebellum, resulting in cerebellar hypoplasia, and most viable kittens subsequently suffered from ataxia (Csiza et al., 1971a; Kilham et al., 1971). CPV infection of neonatal puppies can result in cerebellar disease (Schatzberg et al., 2003), but a more characteristic sign is myocarditis observed generally between 3 and 8 weeks of age, but sometimes up to 16 weeks of age at death. Mortality in affected litters varies between 20 and 100 percent, and disease onset is rapid, being characterized by cardiac arrhythmia, dyspnoea, and pulmonary edema, followed by death (Robinson et al., 1979, 1980a,b; Parrish et al., 1982; Meunier et al., 1984). Affected pups suffer a variety of sub-clinical abnormalities with progressive multifocal necrosis of the myocardium, often with a mononuclear cell infiltrate. Myocardial cells often contain intranuclear inclusion bodies. Lungs may be edematous, most likely secondary to the heart failure. The age dependence of the myocardial infection is probably due to the active division of the myocardial cells only in pups <15 days of age. More rarely, neonatal infections can give a generalized infection with lesions in many different tissues (Lenghaus and Studdert, 1982; Johnson and Castro, 1984). *In utero* infections of cats by FPV or of Arctic foxes by FPV-like virus may result in fetal death and resorption, abortion, or neonatal death (Kilham et al., 1967, 1971; Veijalainen and Smeds, 1988).

## IMMUNITY

The course of infection is acute, with little virus being recovered from tissues or feces by 14 days post infection (Pollock, 1982; Macartney et al., 1984a). The functional immunity against these viruses, which acts both for recovery and to protect against infection, appears to be mediated through serum antibody. T-cell epitopes in the CPV

sequences recognized by dog lymphocytes have been defined within the capsid protein gene (Rimmelzwaan *et al.*, 1990). Colostrum-derived maternal immunity protects against parvovirus infection until serum antibody titers decline to very low levels (Parrish *et al.*, 1982; Pollock and Carmichael, 1982; Ishibashi *et al.*, 1983; Buonavoglia *et al.*, 1992). The role of local immunity in the gut is not known, although levels and classes of antibody in the jejunum collected by cannulation after CPV infection or vaccination suggested that the antibody was being specifically secreted (Nara *et al.*, 1983). However, parenteral administration of anti-CPV antibodies both protects dogs against oral challenge and prevents virus replication in the intestine (Ishibashi *et al.*, 1983; Meunier *et al.*, 1985b).

## CONCLUSIONS

The pathogenesis of CPV and FPV infection largely depends on the requirement of the virus for dividing cells for replication, making the diseases age dependent. However, differences in the susceptibility of cell populations in the bone marrow, the heart, or the cerebellum of the cat and dog also give rise to distinct outcomes of various virus and host combinations.

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# Porcine parvovirus

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Porcine parvovirus (PPV) is a small non-enveloped virus that is highly resistant to environmental inactivation and as a ubiquitous agent affects swine reproductive performance all over the world. PPV infections are considered harmless to adult animals because the promptly produced antibodies efficiently neutralize the virus. However, when seronegative pregnant pigs are exposed to a viremic PPV strain during the first part of gestation, embryonic and fetal death could occur via transplacental infection. Beyond 70 days of gestation, fetuses become immunologically competent; therefore dams infected in this later period usually farrow healthy but seropositive piglets. Isolated PPV strains can be distinguished according to their pathogenicity. Non-pathogenic PPV strains are considered harmless, unless viruses are inoculated *in utero* into the embryos before immunologic competence. Beside reproductive failure problems, PPV infections have been occasionally found associated with dermatitis, nephritis, or enteritis in some cases. Recently, PPV has been shown to be one of the agents in postweaning multisystemic wasting syndrome.

## ETIOLOGICAL AGENT

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The syndrome of stillbirths, mummification, embryonic death, and infertility (SMEDI) was described in 1965 (Dunne *et al.*, 1965). However, the role of PPV as a major cause of embryonic and fetal death in pigs was established later (Joo *et al.*, 1976a; Mengeling and Cutlip, 1975). Pathogens associated with SMEDI syndrome were initially

identified as picornaviruses, but further characterization indicated that they belong to a newly described group of DNA viruses, the *Parvoviridae* family (Cartwright and Huck, 1967; Cartwright *et al.*, 1969; Dunne *et al.*, 1965; Johnson and Collings, 1969). Subsequently, porcine parvovirus had been isolated from pigs with various manifestations of reproductive failure (Mengeling and Cutlip, 1975; Morimoto *et al.*, 1972). All PPV isolates selected for analysis have been found antigenetically similar by hemagglutination (HA), hemagglutination inhibition (HAI), virus neutralization with antiserum (SN), and direct or indirect immunofluorescence microscopy (IF) assays.

## VIRUS PROPERTIES

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The icosahedral capsid of PPV consists of 60 copies of a mixture of structural proteins VP1, VP2, and VP3 with respective molecular weights of 83,000, 64,000, and 60,000 (Molitor *et al.*, 1983). Virion particles (diameter 20–28 nm) contain linear, approximately 5 Kb, single-stranded (minus-strand) DNA genome with palindromic sequences at both ends (Bergeron *et al.*, 1993). Porcine parvovirus is classified in the *Parvoviridae* family, *Parvovirinae* subfamily, and *Parvovirus* genus (Siegl, 1976). Complete virions can be purified at buoyant density of about 1.4 g/ml using cesium chloride (CsCl) ultracentrifugation (Molitor *et al.*, 1983). Viral infectivity and hemagglutinating capability is resistant to heat, various enzymes (DNase, RNase, pepsin, trypsin), and wide pH range. Comparison of four PPV isolates by restriction-endonuclease

digestions indicated only minor differences between NADL-2, NADL-8, Kresse, and KBSH DNA (Molitor and Joo, 1990). Detailed sequence analysis of NADL-2 and Kresse strains revealed few differences between their genome apart from an extra 127 nucleotides repeat in the NADL-2 non-coding region of the right arm (Ranz *et al.*, 1989; Vasudevacharya *et al.*, 1990; Bergeron *et al.*, 1993, 1996). Allotropic determinants of cell tropism were identified at three positions in the VP2 protein (Bergeron *et al.*, 1996). A recent study on samples obtained from fetal tissues of PPV-infected pigs have found variability at the DNA level, in the VP2 protein coding region, among field isolates of PPV (Soares *et al.*, 2003).

## DETECTION AND QUANTIFICATION OF PPV

Cytopathic changes in individual infected cells start to appear at 16–20 hours post infection, but they are usually vague at the terminal dilutions; therefore endpoints of infectivity were previously determined by staining cells for intranuclear inclusions (Cartwright *et al.*, 1969). The full cytopathic effects (CPE) for PPV can be seen after 5–7 days of infection, depending on the cell type used and can be used to determine the TCID<sub>50</sub> (Reed and Muench, 1938). Infectious virus concentration (pfu/ml) also can be determined by plaque assay. The most sensitive method for the quantification of infectious virus is the fluorescent-focus assay, which requires specific antibody and IF microscopy (Mengeling, 1972).

Electron microscopic techniques (EM) can be used for direct counting of intact PPV particles mixed with known concentration of latex beads. However, application of EM on tissue samples is more elaborate for detection of PPV than for viruses with larger size; therefore immune electron microscopy (IEM) may present a choice to visualize viruses through labeled antibody–antigen interaction. HA is a frequently used method for the determination of relative amount of PPV, as the virus agglutinates chicken, guinea pig, mouse, human, monkey, rat, and cat erythrocytes (Cartwright *et al.*, 1969; Mengeling, 1972; Morimoto *et al.*, 1972). Hemagglutination activity of PPV was not found with pig, hamster, quail, sheep, rabbit, horse, dog, and cattle erythrocytes (Cartwright *et al.*, 1969; Mengeling, 1972; Ruckerbauer *et al.*, 1978). HA testing is usually performed with guinea pig erythrocytes in buffers of neutral pH, mainly at room temperature but sometimes at 4°C.

Techniques for detection of PPV DNA are among the most specific methods providing information about the presence of virus in the animal. However, these assays could not differentiate between infectious and inactive viruses. Southern, dot, and slot blot techniques based on nucleic acid hybridization have been used to identify or quantify PPV DNA in infected tissue culture cells and animals (Harding and Molitor, 1988; Gradil *et al.*, 1990; Oraveerakul *et al.*, 1993). Transcription of PPV genes was investigated during

infection of tissue culture cell lines by Northern blot analysis (Oraveerakul *et al.*, 1992). *In situ* hybridization was used to detect viral DNA in tissue samples or for subcellular localization (Oraveerakul *et al.*, 1993; Bolt *et al.*, 1997; Zadori *et al.*, 2001). PCR-based techniques have also been used as very sensitive methods for detection and quantitation of PPV DNA.

Serological methods are based on specific interaction between viral proteins (antigens) and antibodies. Serological tests provide indirect information about the presence of virus, because these assays quantitate antibodies produced by animals reacting to PPV infections. HAI is based on the ability of anti-PPV antibodies to block virus binding to red blood cells. HAI has been used most frequently to detect PPV-specific antibodies, because it is simple, rapid, sensitive, and inexpensive (Cartwright *et al.*, 1971; Joo *et al.*, 1976c). Pretreatment of animal sera by heat inactivation, kaolin, trypsin, and with erythrocytes to reduce non-antibody inhibitors of HA and remove naturally occurring hemagglutinins usually increases the sensitivity of assay (Cartwright *et al.*, 1969; Mengeling, 1972; Morimoto *et al.*, 1972). Antibodies produced in pigs against PPV can reduce the infectivity of virus by binding to the surface of capsids. SN assay, which is more sensitive than HAI, measures the amount of antibodies by their ability to neutralize virus infectivity (Johnson and Collings, 1971; Mengeling, 1972). A modified direct complement-fixation (MDCF) test was also successful to determine antibody titers of different PPV isolates in pig sera (Ruckerbauer *et al.*, 1978). Immunodiffusion and immunoelectrophoresis methods have also been tried; however, enzyme-linked immunosorbent assay (ELISA) is becoming the most applied test to detect PPV-specific antibodies because it is superior in terms of specificity, sensitivity, and speed to the previously described techniques (Hohdatsu *et al.*, 1988; Westenbrink *et al.*, 1989; Jenkins, 1992).

## REPLICATION IN VITRO

Replication of PPV has been studied mainly *in vitro* using tissue culture conditions. Originally, primary cultures and subcultured monolayers of porcine kidney cells, prepared from fetuses or young animals, were used for isolation, propagation, and titration of PPV. Porcine tissue cultures, derived from thyroid gland, kidney, testis, and lung, are more susceptible to PPV than cell cultures prepared from other species (Pirtle, 1974). However, PPV contamination was found sometimes in cultures prepared from porcine tissues. Since PPV is ubiquitous in swine throughout the world, freshly prepared cultures should be tested for PPV contamination using the most sensitive methods that are available (IF or polymerase chain reaction [PCR]). Established porcine cell lines, such as swine testis (ST), pig fallopian tube (PFT), and pig kidney (PK-13, PK-15) cells are also used for propagation of PPV. These stable cell lines are known to be free of PPV contamination; nevertheless the virus can be accidentally introduced into cell cultures

during laboratory manipulations. Contaminated trypsin, derived from pancreas of PPV-infected pigs, was a major cause of virus transmission into cell cultures before suppliers started to test for the presence of PPV. Probably, this was the case for KBSH strain isolated from KB cells, which was one of the several parvoviruses recovered from permanent human cell lines (Siegl *et al.*, 1972).

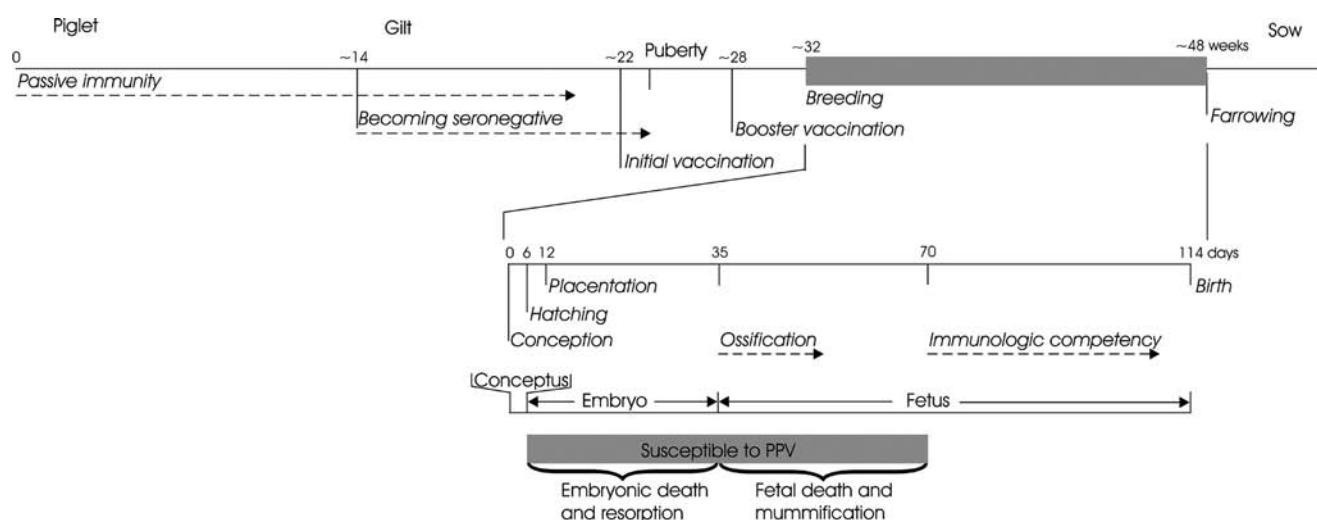
Replication of PPV DNA takes place in the nuclei of infected cells and it is dependent on the mitotic activity of host cells similar to all other members of parvovirus genus (Cartwright *et al.*, 1969; Mengeling, 1972). Replication of virus in porcine host cells induces cytopathic effects (CPE), which may include vacuolation and condensation of the cytoplasm, pyknotic nuclei, rounding up, and disintegration of cells. Hematoxylin-eosin staining can reveal the development of intranuclear inclusions in some cells, which are bodies packed with virus particles in the interchromatin area leaving the nucleoli well defined (Cartwright *et al.*, 1969; Shahrabadi *et al.*, 1982). Depending on the multiplicity of infection (MOI), lysis of cells may be seen after 2–8 days of infection. Sometimes, inhibition of mitosis can be observed before the onset of full CPE when infected cultures are compared with parallel non-infected ones (Mengeling, 1972). Addition of cell culture-adapted virus to rapidly growing cells (freshly seeded, subconfluent monolayer) result in CPE earlier than virus extracted from an infected animal. During the initial isolation of virus (or inoculation at very low MOI), cell culture can become confluent before characteristic signs of CPE start to appear. This could lead to incomplete lysis and only partial detachment of cell fragments from the surface of culture. Small amounts of virus, especially when initial isolation of virus is attempted, may require repeated subculturing of infected cells.

Immunofluorescent microscopy has been extensively used to follow the progress of PPV infection in cell culture (Cartwright *et al.*, 1969; Mengeling, 1972; Siegl *et al.*, 1972). Shortly after addition of high titer suspensions of virus to the culture, viral antigen can be detected inside the cells. Experiments with radiolabeled PPV particles have shown that, at the beginning of infection, viruses bind to the surface of cells and soon enter the endosomal apparatus of cells (Zadori *et al.*, 2001). During the early course of infection, the majority of viruses move from the early endosomes to the late endosomes/lysosomes; this results in perinuclear localization at 2–4 hours post infection. Some of the viruses reach the nucleus after 8 hours, and their entry is immediately followed by the synthesis of new viral protein and DNA, respectively. Nascent viral antigens accumulate in the nuclei of infected cells, but sometimes their amount is sufficient enough to stain both cytoplasm and nucleus with bright fluorescence when labeled antibody is used for detection. Almost all cells of a growing culture contain positive nuclei after 16–22 hours of incubation if high MOI is applied. In the case of low MOI, the few cells that become infected after 22 hours could initiate a second wave of infection with newly produced viruses but the culture should contain proliferating

cells. Consequently, cells should be fixed after 20–24 hours if virus titration is based on IF microscopy. The production of new viruses in the culture medium can be measured by HA, quantitative PCR, and titration on freshly seeded PPV-susceptible cells. Most of the non-permissive cells are as efficient in the uptake of PPV particles as the porcine host cells. However, the viral cycle leading to infection becomes deficient in these cells after PPV reaches the perinuclear area. Infection experiments and transfections with purified PPV genome indicate that permissiveness of cells to virus replication is probably determined by intracellular and intranuclear events rather than selective binding and uptake of PPV (Ridpath and Mengeling, 1988; Oraveerakul *et al.*, 1992).

## PATHOGENESIS

Results of initial experiments infecting pregnant animals with PPV field strains were inconclusive, because they could not reproduce the clinical symptoms of reproductive failure. In the first reports, seronegative piglets or pregnant gilts were infected with PPV strain 3060, isolated from aborted piglets, using oral, intravenous, intramuscular, or intranasal infection routes (Johnson and Collings, 1969, 1971). Viremia was detected in sows between 1–7 days post infection and lasted for 2–4 days; occasionally transient panleukopenia was also observed. Rapid production of antibody (6–8 days post infection) could be detected with HAI and SN, rising to a high titer by 14 days. Development of pregnancy appeared normal; however, specific antibody was detected in newborn piglets. The possibility of immunological tolerance was suspected for litter members from gilts infected before 33 days gestation. Stillborns and mummified fetuses found in a few litters could not be attributed to infection. A similar study was published for PPV strain 59e/63, isolated from breeding herds with SMEDI syndrome (Cartwright *et al.*, 1971). Baby piglets, and sows at various time during gestation were infected by inoculations using intracerebral, intravenous, and oral routes and semen – virus mixture at insemination, respectively. Infections of very young piglets were subclinical, although sometimes rising temperatures, dullness, anorexia, and vomiting have been noted when virus stocks of low passage numbers were used. Viremia was present up to at least 7 days post infection, and virus could be recovered from feces, blood, and most tissues examined. Infection of sows at various stages of pregnancy did not result in reproductive failure; antibody production was detected in some piglets, but the recovery of virus was limited. These studies established that isolated PPV strains can infect swine, and the transmission of virus to offspring occurs via transplacental infection. Although these initial experiments did not prove the etiologic role of PPV in reproductive failure, naturally occurring infections and subsequent experimental studies have found strong association between the disease



**Figure 30.1** Major reference points for the control of pig infection by PPV. Seropositive dams may provide piglets with passive immunity that protects them from PPV infection before reaching the age of puberty. Two consecutive vaccinations are recommended for seronegative gilts before their first breeding and a single dose of revaccination is necessary 4–6 weeks before each subsequent conception. Infection of pregnant animals, that do not possess active immunity acquired either by vaccination or following natural exposure, could lead to reproductive failure. Access of PPV into the embryo can result in death and resorption; the conceptus is protected by the zona pellucida. After 35 days of gestation, fetal death and mummification are the main consequences of PPV infection because early ossification prevents the fetal resorption. After 70 days, fetuses usually survive infection because they are able to produce protective antibodies.

and virus infection. Positive HAI and SN tests and successful recovery of virus demonstrated direct involvement of PPV in the disease.

The effect of infection on the fetus changes with the progress of gestation (Figure 30.1). Several experiments indicated that infection of dams at anytime during the first half of gestation could lead to reproductive failure (Mengeling and Cutlip, 1975; Joo *et al.*, 1976a; Mengeling *et al.*, 1980). These findings were supported by epidemiologic studies (Donaldson-Wood *et al.*, 1977; Mengeling, 1978; Mengeling *et al.*, 1991). Fetuses infected after 70 days of gestation become seropositive and survive infection because they are immunocompetent by this time. Infection of fetuses could occur only 10–15 days after the inoculation of dam by intramuscular injection and somewhat later by the oral route (Joo *et al.*, 1976a; Mengeling *et al.*, 1980). Therefore, infection of dams after 56 days of gestation generally does not cause damage to the fetus (Joo *et al.*, 1976a). Piglets are born but they may be seropositive. The infection of the embryo before 35 days after conception results in death and resorption (Mengeling *et al.*, 1980). After the ossification of skeleton, mummification could occur as a consequence of infection in the fetus between 35 and 70 days of gestation (Mengeling and Cutlip, 1975, 1976; Joo *et al.*, 1976a). Reproductive failure is caused by the direct effect of PPV on a variety of vital tissues and organs, including the placenta (Mengeling, 1992). The virus replicates in the proliferating cells of the fetus. The most apparent features are congestion, edema, hemorrhage, and accumulation of serosanguinous fluids in body cavities because of the damage to the vascular system (Mengeling, 1992).

A crucial question about PPV-induced reproductive failure is how viruses enter the embryo or fetus. No evidence has been found that PPV could infect embryos through the zona pellucida. It was suggested that maternal macrophages are the vehicles of PPV to penetrate the placental barrier (Paul *et al.*, 1979; Mengeling *et al.*, 2000). It was shown that PPV is capable of at least limited replication in lymphoid nodes and immune cells (Choi and Chae, 2000; Krakowka *et al.*, 2000). Further studies are necessary to prove the role of macrophages in transmitting PPV between the dam and its embryos.

## EPIDEMIOLOGY

PPV infection of swine was identified in many countries throughout the world (Molitor and Joo, 1990). While vertical transmission of PPV to prenatal pigs occurs via transplacental infection, postnatal pigs are infected by horizontal transmission, and the usual route is oronasal. Serologic surveys indicate that PPV antibody production is common among the animals and infection is enzootic in most herds. Piglets of immune dams absorb antibodies from the colostrum. Passively acquired antibody protects pigs from infection until it vanishes, which occurs at 26 weeks of age (Johnson *et al.*, 1976; Paul *et al.*, 1982). Susceptible seronegative animals in swineherds can become infected by PPV in several different ways. PPV is a very resistant virus; it may contaminate the pen, sty, and premises over a long period. The virus may remain infectious for months in secretions

and excretions from acutely infected pigs (Mengeling and Paul, 1986). Workers and animals may possibly transport the virus. Rats were proposed as a virus vector, because their blood contained anti-PPV antibodies in PPV-infected farms, and they were seronegative in PPV-free farms (Joo *et al.*, 1976d). However, in experimental studies, rats could only be infected with high doses of PPV using parenteral inoculation or prolonged oral exposure (Cutler *et al.*, 1982a). IF analysis demonstrated that PPV replicated in the small intestine of some of the animals and the virus could be detected in feces and urine. However, experiments for the transmission of PPV from infected rats to seronegative pigs were unsuccessful. This may indicate that rats are not significant vectors, although the experiments were done with a non-pathogenic strain, NADL-2 (Cutler *et al.*, 1982a).

Introduction of acutely infected animals into herds usually did not create immediate problems because most herds were enzootically infected. Some pigs are possibly persistently infected and may occasionally shed virus, but this has yet to be demonstrated (Johnson *et al.*, 1976; Gradil *et al.*, 1990). Separation of gilts for breeding or purchasing a new breeding stock could result in the selection of seronegative, susceptible gilts. Introduction of these animals in a great number into a herd within a short period of time can lead to acute outbreaks of infection (van Leengoed *et al.*, 1983).

It has been suggested that mating with acutely infected boar or artificial insemination with PPV-containing semen might play a role in PPV transmission (Cartwright *et al.*, 1971; Ruckerbauer *et al.*, 1978). HAI test on boars at artificial insemination units showed that a high percentage of them were seropositive and PPV was detected in semen of some naturally infected boars (Cartwright and Huck, 1967; Cartwright *et al.*, 1969; Ruckerbauer *et al.*, 1978). Seronegative boar can become infected either by the oronasal route or during natural mating when virus is in the vaginal mucus. Virus may get to the semen from the male reproductive tract or by external contamination (e.g. virus-containing feces). In experimentally infected boars, the transmission of virus from testicular tissues into the semen could not be confirmed. Apart from the venereal transmission, boars could disseminate PPV infection as physical vectors.

## IMMUNITY

Most seronegative pigs quickly produce antibodies after they are inoculated with PPV (Johnson and Collings, 1969, 1971; Cartwright *et al.*, 1971). In these experimental studies, antibody has been detected by HAI and SN techniques as early as 6–9 days post infection, and the titers rose close to the maximum between 14 and 21 days. An investigation for the persistence of antibodies indicated that HAI titers remained high in infected sows for up to 4 years, despite the absence of reinfection in the commercial herd (Johnson *et al.*, 1976). Besides the stability of actively acquired immunity, it has also been

suggested that latent PPV infection could cause the persistence of detectable antibody levels (Gradil *et al.*, 1990). Before the introduction of regular vaccinations, serological tests indicated that seropositive animals were present in most herds with a varying ratio (Mengeling, 1972, 1978; Johnson *et al.*, 1976; Pointon *et al.*, 1983). High security isolation of herds was necessary to keep pigs free of PPV infection and maintain the seronegative status. However, serological examinations indicated that a significant portion of animals could have antibodies against PPV even in some specific-pathogen free (SPF) herds (Sorensen, 1982). Management practices were designed for placing seronegative gilts in contact with older sows to develop immunity against PPV infection (Johnson *et al.*, 1976). However, groups of seronegative animals who were susceptible to PPV infection continually arose during the management of alternate generations of swine (Cutler *et al.*, 1982b; Pointon *et al.*, 1983). Reproductive failure afflicts mainly gilts but outbreaks have also occurred in herds consisting of only sows indicating that a significant number of older females can occasionally be seronegative (Cutler *et al.*, 1983).

The porcine fetus becomes immunocompetent at about 70 days post conception (Figure 30.1). The diffuse, epitheliochorial placenta does not allow antibody transfer between the mother and the fetus in pigs. Neonate piglet receives protection from the dam by absorbing antibodies from the colostrum and later from milk. Normal sow-reared piglets start to produce immunoglobulins at 10–14 days of age. Colostrum of seropositive dams contains >10 times higher concentration of antibodies against PPV than their respective serum titers. Therefore, the level of anti-PPV antibodies increases dramatically in the sera of seronegative offspring after the first 24 hours of suckling (Johnson *et al.*, 1976). Antibody titers are gradually decreasing by dilution and degradation, resulting in seronegative pigs usually between 22 and 24 weeks of age (Figure 30.1). Anti-PPV antibodies can become undetectable as early as 14 weeks of age but, in some cases, they may persist up to 9 months (Johnson *et al.*, 1976). High levels of passive antibodies prevent infection, and somewhat lower levels can reduce dissemination of PPV from infected pigs (Suzuki and Fujisaki, 1976; Paul *et al.*, 1980). However, sometimes even low levels of anti-PPV antibodies may inhibit the development of active immunity against PPV (Johnson *et al.*, 1976; Paul *et al.*, 1980; Paul and Mengeling, 1986). While vaccinated pigs produce antibodies only against epitopes of structural proteins, animals infected with field virus have anti-NS1 and anti-VP2 antibodies in their sera (Madsen *et al.*, 1997). Specific antibodies against NS1 could differentiate between infected and vaccinated animals.

A weak cytolytic activity of cytotoxic T lymphocytes was observed peaking on days 80–87 in pigs experimentally infected with PPV (Ladekjaer-Mikkelsen and Nielsen, 2002). Moreover, the CD4+ CD8+ T-cell subset of peripheral blood mononuclear cells proliferated in response to PPV, suggesting that cellular immunity may play a role in controlling PPV infection in addition to humoral immunity.

## CO-INFECTION WITH CIRCOVIRUS

Postweaning multisystemic wasting syndrome (PMWS) is an emerging pig disease that was first identified in western Canada in the 1990s, and by now it has been reported worldwide in the swine-raising countries. Clinical symptoms comprise progressive weight loss, respiratory diseases, fever, occasional jaundice, and gross lesions including lymphadenopathy, nephritis, pneumonia and hepatopathy. A novel porcine circovirus (PCV-2), which shows <80 percent nucleotide sequence identity with PCV-1, has been associated with development of PMWS. Several inoculation experiments using PCV-2 alone have resulted in histopathologic lesions without the main clinical syndromes of PMWS. At the same time, laboratory experiments and field studies indicated that PPV co-infection potentiates the effect of PCV-2 in the development of PMWS (Allan *et al.*, 1999; Choi and Chae, 2000; Ellis *et al.*, 2000; Kennedy *et al.*, 2000). Other pathogens such as porcine reproductive and respiratory syndrome virus (PRRSV) have also been shown to promote the manifestation of PMWS in dual infection with PCV-2. Recent studies demonstrated the fundamental role of PCV-2, because infection with the pure virus could induce the clinical signs of PMWS in some of the cesarean-derived, colostrum-deprived (CD/CD) or SPF pigs. The mechanism of how PPV potentiates PCV-2 to cause PMWS in dually infected gnotobiotic swine is not known (Krakowka *et al.*, 2000; Rose *et al.*, 2003). Porcine parvovirus replicates in lymphocytes, macrophages, and monocytes and has been associated with transient immunosuppressive effects (Paul *et al.*, 1979; Harding and Molitor, 1988; Oraveerakul *et al.*, 1993). PPV could advance PCV-2 infection either by reducing immunoprotection or promoting virus DNA replication infecting the same host cells as PCV-2. A cross-sectional study carried out in France to assess risk factors for PMWS affirmed the synergy between PCV-2 and PPV (Rose *et al.*, 2003). Interestingly, when only one shot was administered for the first vaccination of the gilts against PPV, the odds of PMWS were increased, while improving the quality of immune status was protective for PMWS.

## OTHER DISEASES

In the early 1980s, a new strain of PPV (Kresse virus) was isolated from piglets with clinical signs of necrotic and vesicle-like lesions. Generally, pigs were mostly affected on the tongue, lips, snout, and foot at age of 1–4 weeks; sometimes anorexia and diarrhea were also noted (Kresse *et al.*, 1985). Intradermal or oral and intranasal inoculation of PPV-infected crude skin suspension into hysterectomized, colostrum-deprived pigs reproduced the development of the disease. At the same time, inoculation of virus passaged and produced in cell culture resulted in a transient manifestation of the characteristic symptoms, but none of the pigs had all

the signs. Antibiotic treatment improved the sickness but did not always reduce the morbidity. These observations indicated that the disease is probably caused by dual viral and bacterial infection. Experimentally infected pigs produced high antibody titers and PPV could be isolated from a several organs: kidney, tongue, lymph nodes, tonsil, tendon sheath, and foot lesions. It was indicated later that Kresse is a highly pathogenic PPV strain and it has a broad tissue tropism (Choi *et al.*, 1987; Oraveerakul *et al.*, 1993). Depositions of immune complexes were found in skin, kidney, and brain of fetuses infected by Kresse virus (Choi *et al.*, 1987). Porcine parvovirus was found associated with cutaneous lesions in piglets and exudative epidermitis (Whitaker *et al.*, 1990; Kim and Chae, 2004). Although the causative agent of exudative epidermitis is *Staphylococcus hyicus*, triggering factors are probably necessary for the disease to appear. The disease begins with reddening skin and greasy spots generated by odorous secretion of serum; later these patches may spread to cover large areas, causing thickening, cracking, and discoloration. A study on a Swedish-SPF herd did not find the presence of PPV in any of the sick animals (Watrang *et al.*, 2002). Therefore, it is likely that after predisposing factor(s) allow *Staphylococcus hyicus* to infect skin lesions and induce the disease; the environment for PPV becomes more favorable. The damaged skin contains proliferating cells and invading macrophages, which are preferred hosts for porcine parvovirus. PPV infection could exacerbate the clinical signs of the above-mentioned skin diseases.

An association between PPV DNA and inflammatory lesions was found in some cases of non-suppurative myocarditis in piglets (Bolt *et al.*, 1997). Sensitivity of PPV detection was more efficient with PCR analysis (especially when combined with Southern blot hybridization) than with *in situ* hybridization. Porcine cardiac myocytes proliferating beyond the neonatal period were suspected as host cells for PPV. Mild interstitial nephritis could be observed in colostrum-deprived, PPV-infected pigs (Kennedy *et al.*, 2000).

Porcine parvovirus-like particles were also associated with diarrhea in pig (Yasuhara *et al.*, 1989). Notwithstanding the physicochemical similarities, these viruses were antigenically different from PPV. In spite of data on other parvoviruses causing enteric diseases, there is no experimental evidence that PPV could extensively replicate in the crypt epithelium. Factors that may modify the outcome of infection include differential pathogenicity of individual parvovirus strains and infection by other infectious agents. Immune suppression may prolong the elimination of PPV from the body of animals, perhaps enabling a degree of virus amplification in vulnerable proliferating cells.

## IMPACT OF PPV ON TRANSFUSIONS AND XENOGRAFTS

Almost 1 out of 10 hemophilia patients treated with human factor VIII develops persistent inhibitor antibodies, which

render even the increased doses of human factor concentrates ineffective. Porcine antihemophilic factor VIII concentrate (Hyate:C) can be used successfully in the majority of these patients, because it lacks antibody-specific epitopes. Hyate:C (Ipsen Ltd., UK) is produced from pooled porcine plasma collected at designated abattoirs. Therefore, current protocols include tissue culture and PCR screening of porcine plasma, and preparations to reduce the chances of viral contamination during manufacturing. The absence of known infectious agents is confirmed in each batch of final product using rigorous cell culture tests to eliminate the possibility of transmission of viral illness to the human recipients. In 1996, after the implementation of these new revised assays, several product lots were found to contain low levels of PPV and were recalled by the manufacturer. Additional testing confirmed that some previously released lots also contained PPV. Two studies were reported on the assessment of PPV contamination in Hyate:C products and testing of recipients of these lots for the clinical symptoms related to PPV exposure (Soucie *et al.*, 2000; Giangrande *et al.*, 2002). Although PCR and cell culture assays detected PPV contaminations in most of the suspected previously released Hyate:C products, patients did not have PPV antibodies in their blood, tested either with indirect IF or HAI assay. Hyate:C productional personnel and Ipsen plasma collection staff at the abattoir, and recipients of porcine heparin and porcine insulin were also seronegative for PPV. This indicated that human recipients and workers exposed to porcine parvovirus did not become infected.

Even the contaminated Hyate:C samples carried very low amounts of PPV. These viruses are probably complexed with neutralizing porcine antibodies partly because of concentration protocols. Fractionation techniques used for the Hyate:C purification could also reduce the infectivity of PPV. Therefore, the relative safety of Hyate:C application may not apply to xenotransplantation. Healthy organs and subjects of xenotransplants possess mitotically active cells, which are preferable hosts for parvoviruses. PPV as one of the most stable parvoviruses could escape the usual inactivation treatments, and it may become important to prevent its transmission from the xenograft to the human body. If the use of pig tissues as a source for xenografts in humans is permitted, special screening for PPV contamination would then become necessary.

## DIAGNOSIS

Reproductive failure is the only well-established syndrome of PPV infection of pigs. The most common feature of PPV-induced reproductive failure is the appearance of mummified fetuses (Cutler *et al.*, 1983; Mengeling, 1992). Other clinical signs may be smaller litters, irregular returns to oestrus, failure to farrow, abortions, stillbirth, and, less typically, weak piglets or neonatal death. The clinical

manifestation of PPV-induced reproductive failure depends on the gestational stage during which the pregnant animal is infected with the virus. Records of herd history containing data about the proportion of gilts, vaccination practices, and serostatus of breeding pigs may help to consider PPV as the causative agent in the outbreak of reproductive failure (Molitor and Joo, 1990).

Generally, laboratory analysis of samples is necessary whenever the evidence of embryonic or fetal death is observed. Detection of PPV antigen in mummified fetuses or their remnants is usually the best proof of a causative role for the virus. IF microscopy is a reliable and commonly used method to identify PPV in frozen tissue sections of infected fetuses (Mengeling and Cutlip, 1975, 1976). Lung is the most recommended sample if available because it is relatively easy to identify in a mummified fetus and it has minimal autofluorescence (Mengeling, 1978). However, this method is recommended only for fetuses that died at less than 70 gestational days, because antibodies produced in the older fetuses could interfere with the assay. HA has also been used to detect virus in the tissue extracts of mummified fetuses (Joo *et al.*, 1976b). Virus isolation from dead fetuses is not a routine diagnostic procedure because it is time-consuming, there is a danger of laboratory contamination, and infectivity of virus is declining after fetal death. For fetuses that died at >70 days of gestational age, serological procedures are recommended. The time of death can be determined by measuring the crown-rump length (Marrable and Ashdown, 1967). Since maternal antibodies do not enter the fetus, positive tests for PPV antibody in sera of fetuses or stillborn pigs indicate transplacental infection. Sera of neonatal pigs should be taken for test before they nurse. Examination of body fluids of fetal pigs can be a good alternative when serum is not available. Negative results obtained from testing of maternal sera can exclude PPV as a cause of reproductive failure. PCR is a very sensitive method for PPV detection and it can be used when the infectivity of virus in the samples is impaired or when neutralizing antibodies are present.

## PREVENTION

PPV-induced reproductive failure cannot be treated; therefore the infection of pregnant animals should be prevented. Originally, several different management protocols were proposed to prevent *in utero* infections (Johnson *et al.*, 1976). However, shielding of PPV-free breeding stocks from infection is difficult because PPV is a very resistant, prevalent virus, and determination of parvovirus status of each herd into which introduction of new gilts is planned is very complex. Introduction of PPV into an entirely virus-free herd results in an acute outbreak of infection with severe consequences (Donaldson-Wood *et al.*, 1977). Delaying the breeding until the gilts are becoming seropositive may not work in most cases because different areas of farms have varying

levels of PPV contamination, and contact between seronegative gilts and seropositive sows does not always result in the development of immunity in all animals (Pointon *et al.*, 1983; Mengeling and Paul, 1986). Feeding gilts with small quantities of infected feces or mummified fetuses also promises only limited success, because the concentration of infective virus in these materials may be low (Joo *et al.*, 1976b; Mengeling and Paul, 1986). Considering the high immunogenicity of virus and the persistence of immunity in infected animals, vaccination of gilts prior to breeding was assumed as a promising method to assure the prevention of PPV-induced reproductive failure. Vaccines were prepared from inactivated or attenuated viruses, and recently from expressed structural proteins. The efficiency of different vaccines and immunization methods have been tested by measuring antibody titer in pigs, inhibition of viremia and reproductive failure after challenge with live virus, respectively.

Inactivated vaccines have been developed in several laboratories (Suzuki and Fujisaki, 1976; Joo and Johnson, 1977; Fujisaki *et al.*, 1978; Mengeling *et al.*, 1979; Wrathall *et al.*, 1984). Inactivated vaccines against PPV are produced by the chemical inactivation of virus particles isolated from porcine cell cultures. Formalin, beta-propiolactone (BPL), acetyleneimine, and binary ethyleneimine (BEI) diminish the infectivity of PPV without eliminating its antigenic and agglutinative properties. Aluminum hydroxide gel or oil-emulsion adjuvants significantly increase the immunological power of inactivated vaccines. Efficiency of inactivated PPV vaccines and adjuvanting compounds to induce the production of high level of virus neutralizing antibodies can be tested in guinea pig model (Molitor and Joo, 1990). It was shown that high antibody titer after vaccination is required for effective protection against oral infection (Suzuki and Fujisaki, 1976). However, a moderate humoral immune response with HAI titers as low as 10 in vaccinated gilts also prevented the transplacental infection (Mengeling *et al.*, 1979). The mechanism of this prevention is unknown; probably the challenging virus replicated at some sites of exposure resulting in increased antibody production without detectable viremia. Further studies on the level of vaccine and passive immunity indicated that animals with HAI titers >160 are effectively protected from infection and low level of antibodies might render pigs less susceptible to PPV (Paul *et al.*, 1980). According to some studies, animals with residual maternal antibodies can be efficiently vaccinated by inactivated virus (Joo and Johnson, 1977; Wrathall *et al.*, 1987). However, passive antibody titers in the range of 40–80 (HAI) could block immune responses to vaccination but at lower levels, there is no interference (Paul and Mengeling, 1986). Several studies indicate that immunization with inactivated virus could lead to elevated antibody levels, especially when a second booster injection was applied (Joo and Johnson, 1977; Wrathall *et al.*, 1984). Duration of immunity can last for at least 13 months after vaccination (Vannier *et al.*, 1986). Field trials have also confirmed the effectiveness

of killed-virus vaccines, and several companies produce them for veterinary practitioners (Fujisaki *et al.*, 1978; Vannier *et al.*, 1986; Wrathall, 1988).

Modified-live virus vaccines (MLV) have been prepared from attenuated viruses to produce longer-lasting immunity than was achieved with inactivated viruses. NADL-2 strain, which was maintained in cell culture, appeared to be a promising candidate because, after oronasal or parenteral inoculation, it did not cause transplacental fetal infection in seronegative pigs (Mengeling and Cutlip, 1976). Intramuscular vaccination of swine with NADL-2 resulted in higher antibody titers and increased protection against challenge with virulent virus (NADL-8) than oronasal inoculation (Paul and Mengeling, 1984). HAI titers could be observed at low doses (10–100 TCID<sub>50</sub>) and increasing the amount of virus (up to 10<sup>5</sup> TCID<sub>50</sub>) has induced a stronger immune response. Although experiments have indicated that vaccination with NADL-2 did not lead to transplacental infection, inoculation with the necessarily high doses of virus could induce low titer viremia and some virus shedding in the feces (Paul and Mengeling, 1984). Repeated passages of 90 HS field virus in embryonic swine kidney (ESK) cell line at low temperature produced HT<sup>−</sup> strain of PPV with low virulence. Vaccination with HT<sup>−</sup> strain protected swine from infection by orally inoculated wild-type PPV (Fujisaki and Murakami, 1982). However, inoculations of colostrum-deprived, neonatal pigs with attenuated virus by the subcutaneous route resulted in local multiplication of virus in some animals (Kubota *et al.*, 1990). Only a small amount of virus could be recovered in this study from spleen, liver, and mesenteric lymph nodes, and the isolates replicated better at 32°C, thus retaining the signature of the HT<sup>−</sup>/SK strain.

Subunit vaccines produced in bacteria or baculovirus are less expensive than viruses prepared in cell culture. These new generations of vaccines prepared by using recombinant DNA technology are also expected to be safer and may be more environmentally friendly than traditional vaccines. However, PPV structural proteins produced in *Escherichia coli* did not protect animals from reproductive failure when tested by experimental PPV challenge. Antibodies produced by animals did not efficiently neutralize virus *in vitro* (Molitor and Joo, 1990). PPV virus-like particles (VLPs) have been produced in recombinant baculovirus-infected insect cells (Martinez *et al.*, 1992). Injection of pigs with these PPV-VLPs resulted in a similar immunization response as with the commercial vaccine.

While more experimental trials are necessary to address the safety of modified-live virus vaccine candidates and the effectiveness of VLPs, it is accepted that inactivated vaccines are safe, and their application is now common in most countries concerned about PPV-induced reproductive failure (Mengeling, 1992; Molitor and Joo, 1990). It is advised to vaccinate gilts before the first breeding with two initial doses and later at least once a year (Figure 30.1, p. 438).

If PPV infection is noticed, vaccination of seronegative sows and boars is also recommended in that area.

## ECONOMIC ASSESSMENT

The facilities and management system for keeping swine may vary significantly as well as the number of animals in the herd. PPV can persist for years in infected piggeries and eradication through disinfection is extremely difficult as the virus is highly resistant to physical and chemical agents. As the number of susceptible animals fluctuates, PPV infection in the herd can decrease the reproductive performance. Endemic infection may go unnoticed, especially without good record-keeping and careful analysis. Acute outbreaks of PPV infection are readily apparent and the economic cost is more serious (Donaldson-Wood *et al.*, 1977; Morrison and Joo, 1984). Different management methods were recommended prior to the availability of vaccination to avoid reproductive disease. However, these practices were insecure and more expensive than vaccination (long waiting period before breeding to develop immunity or isolation of brought in animals). An alternative method to vaccination is to test the blood of all replacement gilts at 6–7 months of age. On average, 15 percent of replacement gilts do not become infected with the virulent field virus and have low serum antibody titers. These gilts should then be excluded from the breeding herd, thereby reducing the profit. Only proper vaccination can ensure that gilts have protective immunity at mating age. According to one study, the financial loss of PPV infection ranged from \$429.50 to \$1635.09 in a group of 100 replacement gilts that experienced an episode of fetal death during endemic infection (Molitor and Joo, 1990). A loss of \$7260 was calculated for an acute outbreak on a farm with approximately 130 sows at different stages of their reproductive cycle (Morrison and Joo, 1984) (original dollar figures). Prevention of PPV-induced reproductive disease by vaccination could increase the productivity in the pig industry. However, cost analysis of vaccination in field trials is complex because of the high prevalence of virus. It was suggested that vaccination should be used only after considering the absence of PPV in the selected herd to be cost-effective (Wrathall, 1988). The cost-effectiveness of widespread vaccination versus monitoring the antibody status can be decided according to the actual prices of reagents and the size of herds. A decision analysis model was developed to assess the economics of PPV vaccination (Parsons *et al.*, 1986). The modification of this model has used parameters based on data derived from farm studies and serological surveys. According to calculations, a long-term vaccination program is a cost-effective method for the control of both endemic and epidemic PPV infection in a 100-sow pig herd (Parke and Burgess, 1993). There is no need for vaccination against PPV if the pigs are for meat production because losses only occur in pregnant stocks.

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# Waterfowl parvoviruses

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Z. ZÁDORI, J. SZELEI, I. KISS, AND P. TIJSSEN

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In the 1960s, a highly contagious epidemic of a waterfowl disease swept across Europe, causing great economical losses, at the time of the introduction of intensive industrial poultry farming. The disease affected goslings <4 weeks old, often with 70–100 percent mortality. The syndromes were not distinctive, and so veterinarians used various names for the disease according to the different clinical characteristics and pathological abnormalities. It was known as goose influenza in Hungary, virus hepatitis of geese in Germany, goose plague in the Netherlands, infectious myocarditis of geese in Italy and ascitic hepatonephritis in France. To eliminate the discrepancies in nomenclature, the World Poultry Science Association in 1974 designated it Derzsy disease after a Hungarian veterinarian, the late Domonkos Derzsy, who played a crucial role in the research of the disease and the international effort required to control it.

Although the disease came to the forefront of veterinary research only in the second half of the 1960s, records can be found describing the disease as early as the first years of the last century (Levine, 1959). In the earliest studies, the etiological factor was characterized as influenza-like hemophilic bacteria. In later work, Wachnik and Novacki in 1962 identified the causative agent first as a virus with similar properties as duck hepatitis virus. Later several researchers (Krauss, 1965; and Van Cleef and Miltenburg, 1965; Derzsy *et al.*, 1966) isolated an undefined virus from cadavers. Derzsy later tentatively classified the virus as rheovirus (Derzsy, 1967). Finally, Schettler (Schettler, 1971a), based on its behaviour in tissue culture, nucleic acid content, size of the virus, and its biochemical properties, correctly identified the virus as a member of the *Parvoviridae* family. Kisary and Derzsy (1974) confirmed this finding and thereafter the virus was named

goose parvovirus (GPV). Almost 20 years later, the first characterization of the nucleic acid and viral proteins (Le Gall-Recule and Jestin, 1994; Zádori *et al.*, 1994) and the sequencing of the viral genome (Brown *et al.*, 1995; Zádori *et al.*, 1995) confirmed the classification.

Outbreaks of Derzsy disease were reported from several countries within three continents (Europe, Asia, and North America) and, today, there is virtually no country with intensive waterfowl farming free of the disease (Takehara *et al.*, 1995a; Woolcock *et al.*, 2000).

Vaccines were developed in several countries (Hoekstra *et al.*, 1973; Kisary *et al.*, 1978; Takehara *et al.*, 1995b) and GPV seemed to be well under control in most European countries. However, in 1989 in France a new parvovirus emerged with symptoms similar to Derzsy disease and caused great economical losses even in GPV-vaccinated Muscovy duck flocks (Fournier, 1991; Jestin *et al.*, 1991). The virus was named Muscovy duck parvovirus (MDPV).

## VIRUS PROPERTIES

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The virions of the waterfowl parvoviruses (WFPs) are composed of three proteins (VP1–3) of which the ~60 kDa VP3 is the major capsid component representing about 50 of the 60 capsid proteins. The remaining is divided about equally between the ~65 kDa VP2 and the ~81 kDa VP1 minor components (Le Gall-Recule and Jestin, 1994; Zádori *et al.*, 1995). The capsids of GPV and MDPV do not hemagglutinate mammalian or chicken erythrocytes (Kisary and Derzsy, 1974). The virus particles are resistant to various

organic solvents and disinfectants (ether, fluorocarbon, chloroform, 0.5 percent phenol, 1:1000 formalin, sodium deoxycholate) and they cannot be inactivated by low pH (pH 3) or heat treatment (56°C for 30 min) (Schettler, 1973; Kisary and Derzsy, 1974).

WFPs have a single-stranded DNA genome, containing two major open reading frames (ORFs), bracketed by inverted terminal repeats (ITRs) similarly to the adeno-associated viruses and B19 (Astell, 1990). The positive and negative strands are encapsidated in equal frequency and readily anneal to double-stranded DNA upon extraction (Zádori *et al.*, 1994). The genome of MDPV (5132 bp) is slightly longer than that of the GPV (5106 bp). The deletions/insertions are exclusively located in the non-coding region, mostly in the ITRs, while the coding regions contain only substitutions.

The left ORFs of GPV and MDPV, similarly to other parvoviruses, code the non-structural proteins (reps). The rep1 polypeptides of GPV and MDPV show high similarity and their amino acid sequences are around 91 percent identical. The right ORF of WFPs codes the three capsid proteins (VPs). VPs are more divergent than the non-structural proteins, the VP1 polypeptide of MDPV and GPV share 88 percent amino acid sequence identity (Zádori *et al.*, 1995). Contrary to that of AAV2 the VP3 protein of MDPV and GPV is by itself capable of forming capsids (Ruffing *et al.*, 1992; Le Gall-Recule *et al.*, 1996; Kisary, 1999). The majority of the variations inside VP3 are positioned on the predicted capsid surface probably due to the evolutionary pressure by the host immune system. However, the most striking differences in the genome of the two viruses are localized in the VP2 unique region where 35 percent of the amino acids between GPV and MDPV are different (Zádori *et al.*, 1995; Chu *et al.*, 2001). Interestingly, these residues are fully conserved in the different MDPV strains and only minimal variation can be found among GPV strains. The interspecies divergence and species-specific conservation of these amino acids strongly suggest that the VP2 unique region may have a dominant role in the different biological properties of these viruses.

The intraspecies divergence in the amino acid sequence of the VP1 of European and Asian strains of GPV or MDPV does not exceed 5 percent (Chang *et al.*, 2000; Chu *et al.*, 2001). The mutations are in both cases mainly localized at the amino-terminus of the VP3 (amino acids 203–266) and at a potential surface loop at amino acids 482–534 (Chu *et al.*, 2001).

To our knowledge, there is no established cell line that supports WFP replication. GPV replicates in goose and Muscovy duck embryo fibroblasts as well as in primary goose embryo liver or kidney cell cultures (Schettler, 1973). The virus can be propagated in embryonated eggs of goose and Muscovy duck. Although there are some reports to the contrary (Gough and Spackman, 1982), virulent field isolates of GPV frequently cannot be adapted directly to Peking duck embryo (Derzsy *et al.*, 1970; Schettler, 1971c; Hoekstra *et al.*,

1973). However, Muscovy duck embryo-passaged mutant strains of GPV can readily be adapted to Peking duck embryo (Hoekstra *et al.*, 1973; Alexandrov *et al.*, 1999). High passage duck embryo or tissue culture-adapted strains of GPV become attenuated and lose their virulence in neonatal geese and Muscovy ducks (Kisary *et al.*, 1977; Gough and Spackman, 1982; Takehara *et al.*, 1998; Alexandrov *et al.*, 1999). The exact process of adaptation and attenuation is not known but it is probably a consequence of the accumulation of genetic alterations in the genome of the adapted strains. This idea is supported by results of the sequence comparison of the virulent GPV B strain and its attenuated derivative BAV, which revealed several mutations both in the NS and in the VP genes (Zádori, unpublished observations).

## EPIDEMIOLOGY

GPV naturally infects and is pathogenic for domestic geese (*Anser anser*) and Muscovy ducks (*Cairina moschata*), but it is likely that all geese species are sensitive to the virus. Accidental GPV infection of Canada goose (*Branta canadensis*) and snow goose (*Chen caerulescens*) was reported (Schettler, 1971b) in a zoological garden in Germany, and based on a serological survey it seems to be certain that other wild goose species like bean goose (*Anser fabalis*) and white-fronted goose (*Anser albifrons*) have gone through parvoviral infection in nature (Hlinak *et al.*, 1998). Chickens and all the different domestic breeds (Khaki-Campbell, Cayuga, Peking duck, etc.) of mallard duck (*Anas platyrhynchos*) as well as hybrids of Muscovy drakes and Peking ducks (so-called mulard ducks) are resistant to wild-type GPV (Derzsy *et al.*, 1970; Schettler, 1971c; Hoekstra *et al.*, 1973). Derzsy *et al.* (1970) hypothesized, with respect to a field case, that ducks may become long-term symptomless carriers of GPV and disseminate the virus. However, scientific evidence supporting this idea has not yet emerged. Rather, contrary to this theory, mulard ducklings experimentally infected with GPV remained seronegative indicating no virus replication even in crossed animals (Hoekstra *et al.*, 1973).

MDPV was first described after an outbreak during 1989 in France (Fournier, 1991) but it can be tracked back as early as 1985 in Taiwan (Chang *et al.*, 2000). MDPV infects Muscovy ducklings and does not infect goslings (Fournier, 1991; Lu *et al.*, 1993). There are no experimental data, or case reports, which would suggest that European strains of MDPV infect mallard ducks. However, some Asian strains of MDPV have a broader host range. In 1989; an epizootic with atrophic bill syndrome was reported in Taiwan. It affected several kinds of ducks including domestic mallard breeds, Muscovy ducks, and inter- and intraspecific crosses, but geese were spared. The outbreak was diagnosed as a co-infection of MDPV and duck hepatitis virus. Ducklings

from the different breeds experimentally inoculated with the isolated parvovirus alone showed near 100 percent morbidity and high mortality. As the surviving animals became stunted and a high percentage of them developed the marked atrophic bills observed in the field cases, the authors concluded that their MDPV isolates can infect and cause disease not only in Muscovy ducks but also in mallards and in crossbreeds. (Lu *et al.*, 1993).

Although geese are susceptible to GPV throughout their lives, only animals <30 days old show the clinical signs of the infection. In Muscovy ducklings the sensitive interval to MDPV can stretch until 40–50 days (Glavits *et al.*, 1993). The susceptibility to the disease is inversely proportional to the age of the animals during the sensitive phase. Mortality and morbidity also depends on the immune status of the young animals. Among fully susceptible non-immunized animals that contact WFPs in their first days of life, the death rate is frequently 60–80 percent and may sometimes reach 100 percent. Farm losses are the highest in the first year of affection and tend to decrease in consecutive years (Derzsy, 1967; Takehara *et al.*, 1994) because of the emergence of protective antibodies in the progeny of the survivors (flock immunity). Infected animals – even with high titer antibody or without the sign of the disease – continuously shed the virus with feces and become carriers, probably lifelong.

Goslings can become infected through vertical transmission to the egg or contamination of the eggshell (Schettler, 1972). Horizontal infection occurs between siblings through the digestive tract with virus contaminated water and feed. There are no reliable data about transmission via the respiratory system or the conjunctiva. The virus entering in the gastrointestinal tract probably first replicates in the cells of the intestinal wall and then spreads by viremia in the body and reaches other organs, like the heart, the liver, and the skeletal muscle (Kisary, 1993).

## PATHOGENESIS AND PATHOLOGY

In the case of egg transmission, embryos either die during the incubation period or hatch in an infected state depending on the level of yolk-sac antibody. The clinical signs in embryo are edema, hemorrhage, reddening of the skin, and degeneration of the heart and liver (Derzsy, 1967; Schettler, 1971c; Woolcock *et al.*, 2000).

In older animals, the symptoms and severity of the disease vary and largely depend on the time of infection and the level of protective antibody. In unprotected goslings infected with GPV in their first 10 days of life, the first symptoms develop at 5–7 days post infection. The signs include: decreased uptake of water and feed, listlessness, anorexia, polydipsia, and frequently diarrhea. The mortality rate is often 100 percent. Deaths peak around 11–12 days post infection (Schettler, 1971c).

Older geese, 2 weeks old, become less sensitive and have either an acute or chronic disease course. The acute cases

show conjunctivitis, nasal discharge, and diphtheroid pseudomembranes in the oral cavity. Death occurs by an average of 10 days post infection and the death rate is about 60 percent. Some animals show a striking retardation in growth, sometimes just having half the weight of their healthy siblings. They frequently lose their feathers mostly from the wings; the neck, and the back of some animals can be almost completely featherless. Their naked skin is reddened and the uropygeal gland is swollen. Surviving geese grow new feathers but remain stunted. The animals huddle together, their food consumption is decreased and they stay around a heat source even if the temperature is high (Kisary, 1993). Some of the diseased birds have a distended hanging abdomen with marked fluid accumulation in the abdominal cavity (Derzsy, 1967; Schettler, 1971c). Some goslings, infected between their second and fourth week of age, have similar symptoms like their earlier infected mates. Others develop prolonged leg weakness and have reduced body weight, although they usually recover. The total mortality can be around 25 percent in this age group. Geese, 4 or more weeks old, infected with the virus, usually do not show clinical signs of the disease. However, this age resistance can be overcome and disease produced experimentally in 4-week old animals by intravenous administration of high dose GPV. In these animals the death rate is around 10 percent and the clinical manifestations of illness are lameness, feather loss, and decreased food consumption (Schettler, 1971c).

Strain differences can also contribute to an atypical disease presentation. For example, a GPV outbreak was reported among Muscovy ducks in Japan (Takehara *et al.*, 1995a). The characteristic lesions were myositis in the skeletal muscle, and atrophy of bone marrow and thymus. Hepatitis was not detected in any case and the authors concluded that infection by European and Japanese strains of GPV result in altered pathological outcomes.

The presence of virus-neutralizing antibodies decreases the susceptibility of the animal to the GPV and consequently reduces the severity of the symptoms. Commercial hatcheries usually collect eggs from more than one breeder flock, and the level of maternal antibodies can vary even in eggs originating from the same flock. If geese with a different level of antibody are infected in a hatchery, they show the same types of clinical manifestations as their unprotected mates, although the ratio of diseased to healthy animals can differ and largely depends on the antibody level of the individuals. In such flocks, the number of acute cases and the fatalities are usually decreased, clinical signs appear later, and additional infections of the weakened animal with other microbial and viral agents may also play a role in their death (Schettler, 1971c).

The symptoms of MDPV and GPV infections show a lot of similarity but there are some differences (Table 31.1). In the case of GPV, the circulatory problems and the fluid accumulation in the abdominal cavity are among the most conspicuous clinical signs. While they also appear in MDPV-infected animals, their presence is much less obvious.

**Table 31.1** Some differences between GPV and MDPV

Features	GPV	MDPV
Host range	Geese, Muscovy duck	Muscovy duck, duck(?)
Occurrence of disease	First 3–4 weeks	First 6–7 weeks
Distinctive clinical features	Feather loss, fluid accumulation in abdominal cavity	Diarrhea, leg weakness
Distinctive pathological changes	Degeneration of hard muscle, pericarditis, myocarditis, hepatitis	Degeneration of leg muscles and kidney cells, tubulonephrosis, pancreatitis
Protection	GPV vaccines protective only against GPV	MDPV vaccines are protective against MDPV and GPV

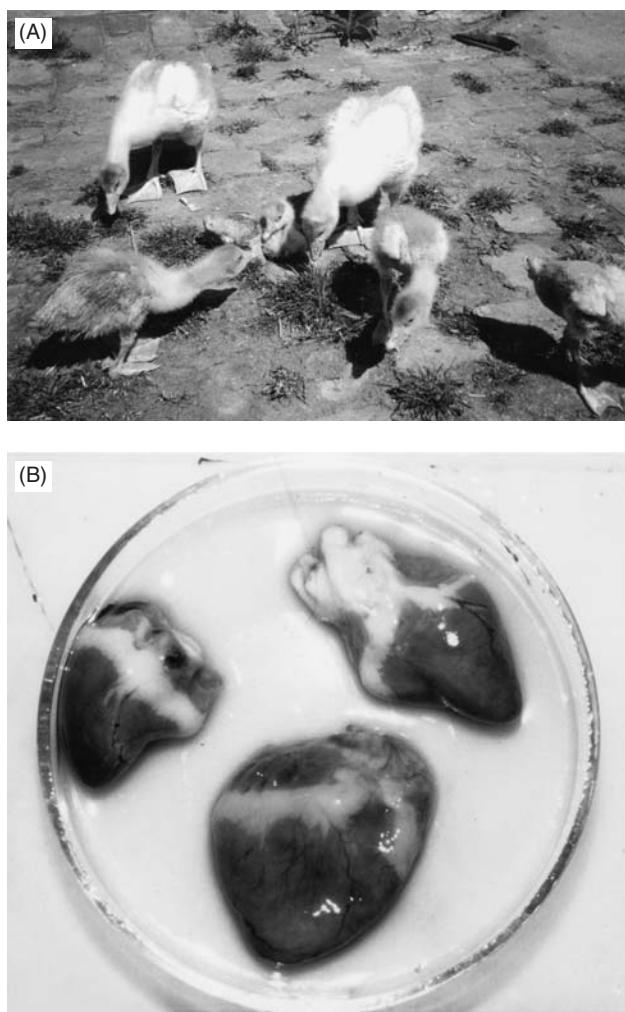
Instead, motion problems, leg weakness, paralysis, and diarrhea are the predominant symptoms of the MDPV infection (Fournier, 1991; Glavits *et al.*, 1993).

Goslings who die in the acute phase of GPV infection may also have hepatitis, coagulative necrosis around the liver capillaries, and inclusion bodies in the Kupffer cells. Usually no changes can be found in the heart and skeletal muscle in these animals.

The most typical lesions in chronic phase animals are muscular degeneration and distension of heart with rounded cardiac apex (Figure 31.1), discoloration of the myocardium, catarrh of the nasal passages and inflammation of the intestinal mucosa. The liver is swollen, hyperemic, and the accumulated fluid in the abdominal cavity contains a large amount of fibrin, which is frequently deposited on the liver and the intestines. Occasionally, edema and hemorrhage can be found in the thigh muscle. The mucous membranes in the oral cavity in the pharynx and in the epiglottis show diphtheroid lesions (Derzsy, 1968; Nagy and Derzsy, 1967; Schettler, 1971c).

The distinctions between MDPV- and GPV-infected animals are also manifest histopathologically. In MDPV-infected animals, degeneration of the cardiac muscle and the inflammation of the pericardium and liver are less frequent, while degeneration and necrosis of the skeletal muscles especially in the thighs and breast are more common. This, together with the neuritis in the peripheral nervous system, explains the leg weakness and paralysis. Tubulonephrosis and pancreatitis are also distinctive characteristics of the MDPV infection. The latter is probably responsible for the high incidence of diarrhea among MDPV-infected ducklings (Glavits *et al.*, 1993).

Secondary infections can further complicate the pathology of the disease. Different *Aspergillus* species were isolated from pulmonary granulomata. As a result of fungal infections, ulcers are sometimes encountered in the esophagus and the gizzard. Isolation of *Salmonella*, *Streptococcus* and *Escherichia coli* species was reported in different cases



**Figure 31.1** Clinical and pathological alterations in Derzsy disease-affected goslings. (A) Some of the siblings are retarded in growth, have swollen uropygeal gland and scanty plumage. (B) Hearts of diseased animals in different stage of distension.

(Derzsy, 1967; Schettler, 1971c, Takehara *et al.*, 1995a). Co-infection with other viruses like rheovirus egg drop syndrome (EDS) and duck viral hepatitis virus can also influence the course of the disease (Coudert *et al.*, 1987; Lu *et al.*, 1993).

Although the pathological side of the disease is well documented, much less is known about the tissue specificity of the virus in its natural hosts. In an electron microscopic study, GPV was demonstrated in the nuclei of cells from the bursa and heart of infected animals (Bergmann, 1987). Roszkowsky *et al.* (1982) detected the virus only in the liver tissue with the immunoperoxidase technique. Alexandrov *et al.* (1999) examined the chorioallantoic membrane of GPV-infected Peking duck and goose embryos as well as the liver and heart of experimentally infected goslings by immunofluorescence tests. They identified the virus in the cells of both the examined organs and the chorioallantoic membrane using different monoclonal and polyclonal

antibodies. Limn *et al.* (1996) and Takehara *et al.* (1998) used PCR to follow the distribution of wild-type and attenuated GPV in experimentally infected birds. They detected both the attenuated and the virulent strain of GPV in every tested tissue, including the brain. However, interpretation of this finding should be cautious because positive PCR results for the brain or other organs do not necessarily mean virus replication. PCR in general is an efficient method to identify a virus but, because of its high sensitivity, it may not be ideal for identification of the target tissue of a virus. (It is almost impossible to dissect an animal without contaminating the organs with each other or with bodily fluids containing the virus.) *In situ* hybridization and immunohistological studies are needed to reveal within which tissues and the particular target cells the virus replicates.

## DIAGNOSTICS

Many different techniques have been employed to develop diagnostic tools for the detection of GPV and MDPV, including virus isolation from tissue homogenate and characterization in tissue culture (Kisary and Derzsy, 1974), virus neutralization test, agar-gel diffusion (Gough, 1984), ELISA (Kardi and Szegletes, 1996), spermagglutination inhibition (Malkinson *et al.*, 1974), immunofluorescence and immunohistochemical methods (Schettler, 1973; Roszkowsky *et al.*, 1982; Alexandrov *et al.*, 1999; Takehara *et al.*, 1999), DNA hybridization (Le Gall-Recule and Jestin, 1995), electron microscopy (Alexandrov *et al.*, 1999; Gough *et al.*, 1981) and PCR (Sirivan *et al.*, 1998; Chang *et al.*, 2000). Their usefulness depends largely on the aim of the investigation. So far there is no immunological test available that distinguishes GPV and MDPV. PCR combined with sequencing or restriction fragment length polymorphism (RFLP) (Sirivan *et al.*, 1998) can be the best choice for quick identification and discrimination of the two viruses especially in Muscovy duck flocks affected with Derzsy disease (where animals are sensitive for both MDPV and GPV).

## PREVENTION AND CONTROL

Great care should be taken to keep uninfected flocks free from virus to avoid outbreaks of Derzsy disease. General animal hygiene rules have to be strictly enforced to reach this goal. Transport vehicles, egg traces, clothing of staff, or any material, which might come into contact with animals with unknown infectious status, should be thoroughly disinfected. However, since WFPs like other parvoviruses are very resistant to chemical and physical agents, once they emerge in an area it is very hard to prevent the infection of unprotected flocks, and they are very difficult to eliminate from already infected farms. So vaccination is essential to prevent further economic loss.

Although some authors suggest otherwise (Gough and Spackman, 1982; Takehara *et al.*, 1995b) active immunization of birds <3 weeks is considered ineffective because they have relatively low immunocompetence at that age (Kisary, 1977). Goslings <20 days old produce antibodies later than do older birds and, even if they were inoculated with the vaccine in their first days, protective antibodies can be detected at 6 days post infection at the earliest, leaving the animals unprotected in their first days of life when they are most vulnerable to the disease (Kisary, 1977). Passive immunization is more efficient in the prevention and control of the disease, because it is able to provide appropriate protection during the first days of the animals' lives when it is most needed. Passive immunity in young birds can be achieved in two ways: by serum inoculation of 1-day-old birds or by active immunization of the layers before the laying season (Derzsy *et al.*, 1970; Hoekstra *et al.*, 1973; Kisary *et al.*, 1978; Kisary, 1993). Serum treatment of goslings was employed for prophylaxis in the 1960s and 1970s due to the lack of appropriate vaccines; 1–2 ml of high titer hyperimmune or convalescent phase sera injected in the egg yolk of the embryonated eggs or intramuscularly in 1-day-old animals generally resulted in satisfactory protection (Van Cleef and Miltenburg, 1965; Derzsy, 1967; Hoekstra *et al.*, 1973). However, the timing of serum treatment is essential (as soon as possible) because the fate of goslings already showing signs of the disease or in the advanced stage of latency cannot be influenced by vaccination. The half-life of the antibody is 5–6 days in goslings and Muscovy ducklings (Kisary, 1977; Takehara *et al.*, 1995b) but yolk-derived antibodies persist longer than exogenous immunoglobulins from hyperimmune sera. Because of this, protective antibody level rapidly decreases in serum-treated animals and (depending on their original value) becomes frequently undetectable after 2 weeks post inoculation (Kisary, 1977; Kelemen *et al.*, 1997). Serum-treated animals are prone to lose their immunity before reaching the age-resistant period, and may then become ill. To avoid this, birds can be reinoculated at around 10 days of age.

Serum treatment is laborious and expensive. Active immunization of laying geese is more economical and it has the additional advantage of reducing the risk of vertical virus transmission (Kisary, 1993). Antibodies are transferred from the layers to progeny via the egg yolk and these parental antibodies can provide the goslings with the necessary protection against GPV during the first weeks of life. In the layers, the protective antibody level continuously decreases towards the end of the laying season, which can result in insufficient protection in later progeny (Kisary, 1993; Kelemen *et al.*, 1997; Polner, 1998). In 1-day-old goslings the neutralizing antibody level should reach the 1/50 value to give full protection through the sensitive period. While at the beginning of the laying season 60–80 percent of the layer's virus neutralizing titer can be measured in the newly hatched offspring, at the end of the season this ratio decreases to only 30–50 percent, probably

due to the drain on the layers. The virus-neutralizing antibody titer has to reach around 1/200 value at the end of the laying season in the parents in order to guarantee full protection in goslings hatched from the late-laid eggs (Kelemen *et al.*, 1997, 1998).

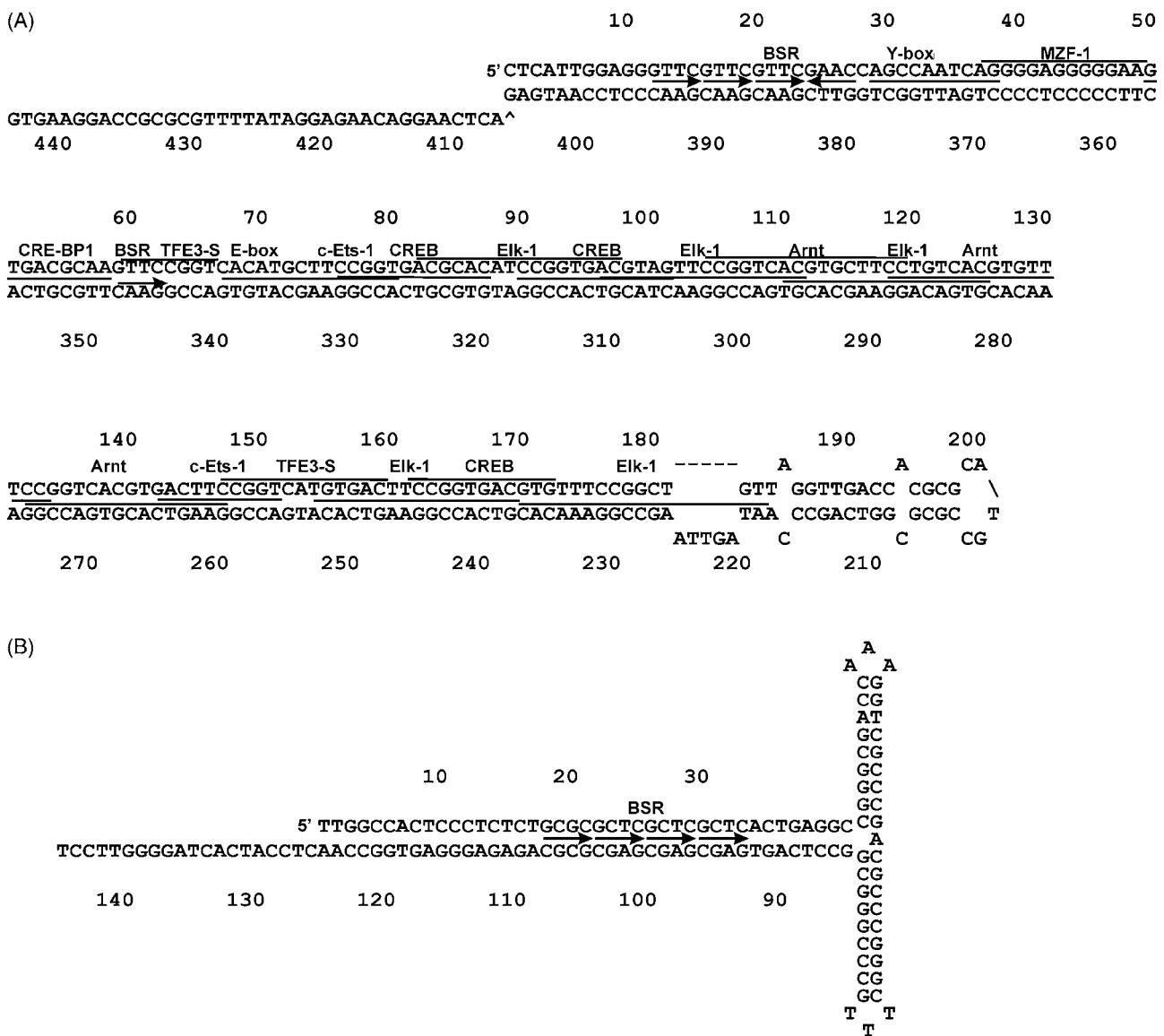
Besides attenuated and inactivated viruses recombinant capsids are also able to induce protective antibody against WFPs (Le Gall-Recule, 1996), and baculovirus expressed VP2 capsid-based recombinant vaccine has been used successfully to immunize laying geese (Kisary and Toth, 1997; Kisary, 1999). Active immunization of the layers with wild-type virus is also protective to the progenies (Hoekstra *et al.*, 1973), although it is contraindicated because these birds are then likely to shed the virus for their entire life. Attenuation of GPV in goose and Muscovy duck fibroblasts as well as in duck embryo has been reported by several groups (Kisary *et al.*, 1978; Gough and Spackman, 1982; Takehara *et al.*, 1998; Alexandrov *et al.*, 1999). Attenuated GPVs, although they cause slight retardation in goose embryo and goslings (Glavits *et al.*, 1991), are not lethal for susceptible goslings. However, they retain the capacity to replicate in geese and induce protective antibody against the wild-type virus. Attenuated virus vaccines were the preferred prophylactic tools during the 1980s and 1990s because of their relatively easy production and their intrinsic safety. Inactivated vaccines were much less widespread primarily because of the difficulty of the economical production of the necessarily large quantity of virus to induce high titer protective antibody levels. Attenuated vaccines have fulfilled their expectations and with their help it has been possible to control the outbreak of Derzsy disease despite their shortcomings. One of them is that the attenuated strains of GPV typically are not able to induce a humoral immune response (Kisary and Kelemen, 1981; Gough and Spackman, 1982), probably because they lost their capability to infect through the gastrointestinal tract. As a direct consequence of this, attenuated vaccines have to be inoculated subcutaneously or intramuscularly and cannot be administered via drinking water or feed. Interestingly, attenuated virus-infected birds, similar to wild-type infected birds, shed the virus from feces for a long time after infection (Gough and Spackman, 1982; Takehara *et al.*, 1998). Another drawback of the attenuated vaccines is that, even if they are administered correctly (twice with a 2-week interval before the laying season), they frequently fail to induce the high antibody levels required for full protection during the entire laying season (Kisary, 1993; Kelemen *et al.*, 1997; Polner, 1998). To prevent viral infection in late-season goslings, the layers have to be revaccinated in the middle of the laying season, which can lead to a drop in egg production (Kisary and Toth, 1997; Polner, 1998) or the goslings have to be treated with hyperimmune sera. Each imposes an additional and undesired financial burden. A possible explanation of the incomplete immune response to attenuated vaccine may be that the already limited ability of the attenuated virus to replicate in young birds becomes

even more restricted in older animals and, consequently, that there is not enough antigen present to induce the desired level of antibody. The results of vaccination programs, in which the combination of attenuated and inactivated or subunit vaccines were used, seem to support this theory. In attenuated virus-primed goose layers, inoculation with the combination of inactivated MDPV and GPV ( $10^6$  EID and  $10^5$  TCID<sub>50</sub>) or 0.1 µg baculovirus-expressed MDPV VP2 capsid induced 1.5–3 times higher neutralizing antibody titers against GPV than did the reinoculation of the attenuated virus (Kelemen *et al.*, 1997). Using the combination of attenuated and inactivated virus (or sub-unit vaccine) in vaccination programs resulted in higher antibody levels in the layers, and consequently full protection could be achieved in the progeny through the full season and expensive serum treatment of the goslings became superfluous (Kelemen *et al.*, 1997; Kisary and Toth, 1997).

All GPV strains are considered serologically identical or similar (Kisary, 1974). However, since MDPV infection did not spare GPV-immunized flocks it was suspected that MDPV could be immunologically different from GPV. Experimental results confirmed this (Fournier and Gaudry, 1992) and revealed an interesting immunological relationship between MDPV and GPV. GPV-induced antibodies give full protection against only GPV, while MDPV-induced antibodies are protective against both viruses. Ducklings hatched from attenuated GPV-vaccinated layers' eggs and being challenged with the two viruses proved much less resistant against MDPV than GPV (Kisary *et al.*, 1994). In virus neutralization tests, the sera of GPV-immunized parents and their ducklings gave at least 10 times higher neutralizing titers against GPV than MDPV (Fournier, 1991). In contrast, goslings hatched from inactivated MDPV-immunized parents were protected against GPV challenge (Kelemen *et al.*, 1997; Kisary and Toth, 1997) and the sera of MDPV-infected Muscovy ducks did not show a significant difference against the two viruses in serum neutralization tests (Fournier, 1991).

## RELATIONSHIP BETWEEN WFPs AND AAVs

Although WFPs are clearly autonomously replicating pathogenic viruses, their nucleotide sequences show relatively little homology to other autonomous parvoviruses. However, they are closely related to the adeno-associated viruses. Based on their genome organization and the phylogenetic analysis of the viral proteins they are classified among members of the *Dependovirus* genus. The most striking differences between the genomes of the so far known AAVs and the WFPs are in the shape and length of the ITRs (Figure 31.2). The ITRs of AAVs are relatively short and Y-shaped without mismatches in the stem structure. Conversely the size and shape of the WFP's ITRs are rather reminiscent of those of the erythroviruses. The ITRs of MDPV, 457 nucleotides



**Figure 31.2** The difference between WFP and AAV ITRs. The ITRs of GPV (**A**) and AAV2 (**B**). The rep binding sites are indicated by BSR and arrows, the transcription factor recognition sites are underlined with the abbreviated name of the factor.

long, are the longest known among vertebrate parvoviruses. The distal 418 nucleotides can fold up to form a U-shaped double-stranded hairpin structure with a proximal 186 bp perfectly-matched stem and a distal imperfectly-matched bubble. Interestingly the arms of the ITRs contain many short 4–10 nucleotide tandem repeats. Many of the repeated motifs match the recognition sequence of known transcription factors (Zádori *et al.*, 1995).

From an evolutionary point of view, the most interesting feature of the WFPs is that, although they are highly pathogenic autonomous parvoviruses, their closest relatives are the helper-dependent non-pathogenic AAVs. Human AAVs, because of this benign character, are popular vectors for gene therapy and their life cycles are extensively studied. The helper functions are necessary at least in three levels of the AAV life cycle where the rep proteins are involved;

second-strand synthesis, mRNA synthesis, and replication itself. GPV rep1 and AAV2 rep78 show 62 percent similarity (Zádori *et al.*, 1995). However, the conserved amino acids are not equally distributed on the polypeptides. The first 228 amino acids of GPV rep1 share around 37 percent identity (58 percent similarity) with the homologous region of the AAV2 rep78 and contains a conserved domain with an active tyrosine implicated in the covalent binding of the parvoviral non-structural proteins to the viral DNA. The core 247 amino acids of the rep1 containing the tripartite nucleotide triphosphate (NTP) binding/helicase motif are 67 percent identical (83 percent similar) to rep78. In spite of the potential zinc-finger domains, the 147 amino acids carboxyl terminal shows the lowest homology between the two proteins with 22 percent identity (32 percent similarity) (Smith *et al.*, 1999).

The function of GPV rep1 was studied in a cell-free *in vivo* system and compared with that of the AAV2 rep78; rep1 indeed contains a helicase activity, which can be abolished by mutating lysine 342. By adding mutant protein to the reaction mix the helicase activity of the wild-type protein was significantly inhibited indicating that multimerization of rep1 might be necessary to this function. rep1 specifically binds to the GPV replication origin containing the GTTC(GAAC)<sub>3</sub> tetramer repeat and exhibits reduced binding to the AAV2 replication origin comprising the binding site of the rep78, the GCGC(GAGC)<sub>3</sub> repeat. The high-affinity binding seems to be essential to initiate origin-dependent replication because rep1 does not mediate that on the AAV2 origin while it does readily on its own GPV replication origin (Smith *et al.*, 1999). Amino terminal domain exchange between rep1 and rep78 revealed that the amino terminal domain of rep1 is not only sufficient for specific origin binding but effectively redirects rep78-mediated origin-dependent DNA replication. A chimeric protein in which the first 235 amino acids of rep78 was substituted with the homologous domain of the rep1-maintained helicase activity and, similarly to the native rep1, effectively mediated DNA replication on the GPV origin, but not on the AAV2 origin. rep1 and rep78 initiated replication very similarly in cell-free DNA replication systems since both required binding to the rep-binding site (RBS), a nicking at the TRS and a helicase activity. The most significant difference was found in the processivity of the two rep protein initiated replications. The rep1 of the autonomously replicating GPV showed high processivity and induced mostly full-length product in the *in vitro* assay, while the rep78 of the helper-dependent AAV showed a lower processivity owing to a lack of helper factors producing mainly short fragments (Yoon *et al.*, 2001). Although these *in vitro* studies shed some light on the different replication mechanism of the closely-related AAV2 and GPV, we are still far from a complete understanding of why AAVs need helper virus and WFPs do not. We note also that some aspects of GPV replication in tissue culture resemble the replication of AAVs. There is no established cell line supporting WFPs replication and, although GPV and MDPV can be propagated well on dividing Muscovy duck or goose primary fibroblasts, the virus dramatically loses infectivity (proportionally to the number of passages) even on the very early second and third passages of these cells. This suggests that host factor(s) supporting virus replication is (are) lost during consecutive cell divisions (Zádori, unpublished observations). Duck plague herpes virus can serve as a helper and can supply missing host factors boosting GPV production even in resting cells (Kisary, 1979), as in the case of AAVs. Human AAVs are extensively studied in tissue culture and were classified as dependoviruses based on their behaviour in tissue culture, but we know very little about their *in vivo* life cycle. The inability of the AAVs to replicate autonomously in tissue culture does not necessarily mean that they are helper-virus dependent in their natural

host. There are some interesting results that may suggest otherwise. Hydroxyurea and other chemical treatments or UV induction of Chinese hamster cells led to independent AAV DNA synthesis and complete infectious cycle of the AAV (Yacobson *et al.*, 1987, 1989). Moreover, autonomous replication of AAV2 was also reported in the granular layers of the organotypic epithelial raft tissue culture system, a model of normal skin, and the authors proposed that AAV is an epithelial-tropic autonomous parvovirus (Meyers *et al.*, 2000). Although these conclusions remain to be verified by other investigators, these experimental data and the close relationship between AAVs and WFPs nevertheless raise some doubts about the *in vivo* helper dependency of the AAVs. The cloning of the AAVs of snakes (Farkas *et al.*, 2004) and especially chicken (Bossis and Chiorini, 2003) can open the way to study AAV replication experimentally in their natural host and to prove whether AAVs require helper function *in vivo* or whether this dependency only occurs *in vitro*.

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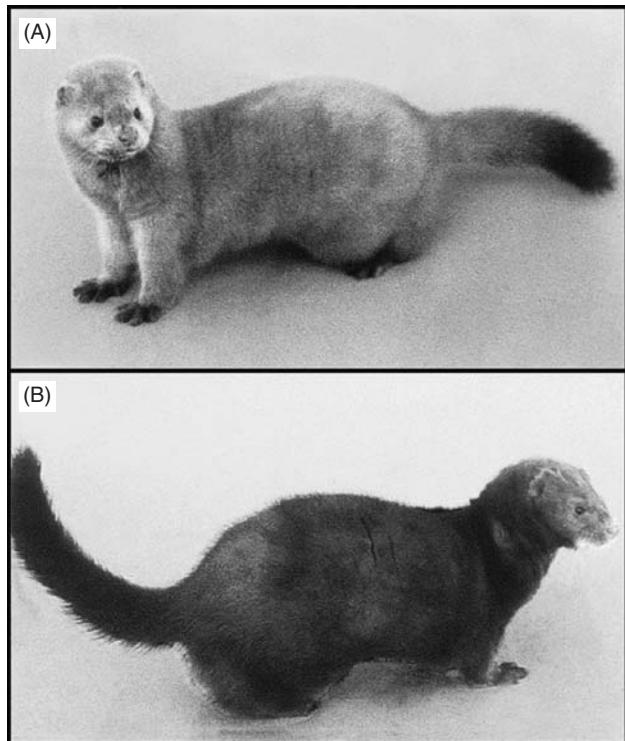
# Aleutian mink disease parvovirus

SONJA M. BEST AND MARSHALL E. BLOOM

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Aleutian mink disease parvovirus (ADV or AMDV) is one of the more unusual of the autonomous parvoviruses in both its replication and its pathogenesis. The virus causes a chronic disease known as Aleutian disease (AD) in mink. As its name implies, Aleutian disease was first recognized in Aleutian mink; a novel coat color mutant of mink that arose spontaneously on an American (Oregon) mink ranch in the early 1940s (Gorham *et al.*, 1976; Henson *et al.*, 1976) (Figure 32.1). This unusual, lighter, shade of fur was a desirable commodity for the fur industry and thus Aleutian mink were bred intensively. However, this new breed of mink was highly susceptible to a previously unrecognized malady characterized by renal disease and an unusual elevation of gammaglobulins (Henson *et al.*, 1962, 1976; Gorham *et al.*, 1976). An infectious agent was identified and named Aleutian mink disease virus (AMDV) (Henson *et al.*, 1962, 1976; Karstad and Pridham 1962). The homozygous recessive mutation that gave the mink their beautiful coats was linked to a rare immune disorder called Chediak–Higashi syndrome. This syndrome is characterized in part by ocularcaneous albinism, bleeding tendencies, a lymphoproliferative syndrome and recurrent bacterial infection from impaired chemotaxis and abnormal natural killer (NK) cell function (Shiflett *et al.*, 2002).

In the mid-1980s, another syndrome of AMDV infection was observed in newborn mink kits (Larsen *et al.*, 1984; Alexandersen, 1986), although the clinical findings were much different than in AMDV-infected adult mink. Mink kits, free of maternal antibody, develop an acute respiratory distress syndrome characterized by fulminant interstitial pneumonia (Alexandersen, 1986; Alexandersen and Bloom 1987; Alexandersen *et al.*, 1987, 1994a). When infected with highly pathogenic strains, the mortality rate is >90



**Figure 32.1** Adult mink. (A) A sapphire mink of the Aleutian genotype. (B) A pastel mink of the non-Aleutian genotype. Both genotypes of mink are vulnerable to infection with AMDV, but the pathogenesis of AMDV in Aleutian mink is the most severe. See also Color Plate 32.1.

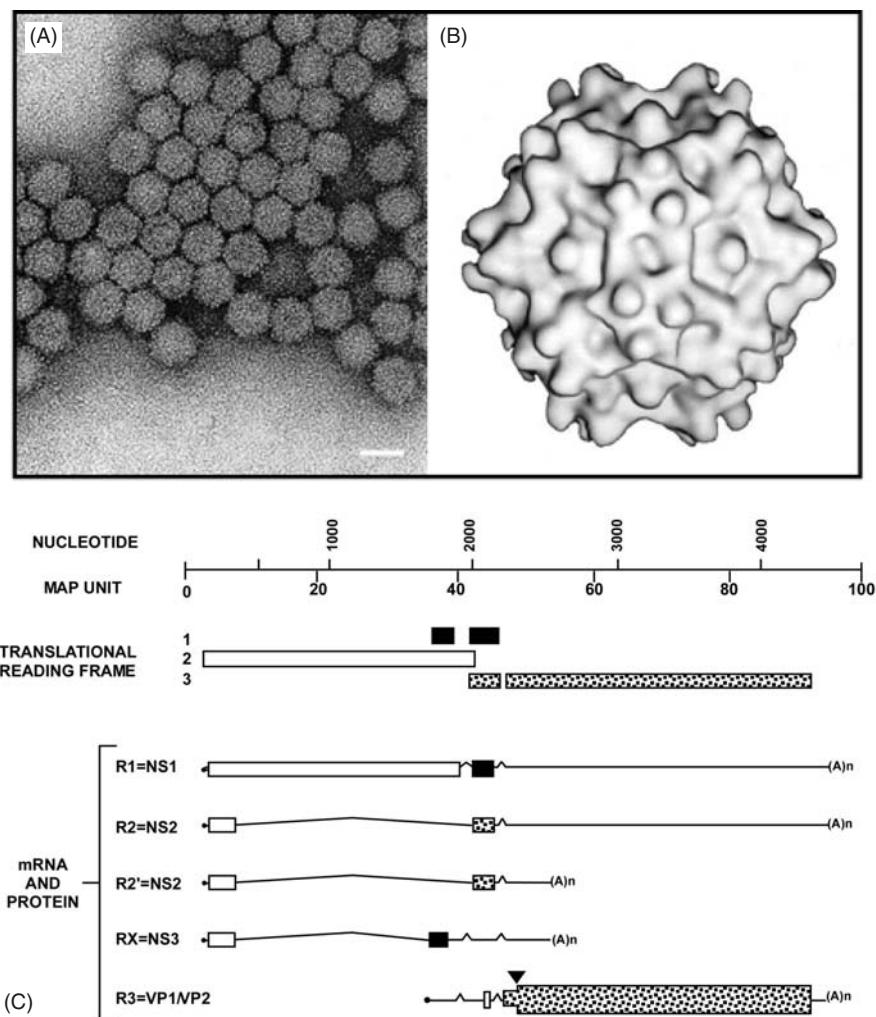
percent with death occurring within 2–3 weeks of infection (Alexandersen *et al.*, 1994a).

Although AMDV was recognized as a significant infection of ranched mink around the world as early as the 1960s, the virus itself was not well characterized until the late 1970s and early 1980s. AMDV was successfully adapted to *in vitro* models of growth at 31.8°C in Crandell feline kidney (CRFK) cells (Porter *et al.*, 1969, 1977; Crandell *et al.*, 1973). The initial isolate derived from the highly pathogenic AMDV-Utah 1 by Porter (AMDV-P) retained pathogenicity through nine serial passages *in vitro*, although the *in vitro* titers were very low (Porter *et al.*, 1969; Bloom *et al.*, 1980). A second isolate of AMDV-Utah 1 by Gorham (AMDV-G) (Bloom *et al.*, 1980) grew very efficiently in CRFK cells but was non-pathogenic, even in Aleutian mink (Bloom *et al.*, 1980, 1990, 1993). Molecular studies on the AMDV-G isolate led to the description of the viral proteins (Bloom *et al.*, 1980, 1982; Porter *et al.*, 1990) and viral genome (Bloom *et al.*,

1983, 1988, 1990; Mayer *et al.*, 1983), and revealed that AMDV is indeed an autonomous parvovirus (Bloom *et al.*, 1980). Studies on AMDV-G also identified a non-virion protein in lysates of infected cells, the first demonstration of a parvoviral non-structural protein (Bloom *et al.*, 1982).

## AMDV – THE INFECTIOUS AGENT

The 25 nm AMDV particle (Figure 32.2A and 32.2B) contains a single strand of DNA that, in >90 percent of particles, is of minus sense (Bloom *et al.*, 1983, 1987, 1993). Its 4748 bp genome (Bloom *et al.*, 1988, 1990) has a similar organization to other autonomous parvoviruses (Cotmore and Tattersall, 1987), in that there is one major right and one major left open reading frame (ORF), several smaller mid-genomic ORFs, and palindromic hairpins at both the



**Figure 32.2** AMDV structure and genomic organization. (A) Electron micrograph of ADV. Bar represents 25 nm. (B) Cryo-electron microscopy reconstruction of recombinant AMDV VP2 capsids to 22 Å resolution. (Reproduced from McKenna *et al.*, 1999 with permission.) (C) Genome organization of AMDV shown as a linear map in nucleotides. The major potential coding regions in each of the three potential transcriptional open reading frames are aligned. The ADV-G mRNAs are depicted along with the proteins for which they encode. The solid arrowhead depicts the start of VP2 on the R3 mRNA. (Reproduced from Bloom *et al.*, 1994 with permission.)

3' and 5' termini (Figure 32.2C). Once inside the cell, the single-stranded genome is converted to a double-stranded replicative form. This replicative form then functions as both a template for viral mRNA transcription and for viral DNA replication, from which the single-stranded genomes are derived for encapsidation.

During the infectious cycle of AMDV four viral mRNAs (R1, R2, R2', and RX) are synthesized from a promoter at map unit 4 (P4), and one mRNA (R3) is synthesized from a second promoter at map unit 36 (P36) (Alexandersen *et al.*, 1988a). R1, R2, and R3 terminate with a polyadenylation signal located at map unit 92 whereas R2' and RX utilize one at map unit 53 (Figure 32.2C); >10<sup>5</sup> copies of viral mRNA are made in a permissively infected cell and all of the RNA species are complementary to the single-stranded virion DNA (Alexandersen *et al.*, 1987, 1988a; Wu *et al.*, 1994).

The two capsid proteins, VP1 and VP2, are encoded for by the 2.3 kb R3 and arise by alternate initiation on the same mRNA (Alexandersen *et al.*, 1988a; Clemens *et al.*, 1992; Christensen *et al.*, 1993a). The coding sequences for VP2 are fully contained within the large right-hand ORF, whereas VP1 contains additional sequences joined to the amino end of VP2 (Bloom *et al.*, 1982; Alexandersen *et al.*, 1988a; Clemens *et al.*, 1992; Christensen *et al.*, 1993a). When expressed in recombinant vaccinia and baculoviruses, the capsid proteins self-assemble into particles with the characteristics of bona fide empty virions (Figure 32.2B) and are transported into the nucleus (Clemens *et al.*, 1992; Christensen, *et al.*, 1993a; Wu *et al.*, 1994; McKenna *et al.*, 1999). Hence, the coding sequence for the capsid genes contains all the sequences necessary for particle assembly and nuclear transport. The capsid sequence also has important roles in host tropism and pathogenesis, which are topics that will be discussed later in this chapter.

There are at least two non-structural proteins produced following AMDV infection, which are derived by complex differential mRNA splicing of the major left ORF and smaller ORFs in the mid-genomic region (Alexandersen *et al.*, 1988a). All of the AMDV non-structural proteins share a common amino terminal portion of 59 residues, but unlike the capsid proteins, the remainder of each of the proteins is unique.

The major non-structural protein (NS), NS1, is encoded by the 4.3 kb R1 mRNA (Bloom *et al.*, 1982; Christensen *et al.*, 1993a, 1995) (Figure 32.2C). This pleiotropic protein has been shown to function very similarly to that of other autonomous parvoviruses, in that it localizes to the nucleus of cells and contains motifs responsible for adenosine triphosphate (ATP) binding and ATPase as well as ATP- or dATP-dependent helicase activity (Christensen *et al.*, 1995). By analogy with NS1 from minute virus of mice (MVM), AMDV NS1 is probably required for virus genomic replication and excision of single-stranded replicative form DNA (Cotmore and Tattersall, 1987, 1988, 1990, 1995). NS1 also functions as a transcriptional regulator by transactivating the P36 promoter and thus controlling the expression of capsid proteins (Christensen *et al.*, 1993b). Furthermore,

NS1 is the primary protein responsible for cytopathology, causing apoptosis in cells transfected with NS1 (Best and Bloom, unpublished observations) or in parvovirus-infected cells (Morey *et al.*, 1993; Ikeda *et al.*, 1998; Rayet *et al.*, 1998). As discussed later in this chapter, this apoptosis has an important and novel role in AMDV replication.

The 2.8 kb R2 and 0.85 kb R2' mRNA encode the 13.4 kDa non-structural protein 2 (NS2) (Cotmore *et al.*, 1983, 1986; Alexandersen *et al.*, 1988b; Porter *et al.*, 1990) (Figure 32.2C). Interestingly R2 is the predominant transcript in AMDV-infected cells, comprising ~70 percent of the total viral transcripts (Storgaard *et al.*, 1997). The function of AMDV NS2 is not as well understood as for AMDV NS1. However, in another parvovirus, MVM, NS2 is a multifunctional protein required for efficient virus replication with roles in capsid assembly (Cotmore *et al.*, 1997) and the egress of progeny virions from the nucleus (Eichwald *et al.*, 2002). MVM NS2 also interacts with a number of cellular proteins (Young *et al.*, 2002; Brockhaus, 1996; Bodendorf *et al.*, 1999) and contributes to cytopathology of infected cells (Daeffler *et al.*, 2003). It is likely that AMDV NS2 has similar functions. In addition, the coding sequences necessary for the production of capsid proteins are present within the AMDV R2 transcript (Alexandersen *et al.*, 1988a) and when expressed in a recombinant baculovirus, the R2 mRNA can direct synthesis of small amounts of capsid protein in addition to NS2 (Christensen *et al.*, 1993a). Whether or not capsid proteins arise from this transcript in AMDV-infected cells is unknown.

The 1.1 kb AMDV-G mRNA, RX, may produce an additional non-structural protein, tentatively denoted NS3 (Alexandersen *et al.*, 1988a). The other mammalian autonomous parvoviruses do not produce a unique NS3, but rather can express several isoforms of NS2 (Cotmore and Tattersall, 1990). AMDV NS3 has been expressed from a recombinant baculovirus (Christensen *et al.*, 1993a), although additional *in vivo* AMDV strains may lack the requisite splicing junctions for RX (Bloom *et al.*, 1994). The functions of NS3, if it is expressed during viral infection, are therefore unknown.

A number of biochemical studies have compared AMDV promoters with those of other parvoviruses, including MVM and mink enteritis virus (MEV), another autonomous parvovirus of mink that also infects CRFK cells (Christensen *et al.*, 1993b; Storgaard *et al.*, 1993, 1997). The AMDV P36 promoter exhibits a lower basal level and is significantly less responsive to transactivation by NS1 than the equivalent promoter of MEV or MVM. In AMDV-infected cells, the total amount of viral mRNA transcripts is 20-fold lower compared with MEV-infected CRFK cells (Storgaard *et al.*, 1997). Furthermore, the predominant transcripts in AMDV-infected cells encode the non-structural proteins, whereas the predominant mRNAs in MEV-infected cells encode the structural proteins (Storgaard *et al.*, 1997). There is no direct evidence that the low expression levels of structural proteins account for the low level of virus replication, but this may be a strategy involved in the restricted and persistent

virus replication observed during AMDV infection *in vivo* compared with the more acute replication of MEV (Storgaard *et al.*, 1997).

## AMDV INFECTION OF WILD MINK POPULATIONS AND OTHER ANIMALS

In addition to mink, a number of mustelids are susceptible to AMDV infection. These include the short-tailed weasel (*Mustela erminea*), fishers (*Martes pennanti*), marten (*Martes americana*), and otters (*Lutra canadensis* and *Lutra lutra*) (Alexandersen *et al.*, 1985; Wells *et al.*, 1989; Manas *et al.*, 2001; Steinel *et al.*, 2001; Stevenson *et al.*, 2001b). Ferrets (*Mustela putorius furo*) are also susceptible to infection with AMDV and develop clinical signs including a wasting syndrome or posterior ataxia and paresis (Ingram and Cho, 1974; Welchman *et al.*, 1993; Saifuddin and Fox, 1996; Stevenson *et al.*, 2001b) in the absence of an immune complex disease. Evidence of infection has been reported in red foxes (*Vulpes vulpes*), the striped skunk (*Mephitis mephitis*), and raccoons (*Procyon lotor*) (Ingram and Cho, 1974; Kenyon *et al.*, 1978; Alexandersen *et al.*, 1985; Oie *et al.*, 1996). Racoons have even been implicated in transmission of disease but do not develop clinical signs of infection (Oie *et al.*, 1996).

AMDV is a potentially significant factor contributing to the decline of wild mink populations across the world (Manas *et al.*, 2001). The European mink (*Mustela lutreola*) is one of the most threatened terrestrial mammals in the world, and infection with AMDV can not only result in mortality, but also in reduced fertility and spontaneous abortions (Gorham *et al.*, 1976; Hansen and Lund, 1988; Broll and Alexandersen, 1996). The accidental and deliberate release of American mink from commercial mink farms in Europe has resulted in competition for resources and the introduction of AMDV into native mink species (Dunstone, 1993). Partial sequencing of the VP2 protein of isolates from *M. lutreola* in Spain suggests that AMDV has diverged from those in North America and other areas of Europe (Manas *et al.*, 2001). This suggests that AMDV may have adapted in its new hosts. However, very little is known about the pathogenesis of these AMDV isolates in European mink and the long-term effects that these viruses will have on wild populations.

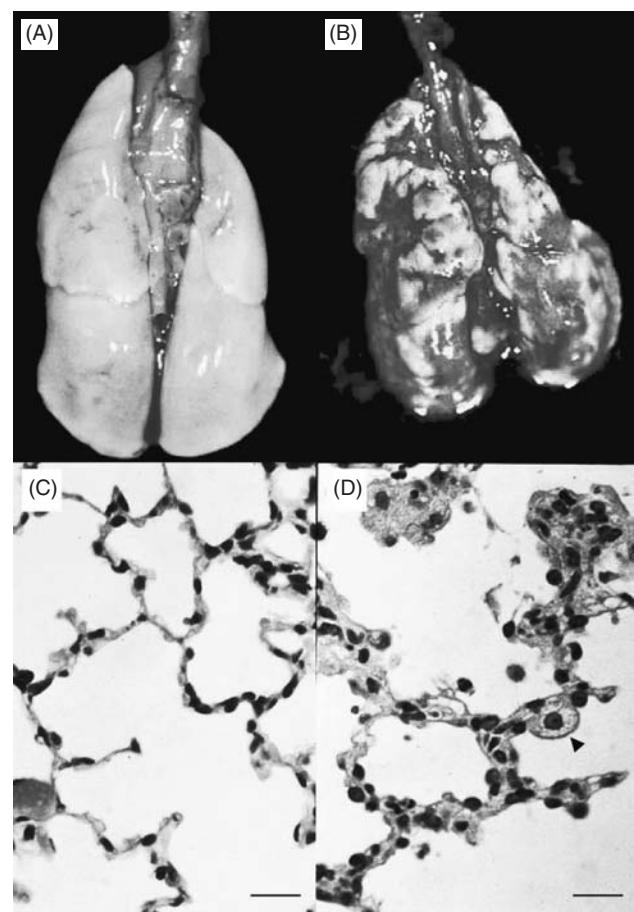
## ALEUTIAN DISEASE – PATHOGENESIS

### Aleutian disease in newborn mink

Transmission of AMDV occurs both horizontally via urine, feces, and saliva, and vertically (Kenyon *et al.*, 1963; Henson *et al.*, 1966; Gorham *et al.*, 1976; Broll and Alexandersen, 1996). Antibody-negative mink kits develop an acute

interstitial pneumonia within 2 weeks of being infected with AMDV (Alexandersen and Hau, 1985; Alexandersen, 1986; Alexandersen and Bloom, 1987; Alexandersen *et al.*, 1987). Within a few days, they succumb to a fatal respiratory distress syndrome involving parenchymal hemorrhage (Figure 32.3), extensive atelectasis and hyaline membrane formation. In the case of infection with highly virulent strains of AMDV, incidence and mortality are >90 percent. Low virulence strains result in between 50 and 70 percent incidence rate and between 30 and 50 percent mortality (Alexandersen, 1986). Those mink kits that survive infection develop typical lesions of the classical adult form of Aleutian disease (AD) regardless of the infecting strain (Alexandersen, 1986).

In mink kits, the primary target cell for virus replication is the pulmonary type II alveolar pneumocyte (Alexandersen *et al.*, 1987) whose normal function is to produce pulmonary



**Figure 32.3** Gross and histological pathology of mink kit lung following AMDV infection. **(A)** Normal lung; **(B)** lung from AMDV-infected Aleutian mink kit demonstrating extensive haemorrhaging; **(C)** histological section depicting the alveolus from an uninfected mink kit; **(D)** histological section of the alveolus from an AMDV-infected kit demonstrating an interstitial infiltrate, interstitial edema and hypertrophy of the alveolar type II pneumocytes (arrowhead). Bars represent 50 µm. See also Color Plate 32.3.

surfactant. Infection of these cells presumably results in abnormal surfactant production and fatal respiratory distress. Approximately 10 percent of the type II pneumocytes become permissively infected, and those cells support production of high levels of viral replicative intermediates, similar to those found in tissue culture (Alexandersen *et al.*, 1987; Alexandersen and Bloom, 1987; Alexandersen, 1990). In addition, the concentration of virus in other tissues and in serum is high, comparable to that found in human patients with acute B19 parvovirus infection, or to animals with other acute parvoviral syndromes (Alexandersen and Bloom, 1987; Bloom *et al.*, 1987). Thus, the disease results from an acute and cytopathic infection similar to that observed *in vitro* with AMDV-G or *in vivo* with other parvoviruses. Hence, infection of CRFK cells with AMDV-G is considered a model useful for the study of permissive replication.

Transplacental transmission of AMDV occurs in neonatal kits from both persistently and acutely infected dams. In particular, persistent infection of pregnant dams has been implicated in increased rates of abortion (Alexandersen, 1986; Hansen and Lund, 1988), absorption of the fetus, or embryonic death (Broll and Alexandersen, 1996). Fetuses or kits from persistently infected dams were examined for sites of AMDV replication. Virus was found in a wide range of fetal tissues, including liver, spleen, connective tissue of the skin, interstitial connective tissue, heart muscle, thymus, bone marrow, lung, and brain, as well as the fetal placenta (Broll and Alexandersen, 1996). Another parvovirus infection resulting in abortion and stillbirth is B19 infection of humans (Bloom and Young, 1999). In the B19-infected fetus, virus can be found in the liver, heart, and skin. The experimental infection of mink dams with AMDV may therefore be a useful model of *in utero* infection with B19.

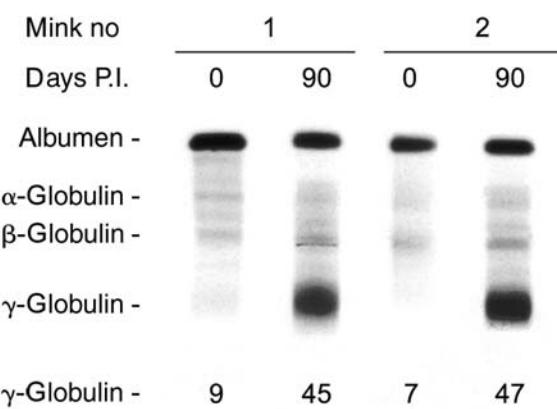
Antibody plays a protective role in AMDV-infected mink kits. The passive transfer of maternal antibody is protective to kits infected after birth, as in these cases the pneumonia is not observed. In addition, kits from seronegative dams experimentally infected with AMDV and treated with anti-AMDV antibody develop an attenuated form of the adult disease and fail to develop clinical pneumonia (Alexandersen *et al.*, 1988c). Kits infected later than about one week after birth have a greatly reduced incidence of pneumonia and again develop a form of the adult disease including rapid induction of antiviral antibody and persistent infection (Alexandersen and Bloom, 1987; Alexandersen, 1990). The role of antiviral antibody in suppressing the induction of AMDV pneumonitis has been further illustrated experimentally by administering antibodies to mink immunoglobulin M (IgM) to suppress anti-AMDV antibody production in infected mink kits. This resulted in a lengthened susceptibility period of kits to acute disease (Alexandersen *et al.*, 1994b). The specific mechanism by which antibody protects mink kits is not clear as the absolute number of infected type II pneumocytes does not differ between anti-AMDV antibody-treated and -untreated kits (Alexandersen *et al.*, 1988c). However, intracellular levels of replicative form

DNA and mRNA were reduced 100- to 1000-fold as compared with those seen in untreated mink (Alexandersen *et al.*, 1987, 1988c). Thus, the antiviral antibody restricts virus replication via as yet unknown mechanisms. The modulation of AMDV infection of kits by antibody is just one example of the important and complex role of antibody in AD.

### Adult or classical Aleutian disease

The classical form of AD is characterized by the development of splenomegaly and lymphadenopathy early after infection, which persists throughout the course of infection (Eklund *et al.*, 1968; Porter *et al.*, 1969). This is followed by a dramatic proliferation of plasma cells and the infiltration of plasma cells and lymphocytes into the liver, spleen, kidney, and even the retina (Eklund *et al.*, 1968; Henson *et al.*, 1976; Porter *et al.*, 1969; Hadlow, 1982; Porter *et al.*, 1983). The hallmark of AD is the unusual serologic reaction following infection. There is a severe, progressive, polyclonal hypergammaglobulinemia (or plasmacytosis) with levels of gamma globulin >30 mg/ml and constituting up to 50 percent of total serum proteins (Henson *et al.*, 1962; Porter *et al.*, 1965; Eklund *et al.*, 1968; Bloom *et al.*, 1975; Aasted and Bloom, 1983; Hadlow *et al.*, 1983) (Figure 32.4).

The outcome in adult mink of infection with AMDV depends on the strain of virus and on the mink genotype. Aleutian genotype mink are uniformly susceptible to infection with almost all strains of AMDV except the non-pathogenic AMDV-G (Bloom *et al.*, 1988, 1990). The susceptibility of non-Aleutian mink is more variable resulting in three patterns of disease: a typical progressive disease (Hadlow *et al.*, 1983; Porter *et al.*, 1969); a persistent, non-progressive infection with slight elevations of gamma globulin and no lesions (Bloom *et al.*, 1975; An and Ingram, 1977, 1978; An *et al.*, 1978; Hadlow *et al.*, 1985); non-persistent,



**Figure 32.4** Hypergammaglobulinemia induced by infection with AMDV. The dramatic increase in gamma globulins is shown as a proportion of total immunoglobulin for two mink prior to infection with AMDV at 0 days and 90 days post infection. See also Color Plate 32.4.

non-progressive infection with clearance of the virus (Larsen and Porter, 1975; Hadlow *et al.*, 1984). All infected mink develop robust titers of anti-AMDV antibody regardless of whether or not chronic disease ensues.

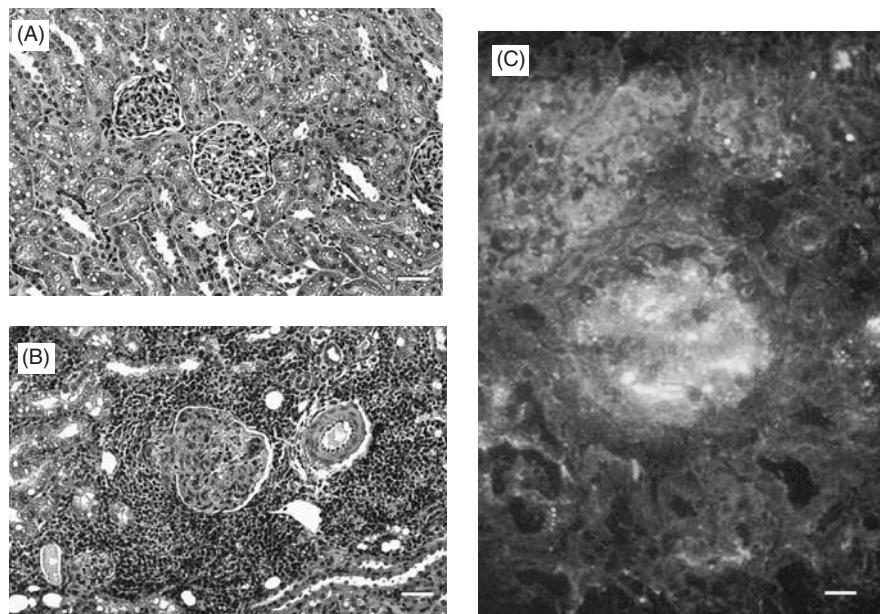
Antiviral antibody can be detected as early as 5 days post infection, and is directed against both capsid and non-structural proteins. In fact, the fraction of immunoglobulin directed against the virus can exceed 50 percent (Aasted *et al.*, 1984; Porter *et al.*, 1984). Anti-AMDV antibody binds but does not effectively neutralize virus *in vivo*, and thus the virus exists in serum as infectious immune complexes (Porter and Larsen, 1967; Hahn, 1984) capable of depositing in the blood vessels and renal glomeruli. Indeed, vaccination of adult mink or the presence of pre-existing antibodies do not protect animals from AMDV infection, but instead leads to an accelerated form of disease upon challenge (Porter *et al.*, 1972; Aasted *et al.*, 1998). Hence, there is a sharp contrast in the role of passive administration of antibody to adult mink, where it exacerbates disease, versus to kits, where it is protective (Table 32.1). This is

probably due to the different target cells of AMDV replication in adults versus kits, and the role that antibody has in enhancement of infection of macrophages. This concept is discussed later in this chapter.

In Aleutian mink, the renal disease observed in AD is not solely due to immune complex deposition (Figure 32.5). Aleutian mink also develop severe interstitial nephritis with infiltration of plasma cells and T cells, and tubulorrhesis (Mori *et al.*, 1991a, 1994) (Figure 32.5). There is evidence of restricted virus replication (in the form of mRNA and replicative form DNA) as early as 10 days post infection in the tubular epithelial layer within the glomeruli, and in cells infiltrating the lesions (Alexandersen *et al.*, 1988b). The infiltration of T cells may reflect an antiviral response in the kidney and contribute to the impairment of renal function (Miyazawa *et al.*, 1994; Mori *et al.*, 1991a, 1994). In non-Aleutian mink, the glomerular lesions as well as the tubular and interstitial pathology are less severe and the levels of detectable viral nucleic acids are lower than in Aleutian mink (Mori *et al.*, 1994).

**Table 32.1** Summary of differences between ADV replication and the role of antibody following infection of mink at different stages

Age of infection			
	Fetal	Kits	Adults
Target cells	Wide range of tissues	Type II pneumocytes	Macrophages, lymphocytes
Virus replication	Permissive	Permissive, cytopathic	Restricted, noncytopathic
Role of antibody	Neither protective nor pathogenic	Attenuating	Pathogenic antibody-dependent enhancement of infection, immune complex formation



**Figure 32.5** Histological changes in the kidney of AMDV-infected adult Aleutian mink. (A) Normal kidney. Bar represents 70 µm; (B) kidney from an AMDV-infected mink demonstrating the extensive infiltration of plasma cells and lymphocytes resulting in a severe interstitial nephritis. Marked glomerular changes are also evident. Bar represents 17 µm; (C) immunofluorescence showing the extensive deposition of mink immunoglobulin in the glomerulus of an infected Aleutian mink. Bar represents 50 µm. See also Color Plate 32.5.

AMDV infection has also been associated with disease of the central nervous system (CNS), including in non-Aleutian adult mink (Dyer *et al.*, 1999). In such cases, mink exhibited signs of lack of coordination, hind limb paralysis, and seizures and death within 3 weeks of the onset of clinical signs. Upon histological examination the mink had a non-suppurative meningoencephalitis with severe infiltration of Gitter cells, lymphocytes, plasma cells, and occasional neutrophils into the hippocampus, cerebellum, and cerebral cortex. This was also associated with damage to neurons, perineuronal vacuolation, and astrocytosis (Dyer *et al.*, 1999). The hypervariable region of VP2 that is associated with pathogenicity (Bloom *et al.*, 1998) was sequenced for the CNS disease-associated isolate of AMDV (AMDV-TH5), but it is unclear if the sequence differences found in this region of the capsid determine neurovirulence of AMDV isolates.

Studies of non-humoral immune responses to infection are limited because mink are outbred animals and specific reagents to cellular markers are limited. Over the course of infection, an increase in CD8<sup>+</sup> T cells is observed in the periphery (Jensen *et al.*, 2003) as well as infiltration of CD8<sup>+</sup> T cells into tissues (Aasted, 1985, 1989), although levels of CD4<sup>+</sup> T cells remain relatively normal (Chen and Aasted, 1997). In terms of cytokine responses, circulating interferon (IFN)- $\gamma$  is increased as early as 2 weeks post infection and is produced by CD8<sup>+</sup> T cells and to a lesser extent, monocytes. Additional cytokine production in response to infection includes interleukin (IL)-4 produced by CD8<sup>+</sup> T cells, IL-8 produced by monocytes (Jensen *et al.*, 2003) and IL-6 by infected macrophages (Bloom *et al.*, 1994). Thus, cytokines from both Th1 and Th2-type responses are observed (Murphy and Reiner, 2002). The increases in IL-4 and IL-6 production (Th2) are consistent with the development of plasmacytosis, the hallmark of AD. Interestingly, NS1 of B19 has been shown to transactivate the promoter of IL-6 (Moffatt *et al.*, 1996; Mitchell, 2002), and AMDV NS1 may act similarly (Jensen *et al.*, 2003). Another interesting point is that the AMDV genome contains three copies of CTGGGA, a sequence identical to the consensus sequence for an IL-6-responsive enhancer element (Heinrich *et al.*, 1990). One of these copies is positioned approximately 700 bases from the 5' transcriptional start of R3 (capsid protein mRNA) and another is embedded in the 5' non-coding region of R3 (Bloom *et al.*, 1994). Perhaps IL-6 production plays a pivotal role in AMDV pathogenesis through the perturbation of humoral immune responses, as well as the modulation of viral gene transcription in infected macrophages.

## VIRUS REPLICATION IN MACROPHAGES

AMDV-Utah infection of Aleutian mink results in rapid replication of the virus in lymphoid tissue, reaching peak levels of  $>10^8$  infectious doses per gram of tissue by 10 days after infection. Virus is concentrated in tissues rich in

macrophages, including the lymphoid system and the liver (Porter *et al.*, 1969). However, the virus is present in the cytoplasm of infected cells, suggesting that it has either been sequestered there (Porter *et al.*, 1969), or it is undergoing a restricted type of replication. This is in marked contrast to the infection of type II pneumocytes of mink kits, in which viral antigen is readily found in the nucleus, an indication of *de novo* viral expression. At later times during infection of adult mink, the virus levels decrease significantly but the virus is seldom cleared (Larsen and Porter, 1975). Other strains of AMDV replicate in the same tissues as AMDV-Utah, but the titers are somewhat lower and systemic dissemination occurs at slower rates (Eklund *et al.*, 1968; Hadlow *et al.*, 1984, 1985).

Due to the fact that infectious virus exists in immune complexes and is probably sequestered inside macrophages, the demonstration of virus presence by DNA or virus protein is not sufficient proof of virus replication at a particular site (Bloom *et al.*, 1994). Instead, *in situ* hybridization and Southern blot are used to determine where replicating virus is located in an infected animal. Using these techniques, it has been shown that replication of AMDV occurs at 100- to 1000-fold lower levels in adult mink compared with replication in mink kits (Alexandersen *et al.*, 1987, 1988b; Bloom *et al.*, 1989; Mori *et al.*, 1990, 1991b), although in the adults, type II pneumocytes are not infected. Instead, virion DNA is located in the reticuloendothelial system including cells resembling follicular dendritic cells and macrophages (Alexandersen *et al.*, 1988b; Mori *et al.*, 1991b; Kanno *et al.*, 1992). Immunoglobulin and virion antigen co-localize with virion DNA suggesting that some virus may be present as immune complexes bound to follicular dendritic cells (FDC) (Race *et al.*, 1986; Alexandersen *et al.*, 1988b; Mori *et al.*, 1991b). A similar distribution of human immunodeficiency virus (HIV) has been observed in infected lymph nodes, with FDC a likely reservoir of that virus (Armstrong and Horne, 1984; Laman *et al.*, 1989).

The actual tissue and cellular localization of sequestered versus actively replicating virus is difficult to ascertain in adult mink. This is because the replicative intermediates are present in greatly reduced amounts compared with infected mink kits or tissue culture. However, there can be relatively high amounts of single-stranded virion DNA (Bloom *et al.*, 1983, 1987, 1989; Alexandersen *et al.*, 1987, 1988b; Mori *et al.*, 1991b; Kanno *et al.*, 1992). Evidence suggests that a small percentage of macrophages support virus replication and gene expression (Alexandersen *et al.*, 1987, 1988b; Mori *et al.*, 1991b) and that FDC and lymphocytes (Jensen *et al.*, 2000) may also be targets of replication. Importantly, the number of cells containing sequestered virion DNA is at least 10 times greater than the number containing AMDV RNA, suggesting that not all cells containing AMDV support permissive replication (Alexandersen *et al.*, 1988b; Mori *et al.*, 1991b; Kanno *et al.*, 1992, 1993b).

At later times in infection when the immune disorder is more prominent, the amount of virus is further reduced

and the detection of viral sequences is even more difficult (Alexandersen *et al.*, 1988b; Mori *et al.*, 1991b). Virion DNA remains detectable sequestered in the reticuloendothelial system, but only macrophages appear to support any active virus replication (Mori *et al.*, 1991b). In addition, white blood cells, bone marrow, kidney, liver, and spleen also contain viral sequences (Kaaden *et al.*, 1986; Alexandersen *et al.*, 1988b; Haas *et al.*, 1988). Hence, it appears that at all times following infection, AMDV replication and gene expression is restricted at a low level compared with that observed following permissive infection of mink kits or tissue culture (Bloom *et al.*, 1994).

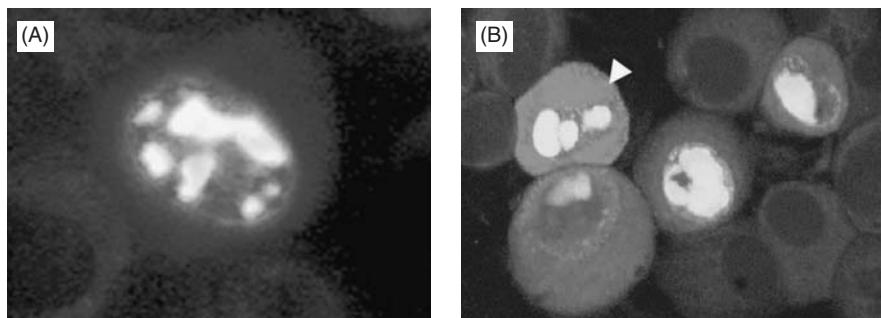
## VIRUS-CELL INTERACTIONS

### Infection of macrophages by AMDV

Macrophages have a central role in the replication and pathogenesis of AMDV infection of adult mink, as they are the primary sites for virus replication and also for the sequestration of virus; 20–30 percent of phagocytic cells isolated from infected mink contain viral DNA while only 1–2 percent have viral mRNA and therefore are supporting virus replication (Kanno *et al.*, 1992). In addition, infection of primary macrophages from uninfected mink with AMDV results in approximately 2 percent of cells containing viral antigen in the nucleus of cells (Kanno *et al.*, 1993b). The production of infectious virus from primary macrophage cultures is not readily demonstrated. Thus, this infection is not fully permissive, but is rather restricted. Infection of mink lymph node histocultures results in the infection of macrophages and lymphocytes similar to that observed *in vivo* (Jensen *et al.*, 2000). In these histocultures, the production of viral replicative intermediates indicated that infection is restricted, although infectious progeny could be recovered. This may simply reflect the mixed population of cells infected. The histocultures are potentially a very useful

model to further dissect virus-cell interactions, immune responses and virus-induced cell death.

A number of studies on AMDV replication in macrophages have also been undertaken in macrophage cell lines. The human monocytic cell line, K562, is susceptible to infection by AMDV (Kanno *et al.*, 1993b). Similar to infection of primary macrophages, replicative intermediates can be detected, although infectious virus is not produced, suggesting that infection of these cells is restricted. In a direct comparison of replication between AMDV-infected CRFK and K562 cells, it was shown that replicative compartments in the nucleus are formed in both cell types and consist of NS1, NS2, and viral DNA (Oleksiewicz *et al.*, 1996, 1998). Also in the nucleus, but excluded from the replicative complex, are VP proteins localized in a shell-like distribution around the periphery (Figure 32.6). As replication proceeds, NS2 becomes gradually localized in the cytoplasm of infected cells. The main difference between infection of CRFK (permissive) and K562 (restricted) cells is that cytoplasmic localization of NS2 was 4- to 10-fold more restricted in K562 cells. The similarity between replication of AMDV in CRFK and K562 cells may indicate that restriction of infection occurs at a late stage (Oleksiewicz *et al.*, 1998) following DNA replication and protein expression (Kanno *et al.*, 1993a,b; Dworak *et al.*, 1997). As NS2 has roles in capsid assembly and export from the nucleus, it may be that one level of restriction operates by preventing virion egress from the nucleus of infected cells, resulting in reduced virion maturation and the subsequent failure of infectious progeny production observed in these cultures. Indeed, NS2 of MVM is required for permissive replication, efficient transcription and virus production in some cell types but not in others (Naeger *et al.*, 1990, 1993), suggesting that interactions of NS2 with specific cellular factors may mediate persistence. The differences observed in cytoplasmic NS2 expression between restricted and permissive infections are clearly an interesting area of further research, particularly as the transcripts encoding for NS2



**Figure 32.6** Localization of AMDV proteins in infected CrFK cells. (A) AMDV-infected cell with nuclear staining of the replicative complex containing NS1 (green) and NS2 (red). Capsid proteins are localized around the nucleus periphery (blue). (B) AMDV-infected cells showing localization of capsid protein (red) around the nucleus periphery and in the cell cytoplasm (arrowhead). The nuclear localization of NS1 is shown in green. Yellow represents co-localization of both capsid and NS1. See also Color Plate 32.6.

are the most abundant transcript produced in an AMDV infected cell.

### Antibody-dependent enhancement of infection

One of the very interesting aspects of AMDV infection is that such a great proportion of antibody present in the serum of infected animals is specifically directed against the virus. As has already been noted, this antibody can modulate the disease in mink kits, and is involved in the pathogenesis of disease via the formation of complexes with the virus itself. Another dimension to the role of antibody bound to virus is that it can then facilitate the entry of AMDV into macrophages via antibody binding to cellular Fc or complement receptors (Kanno *et al.*, 1993a). This promotion of virus infectivity by antibody is termed antibody-dependent enhancement of infection or ADE (Porterfield, 1986).

Using K562 cells or primary macrophages, the pre-incubation of AMDV with serum from infected mink increases the infectivity up to 10-fold (Kanno *et al.*, 1993a). For this to occur, the Fc portion of the antibody is required. In addition, blocking the Fc receptor on K562 cells with a specific monoclonal antibody abrogates such infectivity. Once inside the macrophage, antibody may influence persistence by restricting virus replication, similar to that observed in infected mink kits treated with anti-AMDV antibody. However, infection of macrophages with AMDV can occur at low levels in the absence of antibody. Hence, an early infection of macrophages is possible *in vivo*, which may be a critical determinant in the early virus replication, as well as perturbation of the immune response.

### Capsid–antibody interactions

As intimated throughout this chapter, the interactions between virus and antiviral antibodies have a critical role in the pathogenesis and even the replication of AMDV. Antibodies can have mitigating effects on AMDV infection, as they can neutralize infection of CRFK cells *in vitro* (Stolze and Kaaden, 1987; Kanno *et al.*, 1993a) and can protect mink kits against fatal pneumonitis associated with infection of the type II pneumocytes. However, antiviral antibody exacerbates disease through the formation of infectious immune complexes resulting in renal disease. Antiviral antibody also facilitates infection of macrophages bearing Fc receptors via ADE, establishing persistent infection and perhaps affecting the immune responses orchestrated by these cells (Bloom *et al.*, 1994).

The AMDV capsid consists of 60 individual capsid sub-units. Each unit consists of 90 percent VP2 and 10 percent VP1. The structure of the AMDV capsid has been resolved to 22 Å resolution using cryoelectron microscopy (Figure 32.2B; McKenna *et al.*, 1999). The AMDV capsid structure

shares canonical features with those solved for the other autonomous parvoviruses including canine parvovirus, feline panleukopenia virus, and minute virus of mice (McKenna *et al.*, 1999; Bloom *et al.*, 2001). However, the AMDV capsid contains three knob-like mounds decorating the icosahedral 3-fold axes of symmetry and wider ridges that separate the dimple-like depression at the icosahedral 2-fold axes from the canyon-like depression surrounding the icosahedral 5-fold axes (Bloom *et al.*, 2001). The differences can be largely accounted for by short stretches of amino-acid sequences inserted into the flexible loop segments of VP2 protein molecules in AMDV compared with the other parvoviruses (Chapman and Rossmann, 1993; Bloom *et al.*, 1997; McKenna *et al.*, 1999). Interestingly, the structure of the AMDV capsid is strikingly similar to that of the defective parvoviruses AAV5 and AAV2, despite sharing only ~20 percent similarity in amino-acid sequences (Walters *et al.*, 2004).

The AMDV capsid houses immunodominant epitopes that map adjacent to the threefold axis of symmetry. Specifically, the major immunodominant epitope, demonstrated *in vitro* and recognized by antibodies generated in AMDV-infected mink, is contained within VP2 residues 428 to 524 (Bloom *et al.*, 1997; Costello *et al.*, 1999). Interestingly, polyclonal and monoclonal antibodies generated toward the peptide sequence represented by VP2:428–446 mediate aggregation of virus particles, neutralization of infectivity to CRFK cells and ADE of K562 cells. Structural modeling determined that surface-exposed residues contained within VP2:428–446 are readily accessible for antibody binding (Bloom *et al.*, 2001). Thus, one amino acid sequence of the capsid can be implicated in all antibody-mediated facets of AMDV pathogenesis. The observation that antibodies to the immunodominant peptide can mediate both neutralization and ADE may help to explain why capsid-based vaccines exacerbate disease rather than protect (Aasted *et al.*, 1998).

Sequences within the AMDV capsid also mediate host range, in the absence of any antibody. Sequence comparisons between AMDV-G and other strains, including the highly pathogenic AMDV-Utah 1, reveal a number of differences, most of which map to the capsid gene. A short stretch of sequence of <20 amino acids at map unit 64–65 has the greatest amount of divergence and hence is called hypervariable (Bloom *et al.*, 1998). The development of a replication-competent molecular clone of AMDV-G (Bloom *et al.*, 1990, 1993) enabled these differences to be examined through the construction of chimeric viruses between the AMDV-G molecular clone and portions of AMDV-Utah (Bloom *et al.*, 1990; Fox *et al.*, 1999; Stevenson *et al.*, 2001a). Remarkably, changes at one of two capsid residues from the AMDV-G sequence to the AMDV-Utah sequence were required to alter host range and confer both the ability to replicate in mink and to cause disease. These were residues I352V and H534D where isoleucine and histidine are the AMDV-G residues and valine and aspartic acid represent the AMDV-Utah residues (Fox *et al.*, 1999;

Stevenson *et al.*, 2001a). Host-range is determined by the capsid sequences of canine parvovirus and feline pan-leukopenia virus via interactions with the cellular transferin receptor (Govindasamy *et al.*, 2003; Hueffer and Parrish, 2003; Hueffer *et al.*, 2003). Although the AMDV-receptor on non-Fc-bearing cells is not yet known (Fox and Bloom, 1999), the capsid residues identified as important for AMDV host range may also be important in host-cell receptor interactions.

### Virus-induced apoptosis

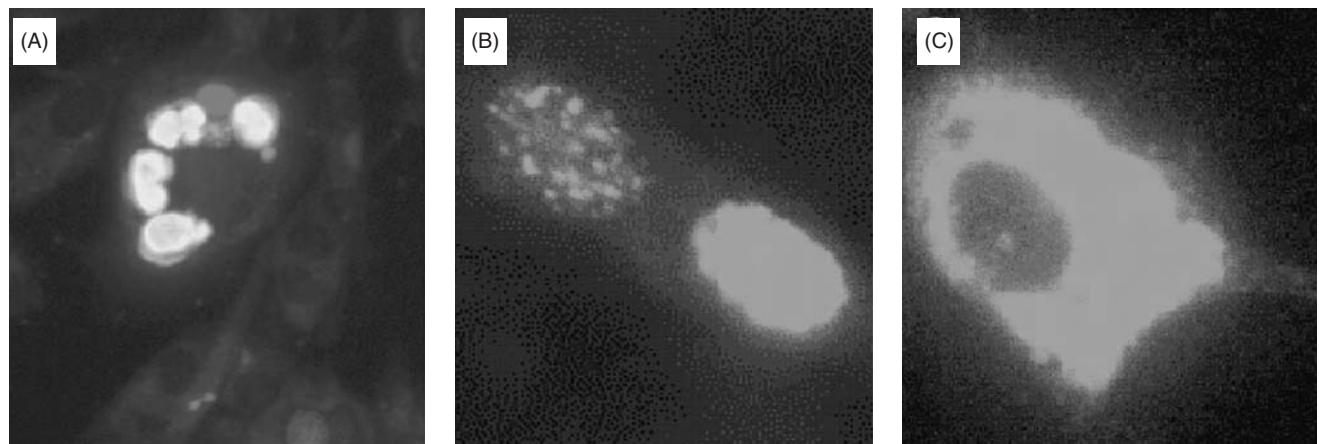
Similar to infection with other autonomous parvoviruses, permissive infection of AMDV in CRFK cells induces cell-cycle arrest (Oleksiewicz and Alexandersen, 1997) and caspase-dependent apoptosis (Best *et al.*, 2002). NS1 itself is also highly toxic to cells causing caspase-dependent apoptosis following transfection into CRFK cells (Figure 32.7A). Caspases are a family of cysteine proteases that have a crucial role in the step-wise dismantling of apoptotic cells (Saikumar *et al.*, 1999; Wolf and Green, 1999; Degterev *et al.*, 2003). The addition of caspase inhibitors to AMDV-infected cells blocked the apoptotic cascade (Best *et al.*, 2002). Surprisingly, inhibition of specific caspases also resulted in a dramatic decrease in virus yield suggesting a requirement for caspase activity during permissive replication (Best *et al.*, 2002). It has recently been demonstrated that AMDV NS1 is cleaved by executioner caspases early in virus replication (Best *et al.*, 2003). Caspase-mediated cleavage of NS1 occurs at two sites, immediately following residues D227 and D285 (Best *et al.*, 2003). Mutation of these NS1 caspase-cleavage sites abrogated virus replication by severely impairing translocation of NS1 to the nucleus of infected cells (Figure 32.7B and 32.7C). However, the C-terminal caspase-cleavage product was capable of translocating efficiently into

the nucleus following expression. Furthermore, the mutated, full-length NS1 was able to translocate to the nucleus in the presence of the C-terminal product. As parvovirus NS1 molecules must oligomerize in order to translocate to the nucleus, it is likely that caspase-cleavage of a portion of AMDV NS1 generates an abbreviated NS1 molecule capable of binding the full length NS1 and taxiing it into the nucleus.

This raises the question of how a virus that can readily cause apoptosis in infected cells can initiate a restricted, persistent infection. One possibility by which this could occur is through the tight regulation of caspase activity in the infected cell (e.g. in macrophages) (Best and Bloom, 2004). If caspase activation and apoptosis are limited after AMDV infection *in vivo*, nuclear translocation of NS1 may be limited. This would lead to restricted function of NS1 in DNA replication and control of viral gene expression leading to the production of low levels of replicative intermediates. The tight regulation of caspase activity by the cell or by AMDV may therefore be a mechanism involved in the restriction of virus replication, and may contribute to the persistent infection of adult mink. It is not currently known if NS1 of any of the other autonomous parvoviruses is cleaved by caspases, although replication of MEV is not dependent on caspase activity (Best *et al.*, 2002). At least one caspase-cleavage site of NS1 is conserved in all isolates of AMDV studied to date including AMDV-Utah (Gottschalk *et al.*, 1994), implying that caspase cleavage of NS1 is an important event in the replication of both pathogenic and non-pathogenic viruses.

### PERSPECTIVES

The constellation of a complex pathogenesis, the perturbation of the immune response, the involvement of anti-AMDV antibody in infection, and the requirement for caspases



**Figure 32.7** The role of caspase activity in nuclear localization of NS1. (A) Wild-type NS1 expression (red) following transfection causes apoptosis demonstrated by TUNEL (terminal deoxy-uridine nick end labeling) (green) that is dependent on caspase activity. (B) Wild-type NS1 (green) is expressed in the nucleus of transfected cells. (C) Following pretreatment of cells with broad-spectrum caspase inhibitors, wild-type NS1 (green) localizes in the cell cytoplasm. See also Color Plate 32.7.

during permissive replication make AMDV a unique infectious disease. These unique properties make AMDV a good model system to examine aspects of viral pathogenesis for other interesting infectious diseases. For example, B19 infection presents as both acute and chronic disease (Bloom and Young, 1999). Similar to AMDV, the acute syndrome of B19-induced red cell aplasia is a direct effect of cytopathic virus infection of a select target of cells. Also reminiscent of AMDV, some syndromes of B19 infection are associated with immune complex formation and deposition, including autoimmune responses such as arthritis, rash, purpura, and polyarteritis nodosa. In addition, B19 NS1 is a strong inducer of IL-6 expression, and increased levels of circulating cytokines such as IFN- $\gamma$ , IL-6, and IL-8 are observed in chronically affected patients (Nigro *et al.*, 2000; Mitchell, 2002). Hence, the immune response of mink following AMDV infection is a potentially useful model for the study of human B19 infection.

NS1 transcripts are produced at greater levels than VP transcripts during permissive AMDV replication (Storgaard *et al.*, 1997). Although this observation is opposite of that observed during the replication of acutely infecting parvoviruses such as MEV (Storgaard *et al.*, 1997) and B19 (Liu *et al.*, 1992), restricted replication of B19 is also characterized by a change in virus gene transcription. In non-permissive cells, the mRNA for B19 NS1 is the most abundant species whereas the structural mRNAs are the most abundant transcripts during permissive replication (Liu *et al.*, 1992). Expression of NS1 in non-permissive cells may be directly cytotoxic or induce inappropriate cellular protein expression such as cytokines, and thereby contribute significantly to pathogenesis of AMDV or B19 infections (Bloom and Young, 1999).

AMDV infection also raises intriguing questions concerning restriction of virus replication and viral persistence. Restricted replication of AMDV occurs in macrophages and is likely to be influenced by numerous factors. To date, the presence of antiviral antibody and activation of caspases in the infected cell have been shown to be involved in restriction of permissive replication. In mink kits, the presence of antibody restricts the production of replicative intermediates in infected type II pneumocytes without reducing the number of cells infected (Alexandersen *et al.*, 1988c). Inhibition of caspase activation in infected CRFK cells also restricts the levels of viral replicative intermediates by preventing nuclear localization of NS1 (Best *et al.*, 2002). Ultimately, both antibody and caspase-mediated mechanisms of restriction may affect the downstream viral transcription events and viral DNA replication, leading to low production of infectious virus progeny.

Finally, AMDV infection of mink is a relevant model of infection for other unrelated, but medically important, viruses. Examples of this relevance include infection with dengue virus, which utilizes ADE of infection in macrophages (Halstead, 2003). HIV can be found in immune complexes retained on the surface of dendritic cells that may be a

continued source of infectious virus and stimulation of the immune response (Burton *et al.*, 2002). Hepatitis C virus uses caspases for the cleavage of its non-structural protein, NS5A, which translocates following cleavage to the nucleus and has increased transactivating abilities (Satoh *et al.*, 2000). The relative genetic simplicity of AMDV makes it a useful model to study these attributes, and how they may apply to other models of virus infection where the virus itself is more complex. Despite its inherent limitations, the fact that the *in vivo* model for the study of AMDV is its natural host is also invaluable for the study of host–virus relationships.

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# Minute virus of canines (canine minute virus) – the virus and its diseases

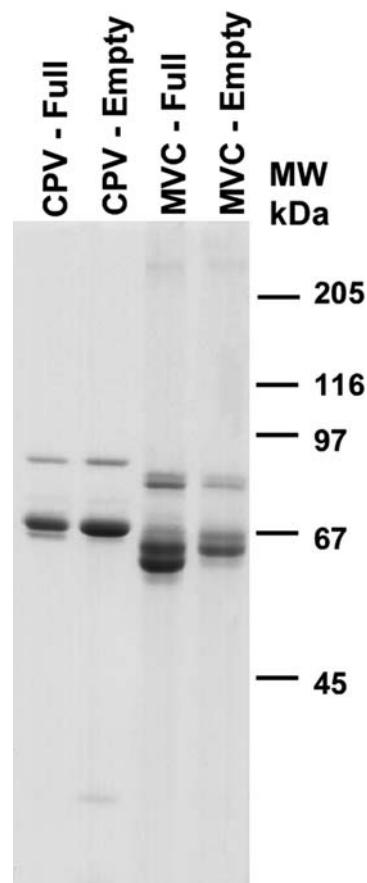
COLIN R. PARRISH

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The minute virus of canines (CnMV) – also known as canine minute virus – was first described as an isolate from the feces of a healthy dog in the USA (Binn *et al.*, 1970). That virus was grown in the Walter Reed canine (WRCC/3873D) cell line, and had the properties of a parvovirus, including small size (20–21 nm diameter) and the presence of virions in infected cell nuclei (Binn *et al.*, 1970; Macartney *et al.*, 1988; Harrison *et al.*, 1992). After canine parvovirus type-2 emerged in 1978, it was seen to differ from the CnMV antigenically and genetically based on antibody cross-reactivity and restriction enzyme analysis of the viral DNA (Macartney *et al.*, 1988), and more recently by DNA sequencing (Schwartz *et al.*, 2002).

## CnMV AND DISEASE

The distribution of CnMV and its association with canine diseases are currently not fully understood. Serological testing suggests that CnMV is widespread in dogs in the USA, with over 50 percent positive samples in the studies reported (Binn *et al.*, 1970; Carmichael *et al.*, 1994), although in Japan serological testing indicated that about 5 percent of the dogs were positive by the hemagglutination inhibition test (Mochizuki *et al.*, 2002). For many years no disease was known to be associated with CnMV, and it appears that most infections with CnMV are subclinical. However, diseases that have since been associated with virus infection include fetal infections leading to reproductive failure and neonatal respiratory disease (Carmichael, *et al.*, 1991; Harrison *et al.*, 1992; Jarplid *et al.*, 1996), which appear similar to fetal or neonatal diseases caused by many parvoviruses. In other



**Figure 33.1** Proteins found in CnMV full (DNA containing) and empty virions, compared to the proteins of canine parvovirus (CPV), showing the additional protein forms in the MVC capsids. Size standards are shown in kDa.

cases the virus genome was detected by polymerase chain reaction (PCR) in fecal specimens of puppies suffering from a variety of diseases, but in each case other viruses were also isolated making it unclear what role the CnMV played in the disease (Mochizuki *et al.*, 2002). In some cases the virus was associated with enteritis in puppies or older dogs (Binn *et al.*, 1981; Mochizuki *et al.*, 2002), although again the causal relationship is unknown.

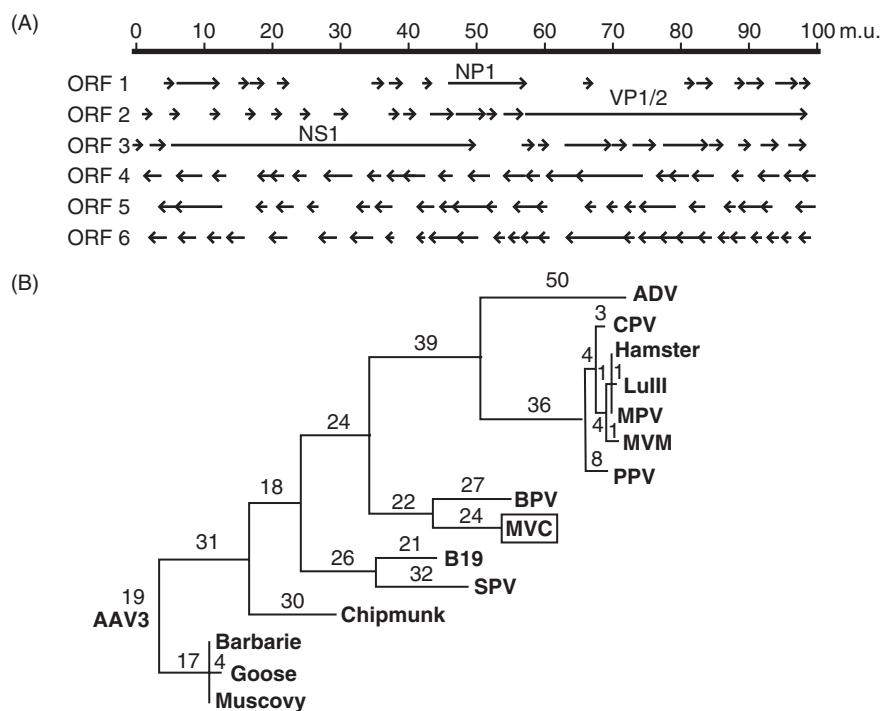
CnMV is restricted in its ability to replicate in cells in tissue culture, with infections being seen most reliably in the WRCC/3873D cell line, sometimes in Madin Dorby canine kidney (MDCK) cells, and also in some primary canine lung cultures from fetal pups (Carmichael *et al.*, 1991; Mochizuki *et al.*, 2002). Until recently there were few sensitive diagnostic tests widely available, but the recent development of PCR-based tests for the viral nucleic acids allow ready detection of the virus. However, these tests do not appear to be showing a significant number of CnMV-related diseases (Mochizuki *et al.*, 2002; Schwartz *et al.*, 2002).

## VIRAL PROPERTIES

Purified CnMV capsids contained a number of protein forms, with a minor and major form representing the VP1 and VP2 proteins (Figure 33.1). The major form of the larger proteins (VP1) had an estimated size of 81 kDa, while the

small (VP2) forms were 67 and 63 kDa, respectively. VP3 (61 kDa) was present in the full virus capsids, and probably results from cleavage of VP2 at arginine 19 in the VP2 sequence, similar to that reported for other parvoviruses (Figure 33.1) (Tullis *et al.*, 1992; Weichert *et al.*, 1998). Smaller amounts of other proteins were also seen, but their identities are not yet known, although they may derive from alternative splicing of various mRNAs (messenger RNA) (see below).

The almost complete sequence of the genome of CnMV was obtained from plasmid clones or directly from viral replicative form (RF) DNA, and that covered 5097 bases, and restriction analysis of the RF DNA showed a total genomic length with extended palindromes of about 5390 bases (Schwartz *et al.*, 2002). The turn-around and extended forms of the 3' end differed in length by about 80 bases, and those of the 5' end by about 75 bases, indicating that those palindromes contained about 160 bases and 150 bases, respectively. The CnMV sequence was most similar to the bovine parvovirus (BPV), with which it shared 43 percent DNA sequence identity (Schwartz *et al.*, 2002). When a conserved region within the translated sequence of the NS1 gene was compared with the equivalent sequence from other parvoviruses, CnMV was again closely related to BPV, with the next closest relatives being the erythroviruses (human B19 and simian parvoviruses) (Figure 33.2). Among the viruses of vertebrates there are at least three distinct



**Figure 33.2** (A) Open reading frames in the sequence of MVC shown as arrows. Labels indicated the proposed NS1, NP1, VP1, and VP2 sequences. (B) Phylogeny of representative parvoviruses showing the relationship of MVC to the other viruses. A conserved sequence of 149 residues of the CnMV NS1 was aligned with the sequences of the other viruses indicated and the most parsimonious phylogeny determined. The numbers indicate the number of residues on each branch.

(A)

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MVC MAPPNRKPG--GWVVPGYKYLGPFNPLDNGTPINKVDKAAQKHDFAYQSINKGENPYLN
BPV MPPTNKANSKKGLTLPGYNYLGPFNLFAGAPVNKADAARKHDFGYSDDLKEGKNPYLY
MVC FNKADSDFIEDLKNDSSFAGWLGSNFLRKRLAPHLKENTPPAKRVAGNSRQDRAQKRK
BPV FNTHDQNLIDELKDDTSFGGKLARGVFQIKKALAPAL-----PGTSKGG----DRAALKR
MVC LYFARSNKNQSKQARMEP-----QETENTIEAD-AG-----IAGRAGGGGGPGGGGS
BPV LYFARSNKGAKKANREPAPSTSQQNMEVSNNDIPNDEAGNQPIELATRSVVGSGSVGGGG
MVC GGGSGVGVSTGGWEGGTLFGDNRVITVNTRQWYAPIYNGHRY-TKLEGTGNTFWK--GIK
BPV RGGSGVGVYSTGGWTGGTIFSENIIVVTKNTRQFICDIKNGHLYKSEVLNTGDTAHRQYAIT
MVC TPWGYFNFNAYDSHFSQDWQRILTNEYRRWRPKMMVKIYNLQIKQVVTIQLG--DTLYNN
BPV TPWSYFNQNQYSSHFSQNDWQELVNDYERFRPKAMIVRVYNLQIKQIMT-DGAMGTVYNN
MVC DLTAGVHIFCDGSHQYPYSQHPWDAGTMPELPYKVWLLENYGYFQFQGDLIDTSVDGGSP
BPV DLTAGMHIFCDGDHRYPYVQHPWDDQCMPPELPSIWEPLQYAYIPAPISVVVDNNT-----
MVC DVENVEKXIAKSAPFYILEANHEVLRGEETNFHFNFDCGW-VNNDRAYCPLQADFNPL
BPV -TNTVEEHLKGVPYMLENSDEHEVLRNGRIYRIVIQL--WRLRMDRKQHHIQHASDDV
MVC VKT---RRYFATRNNYNNSGKFVYTRYSPYNKPSQWMPGPSLGYIGNTQSAATREQALG
BPV QSTGQKQKNLLIQRKTQPNKQRF---QNAALRTSNWMSGP--GIARGTHNATLQTQSAG
MVC P-VTVVTAPPGTSAYTAFTEQQSNTNQQSASNATWSGYDVSPVNCARSGF-DKIGLAYDS
BPV ALVTMVTN--GADV-----SGVRRAVRVGYSTDPYIYGQQQPESDLLRLRY-S
MVC APESELEEKISIRDIDNDMSRWGQVFVQDGTNKEISNDNTGQGGNTRQNMELKNVWMFP
BPV ASAAEQQQN---PILENAAR--HTFTREARTKLITGSNGADG-----DYKEWWMLP
MVC NQAWDSTPISRDFPIWVKSPTDKHTLFDSSDGTLPMSPHPGTIFVKVAKIPIPTQTNTD
BPV NQMWDsapisryNPiWVVKPRVNRKTLTDQGSIpmshppgtifIKLARIpvp--GNGD
MVC SYLTLYVTGQVTCTIEWEVERFMTKNWRPESKNDVSSFR-DAFLYTVGADGTNTPERFL
BPV SFLNIYVTGQVSCEVVWEVEKRGTKNWRPEYMHSATNMSVDA--YTINNAGVYAGAVQNA
MVC EGMPTRRGINKTLE
BPV DVMQTRFNHHKVLE

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(B)

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MVC SIGPHNH-----LLRPSNMSTRHMS-----KRSKARSRSRSPQD-----
BPV IPFQKNQKKGSGIWLERTPSQKHRRVTLQLPKELAWRKVSRGMERSRSRSPRETGSTSSRDK
MVC -----SRRP---NGERDLGSFHRGWTRNQSSSTVSR--QSTGRKTT-
BPV SDADWSERRREERTRTWKSRSPIRARGERSWGSR-RSREKNQSSSTASRPYQKATRKETA
MVC -----PMHFVNEHKRSRSKEPLPAFCGFYWHSTRILARC GTDWIFNEGKPKFQSVCHENK
BPV TKKTKHTPFNVFAHRALSKTDLQ-FCGFYWHSTRILASKGTNEIFNGLKQSFQSKAIDGK
MVC ITWDQCREMLFEFKKTIDQKYRNIMYHMGRGGFCQKCCYWDDVYTKHLANVNDV-LTQDL
MVC LDWEGVRELLFEQKKCLDTWYRNMMYHFALGGDCEKCNYWDDVYKKHLANVDTYSVAEEI
MVC SDAEMLSAAMEVDGSSEE
BPV TDSEMLEAAEAVDAANQE

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**Figure 33.3** Alignments of CnMV VP1, VP2, and NP-1 sequences (top) with those of BPV (lower). (A) The VP1/VP2 ORF. The conserved phospholipase A2-specific residues in that sequence are indicated. Closed circles indicate the active site motif (HDXXY) and the closed squares indicate the potential  $\text{Ca}^{2+}$  binding loop residues (YXGXG). The arrow indicates the predicted start of the VP2 sequence. (B) The NP-1 ORF, which appears to be unique to the MVC and BPV viruses.

clades, one of which includes the erythroviruses (B19 and similar viruses from primates), as well as the more distantly related BPV and CnMV. However, CnMV is still only distantly related to the other viruses, indicating that it diverged in the distant past.

Large open reading frames (ORF) within the left and right halves of the genome encoded the NS1 and the VP1 and VP2 proteins, respectively, while a shorter ORF in the middle of the genome overlapped with the NS1 protein, and showed greatest similarity to the predicted NP-1 protein of BPV (Lederman *et al.*, 1984). The NS1 sequence encoded a 717 residue protein with a predicted molecular weight of 81.9 kDa, which shared 32.6 percent sequence identity with the NS protein of BPV. A conserved core of the NS1 that contained sequences identified as being involved in the nucleotide binding and DNA nicking activities of the protein (Jindal *et al.*, 1994; Corbau *et al.*, 2000). However, sequences identified in the MVM NS1 as a metal coordination site (histidines 127 and 129), and to be part of the active site (tyrosine 210) could not be clearly identified in the NS1 of CnMV or BPV. The right hand ORF encoded VP1 and VP2 proteins of 78.9 and 63.9 kDa respectively which initiated at methionine codons in the same reading frame, at nucleotides 2933 and 3329 in the sequence (Figure 33.3A). The 21.7 kDa protein encoded by a third ORF was 39 percent identical with the NP-1 protein predicted from the BPV sequence (Figure 33.3B) (Lederman *et al.*, 1984), but showed no homology to any other protein in the databases. The first 188 nucleotides of the NP1 ORF overlapped with the C-terminal sequence of NS1 in an alternate reading frame. The two capsid proteins appear similar to the VP1 and VP2 proteins of the other parvoviruses, but no evidence was found of a spliced product within the VP1 gene, indicating that the two proteins are likely encoded through translation of one message at two ATG codons to give VP1 and VP2. Conserved sequences that are associated with the active site of a phospholipase A2 activity of the VP1 unique region of several parvoviruses were present in the CnMV and BPV sequences, suggesting that that is a highly conserved function of the protein (Zadori *et al.*, 2001).

RT-PCR (reverse-transcriptase PCR) was used to isolate spliced mRNA products from CnMV-infected cells, using two different pairs of PCR primers flanking the introns described for many parvoviruses. For the RT-PCR spanning the region around the beginning of the VP1 ORF, the only product detected was the intact 745 base pair sequence, and no splices of these sequences were detected. For the 368–3346 nucleotide region of the genome, the predicted full-sized product was detected, as well as products of ~1100 nucleotides and ~400 nucleotides. The ~1100 nucleotide product had two splices that fused two sequences within NS1 and part of the NP1 ORF. The ~400 nucleotide product showed at least two sequences – in one of those, 26 residues from the N-terminus of NS1 were fused to the VP1 ORF, while the other fused the NS1 ORF to an alternative ORF overlapping that of NP1. Whether the NS1-VP1 fusion is formed in any quantity was not

determined, but that may be the origin of the alternative VP1 forms recovered from the viral capsids (Figure 33.1). The mRNA transcripts identified by RT-PCR between primer sites around nucleotides 390 or 2618 and 3325 showed a variety of possible mRNA forms producing a number of proteins. The significance of those spliced mRNA is not known, but they appear similar to those described for the Aleutian mink disease virus and the human parvovirus B19, where a variety of spliced products have been reported, but for most of which the functions have not been defined (Ozawa *et al.*, 1987; Alexandersen, *et al.*, 1988; St Amand, 1991).

In summary, the CnMV is a widespread infection of dogs, but is rarely associated with disease in dogs. The genome of the virus is most similar to the BPV, but it shows a number of differences even from that virus.

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# Bovine parvovirus

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The progeny of viruses initially isolated from cattle by Abinanti and Warfield (1961), the HADEN (hemadsorbing enteric) strain, is the type strain of bovine parvovirus (BPV). It is classified in the genus *Bocavirus* (along with minute virus of canines (CnMV), subfamily Parvovirinae, family *Parvoviridae*. (See Chapter 1 for further discussion.) In this chapter we will review the structural features of BPV and the characteristics of infection *in vitro* and *in vivo*.

## FEATURES OF THE VIRUS AND ITS GENOME

BPV is a small, naked icosahedral virus. Its genome contains 5517 nucleotides (Chen *et al.*, 1986, 1988). There are three coded capsid proteins, VP1 (80 KDa), VP2 (72 KDa), VP3 (62 KDa), along with VP4, (60 KDa), which is likely a proteolysis product of VP3 (Johnson and Hoggan, 1973; Lederman *et al.*, 1983); none of the virion proteins is glycosylated or phosphorylated. These proteins surround predominantly negative-strand DNA, although a variable but small amount of positive strand (~10 percent) can be found within a separate capsid (Chen *et al.*, 1988). The BPV termini contain self-complementary sequences; the left terminus can fold into a T-shaped 'stem-plus-arms' structure, which is 150 nucleotides in length, while the 173 nucleotides right end can assume a U-shaped conformation. The left hairpin contains unpaired/mismatched nucleotides in the duplex stem forming a bubble, not found in the right hairpin. Parvoviral termini have been found in two different sequence orientations, flip or flop, which are inverted complements of each other. The proportion of each of these conformations is strand-specific for each terminus of the viral genome. The left end of the BPV minus strand is 90 percent flip and 10 percent flop, while the right terminus is

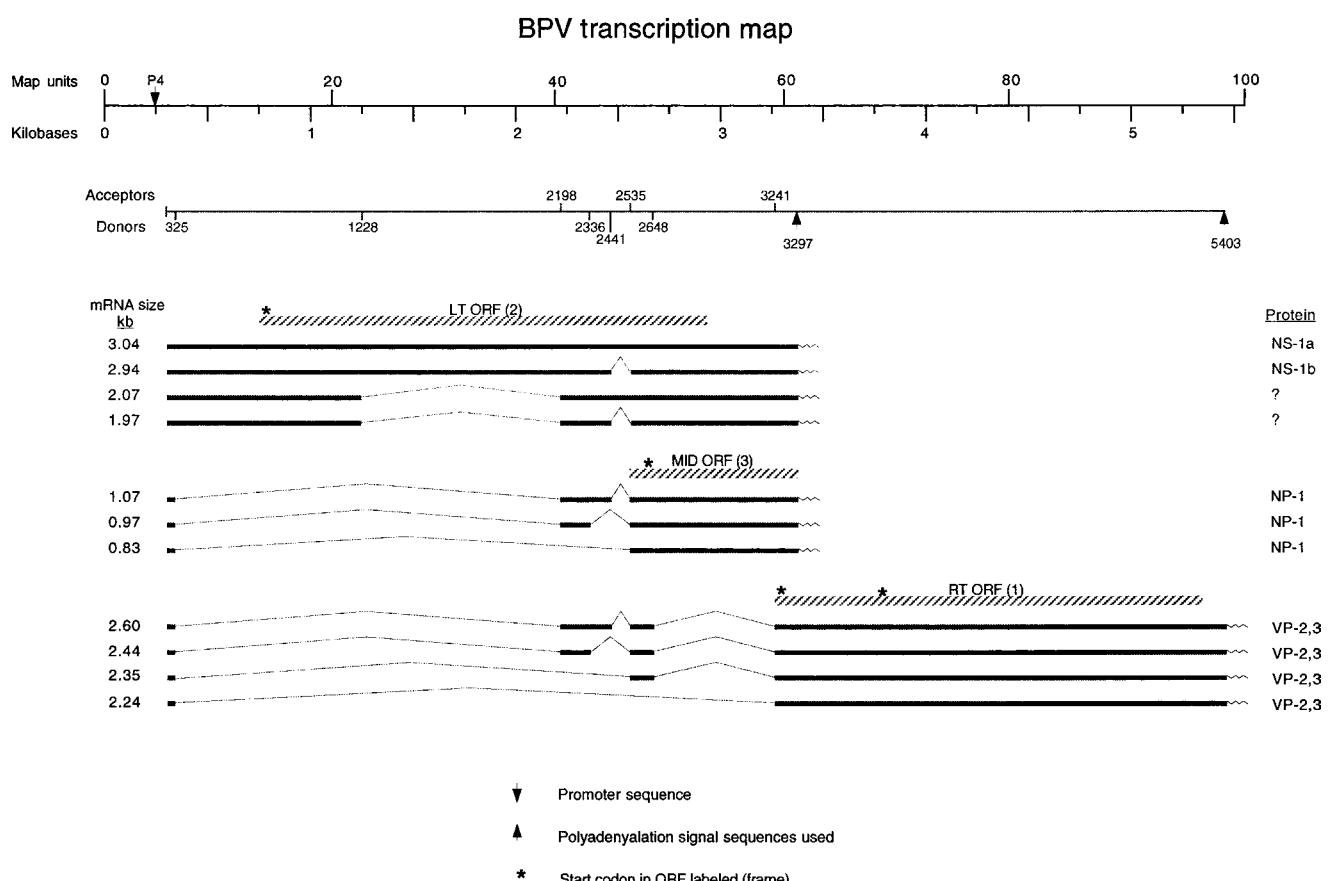
equally flip and flop. For the plus strand, the flip/flop ratio at the left end is 1:1 and 1:3 at the right end (Chen *et al.*, 1988).

Analysis of the genomic sequence (Chen *et al.*, 1986) reveals three open reading frames (ORFs); the left ORF probably codes for two phosphorylated proteins (75 and 83 KDa) that are equivalent to the NS-1 proteins of viruses in the family Parvovirus (Lederman *et al.*, 1987); the mid-ORF may code for the third phosphorylated non-structural protein, NP-1 (28 KDa) (Lederman *et al.*, 1984), while the right ORF contains the sequences for the overlapping capsid protein genes. There are three putative promoters at map unit 4, 13, and 38, identified by their core and surrounding sequences. Polyadenylation signals that might be functional are located at map units 62, 65, and 98.

Recently, two new BPVs (BPV-2 and BPV-3) have been reported (Allander *et al.*, 2001); more precisely, amplification products with some relation to BPV sequences were produced from DNase-treated bovine serum. The nucleotide sequence of each of these putative viruses differs greatly, not only from the other, but also from BPV-1. These amplified products contain two, rather than three ORFs, whose deduced amino-acid sequences each contain consensus sequences found in the capsid and non-structural proteins of other parvoviruses.

## BPV INFECTION *IN VITRO*

The replication cycle of BPV has been studied most often in parasympathetic primary bovine fetal lung (BFL) cells, and to a lesser extent in embryonic bovine tracheal cells; replication in other cells, including transformed cells, has been weak or non-existent. To initiate infection, BPV may bind to glycophorin A (Thacker and Johnson, 1998). The



**Figure 34.1** Transcription map of bovine parvovirus. The ruler marked in map units and kilobases represents the genome of BPV. P4 marks the promoter used for the mRNAs described. The nucleotide positions of splice donors and acceptors identified are marked. Thick lines denote sequences included in mRNAs while thin, angled lines denote sequences not present in mRNAs.

virus interacts with erythrocytes through this protein, accounting for the hemagglutinating properties of the virus, although the presence of glycophorin A on the surface of permissive cells or binding of the virus to the cell-associated protein has not been shown. In BFL cells, mRNA for non-structural proteins can be detected at 14 hours post infection, while mRNA for capsid proteins is seen at 20 hours. DNA replication occurs at 24–48 hours and non-structural and capsid proteins migrate to the nucleus, possibly using nuclear localization signals (Lederman *et al.*, 1986), where the virus is assembled.

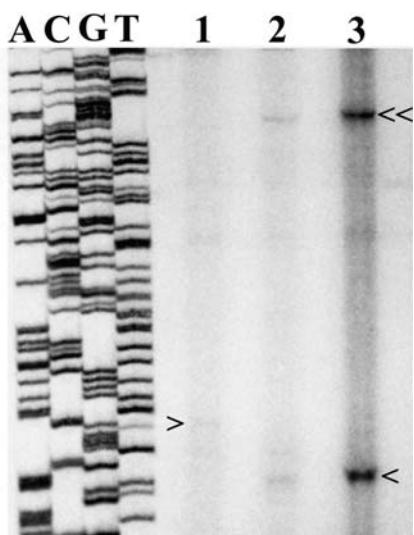
## BPV transcription

Because of the low density of primary cells, along with possibly a low level of transcripts, obtaining a transcription map of the virus required reverse-transcriptase PCR (RT-PCR) using primers in regions deduced from the genomic sequence to be within ORFs. Eleven mRNAs are transcribed during BPV infection of bovine fetal lung (BFL) cells (Figure 34.1). All BPV transcripts appear to originate from the promoter at map unit 4. A primer extension assay (Figure 34.2) identified

nucleotide 275 as the start of transcription for the viral mRNAs. Nucleotide 275 is an A residue but there is a G residue at nucleotide 271. We suggest that nucleotide 271 is the cap site, with incomplete extension due to interference by the cap itself. Two polyadenylation signals are used: one at map unit 62 for left and mid-ORF transcripts and one at map unit 98 for the right ORF transcripts.

The 3.04 kb unspliced message could encode an 81 kDa polypeptide of 726 amino acids. The 2.94 kb spliced message has the capacity to code for a 77 kDa polypeptide of 695 amino acids. These mRNAs seem likely to encode the two forms of NS1 observed in BFL cells after infection or *in vitro* translation reactions programmed by RNA from infected cells (Lederman *et al.*, 1987). Polypeptides of 45 kDa and 41 kDa could be produced from the 2.07 and 1.97 kb mRNAs transcribed from the left ORF. Proteins of these molecular weights have been observed by immunoprecipitation of radiolabeled BPV-infected cells with anti-capsid antibodies.

The mid-ORF is expected to encode the NP-1 protein. While mid-ORF transcripts are of three sizes (1.07, 0.97, and 0.84 kilobases), only one polypeptide of 24 kDa could be encoded by any of them, since the first ATG codon in



**Figure 34.2** Primer extension assay using BPV-infected BFL cell RNA. ACGT: sequencing reactions using M13 DNA as template. **Lane 1:** sequencing reaction using M13 DNA as template. **Lane 2:** product of extension reaction (194 nucleotides, single arrowhead) using primer MORF $(-)$ , complementary to BPV nucleotides 2679–2656. **Lanes 2 and 3:** products of extension reaction (295 and 182 nucleotides, double arrowheads) using primer RORF $(-)$ , complementary to BPV nucleotides 3373–3354. The products in lanes 2 and 3 used RNA harvested 20 hours post infection as template while the products in lane 4 used RNA harvested 24 hours post infection.

this ORF is 3' to the most downstream splice acceptor in all these messages.

All of the right ORF messages could be translated to produce the virion proteins VP2 and VP3 of 75 kDa and 60 kDa, respectively, using ATG codons at nucleotides 3286 and 3697, respectively. The 2.44 kb right ORF mRNA could encode the 80 kDa VP1 protein by initiating translation in the left ORF at the ATC start codon at 2246, continuing in the mid-ORF between nucleotides 2535 and 2648, and completing translation with right ORF sequence, overriding the termination codon at 3247 by suppression or ribosomal frameshifting. This assignment explains the immunoprecipitation of the 45 kDa protein using antibodies produced against capsid proteins (Lederman *et al.*, 1983) and the similarity in the size of chymotryptic cleavage products of VP1 and NP-1 (Lederman *et al.*, 1984).

### Sequences involved in BPV DNA replication

The secondary structure of the viral ends is remarkably conserved across parvoviruses. Mutation of the termini showed that the secondary structure of the arms of the AAV terminus is more important than its primary sequence for replication (Lefebvre *et al.*, 1984; Bohenzky *et al.*, 1988). Of particular interest is the presence of mismatched/unpaired nucleotides, forming a bubble, in the

stem of the left hairpin; this bubble is present in the genomes of rodent parvoviruses and is required for DNA replication (Cotmore and Tattersall, 1994) but no mismatched nucleotides are present in the stem of the B19 or AAV hairpins.

To analyze the role of these unpaired/mismatched nucleotides on the parvovirus life cycle, two ‘bubbleless’ clones containing completely base-paired stems were constructed. The clone referred to as ‘BLOP’ (for ‘Bubbleless Left flOp Plus’) contains five ‘extra’ nucleotides that are complementary to the unpaired nucleotides of the BPV wild-type sequence and has two mutated bases. The other clone, referred as ‘BLOM’ (for ‘Bubbleless Left flOp Minus’) lacks five of the unpaired nucleotides found in the BPV wild-type sequence and has two mutated bases (Figure 34.3). The ability of these clones to replicate was analyzed after electroporation of BFL cells by observation of cytopathic effect (CPE), indirect immunofluorescence (IFA), and transient DNA replication assays.

No CPE was observed for the BLOP clone, even by 12 days after electroporation of BFL cells. CPE was first observed in cells transfected with the BLOM clone 6–7 days after electroporation and the cell layer was completely lysed after 12–13 days. In comparison, BFL cells electroporated with the wild-type, infectious clone of BPV, pVT501 (Shull *et al.*, 1988), showed CPE at 3–4 days, and were completely lysed by 5–6 days. Blind passage revealed that the BLOM clone produced infectious viruses since CPE appeared on fresh cells after a few days, while no CPE could be observed for BLOP even 7 days after infection.

In an IFA assay to detect the presence of virally-coded proteins in the electroporated cells, cells transfected with BLOM and pVT501 both showed positive nuclear fluorescence using rabbit serum against BPV capsids (0118) and a calf serum containing antibodies against the structural and non-structural proteins (calf 86). The number of positive cells was much lower in cells electroporated with BLOM than with pVT501. No positive immunofluorescence was seen using cells transfected with the BLOP clone.

The replication of the mutant BPV clones was studied by isolating low molecular-weight DNA from the cells 5–6 days after electroporation. The genomic clone pVT501 was replicated as indicated by the presence of DpnI-resistant monomer and dimer replication intermediates (Figure 34.4, lane 4). Little monomer-length DNA was obtained from the BFL cells electroporated with the BLOM clone (lanes 5, 6); the amount of newly-synthesized DNA was about 8 percent of that found with pVT501, assessed by densitometric comparison of the intensity of the 5 kb bands. Doubling of the amount of BLOM used in the electroporation resulted in a decrease rather than an increase in DNA replication (lanes 7, 8). The BLOP plasmid was not replicated since no genome-length DNA was detected after DpnI digestion of the Hirt DNA (lanes 9, 10) even after electroporation with twice the amount of plasmid DNA (lanes 11, 12). Our results suggest that the bubble in the left hairpin

## (A) FLIP

A  
 80—A G  
 G C  
 C G  
 G C           Stu I  
 C G           |  
 G CGTAA C TT CCGG ATTA GCCCGTCGGTTATACCACCTACGTCTTACTCATTATTTTATATCTCGGACAC -- 5'  
 C  
 A TCGCATT A T GGCC --- CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT 3'  
 G C — |  
 C G           Sma I    40           |  
 G C  
 G C  
 C G  
 G A  
 C

## (B) FLOP

G  
 C T  
 G C  
 C G  
 C G  
 80—G C           Sma I  
 C G           |  
 T AGCGTAA T A CCGG --- GCCCGTCGGTTATACCACCTACGTCTTACTCATTATTTTATATCTCGGACAC -- 5'  
 G  
 C GCGCATT G TAAT GGCC TAAT CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT 3'  
 G C           |  
 C G           Stu I    40           |  
 G C  
 C G  
 T C  
 T

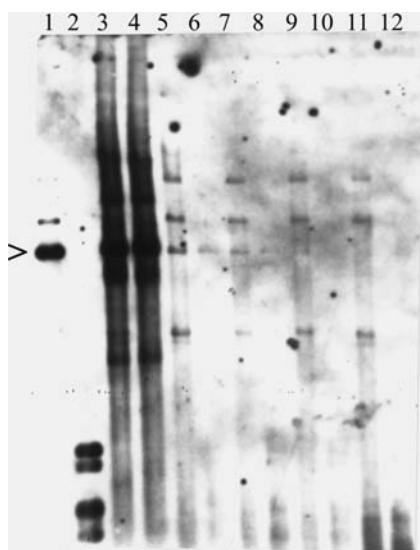
## (C) BLOP

G  
 C T  
 G C  
 C G  
 C G  
 G C           Stu I  
 C G           |  
 T AGCGTAA C TT CCGG ATTA GCCCGTCGGTTATACCACCTACGTCTTACTCATTATTTTATATCTCGGACAC -- 5'  
 G  
 C GCGCATT G TAAT GGCC TAAT CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT 3'  
 G C           Stu I  
 G C  
 C G  
 T C  
 T

## (D) BLOM

G  
 C T  
 G C  
 C G  
 C G  
 G C           Sma I  
 C G           |  
 T AGCGTAA T A CCGG --- GCCCGTCGGTTATACCACCTACGTCTTACTCATTATTTTATATCTCGGACAC -- 5'  
 G  
 C GCGCATT A T GGCC --- CGGGCAGCCAATATGGTGGATGCAGGAAATGAGTAATAAAAATAT 3'  
 G C           Sma I  
 G C  
 C G  
 T C  
 T

Figure 34.3 Structure of 'bubbleless' BPV termini. Nucleotide sequence of BLOP and BLOM mutants and of wild-type termini.



**Figure 34.4** Analysis of low molecular weight DNA 5–6 days after electroporation of BFL cells with mutant 'bubbleless' clones. **Lane 1:** Sall fragment of pVT501 (Shull *et al.*, 1988), the genomic clone of BPV. **Lane 2:** Dpn-digested pVT501. **Lanes 3–12:** Hirt DNA extracted from cells electroporated with 'bubbleless' circular plasmids. **Lanes 3 and 4:** 10 µg pVT501. **Lanes 5 and 6:** 10 µg BLOM. **Lanes 7 and 8:** 20 µg BLOM. **Lanes 9 and 10:** 10 µg BLOP. **Lanes 11 and 12:** 20 µg BLOP. DNAs in lanes 4, 6, 8, 10, and 12 were treated with DpnI prior to electrophoresis. To standardize the Hirt isolation procedure, the number of cells harvested at the beginning of the procedure was determined, and the efficiency of the DNA recovery during the extraction was monitored by adding a known amount of DNA of unrelated sequence to the harvested cells. The internal standard DNA (insertion element IS 1141 contained in *Mycobacterium avium* plasmids [Via, 1993]) was detected by dot-blotting the Hirt DNAs with a digoxigenin-labeled *Mycobacterium* probe. The Hirt DNAs were hybridized with a BPV-specific probe and chemiluminescent signals were quantified with a laser densitometer.

of BPV is not required for genome replication, although specific sequences within the mismatched/unpaired region are required to produce progeny genomes. The point within the replication cycle affected by the bubble has not been addressed by these experiments.

## BPV INFECTION *IN VIVO*

The symptoms of BPV infection are diarrhea and reproductive failure, along with respiratory infection and conjunctivitis. In cattle herds, horizontal transmission is by the fecal–oral route and vertical transmission affects the fetus. The symptoms reflect the requirement that host cells pass through the S-phase of the cell cycle for replication, since gut and embryonic cells divide rapidly. The effects of BPV in cattle can be better delineated after experimental administration of the virus. Such studies confirmed enteritis and spontaneous abortion as sequelae of infection and found that BPV

infection of animals with coincident coccidial disease resulted in more severe symptoms (Freeman *et al.*, 1986). This effect may be the result of coccidial-induced increased mitotic activity providing a greater number of permissive cells. Other factors promoting BPV-caused disease are weaning stress and poor management practices (Durham *et al.*, 1985).

## GEOGRAPHIC DISTRIBUTION AND FREQUENCY OF BPV INFECTION

BPV infection has been seen worldwide. This virus has been isolated in Algeria, Australia, Austria, England, Japan, Russia, the United States, Trinidad (Adesiyun *et al.*, 2001), and Germany (Elschner, 1995).

Surveying diseased animals for evidence of the virus as an indicator of the prevalence of BPV infection yields numbers so low that they appear to be artifactual. Only two isolations of BPV were obtained from nearly 9000 bovine abortions and stillbirths in South Dakota between 1980 and 1990 (Kirkbride, 1992). Biermann *et al.* (1989) found parvoviruses in 0.5 percent of almost 600 calves by electron microscopy while Elschner (1995) found BPV in five of 117 fecal samples from cows in Thuringia by solid phase immuno-electron microscopy. On the other hand, if infection is monitored by presence of anti-BPV antibodies, the numbers are dramatically different. All 12 herds surveyed in a study in South Carolina had cows whose sera tested positive for the virus, and these cows, not surprisingly, had increased reproductive problems, such as embryonic mortality and more services per conception than seronegative cows (Barnes *et al.*, 1982). In Ontario, Canada, approximately 1100 animals in 29 herds were tested twice, the tests being 1 year apart. Initially, 49 percent of cows and 96 percent of herds were BPV-positive, the criterion being hemagglutination inhibition (HI) titers of >1/32. After a year, all the herds and 86 percent of the cows were seropositive (Sandals *et al.*, 1995).

The reason for the discrepancy between the results obtained with these two types of assay is not clear. Attempting to demonstrate the presence of virus particles may be affected by the presence of non-specific inhibitors, hemolytic material, or dilute virus. On the other hand, HI tests may give an overestimate of infection due to the presence of cross-reacting antibodies or non-specific inhibitors of hemagglutination. Nonetheless, BPV is probably a major agricultural pathogen, even though the US Department of Agriculture does not track its prevalence.

## IMMUNOLOGICAL CROSS-REACTIVITY OF BPV

BPV capsids are not immunologically related to members of the genus *Parvovirus* or to goose parvovirus (Mengeling *et al.*, 1986), but are related to B19. Mengeling and Paul (1986)

screened human sera for the presence of antiparvovirus antibodies. They found that 25 percent of a panel of human sera were positive by IFA when tested against BPV-infected cells. These sera were thought to contain antibodies to B19 that cross-reacted with BPV rather than antibodies to BPV itself. Lederman and Naides (unpublished) confirmed this conjecture; they found that human sera containing antibodies directed against proteins of parvovirus B19 immunoprecipitate BPV proteins from radiolabeled cell lysates. An antibody raised in rabbits against the capsid protein gene of B19 expressed in bacteria (a gift of S. Cotmore), reacted with a BPV capsid protein in a Western blot. Conversely, sera that were confirmed B19-antibody negative by an enzyme-linked immunosorbent assay (ELISA), immunoprecipitated BPV capsid proteins, suggesting that humans can develop antibodies to BPV after exposure to the virus, perhaps when present in contaminated milk or milk-products (Naides and Lederman, unpublished). Cesarean-delivered, colostrum-deprived (CD/CD) pigs developed neutralizing antibodies to BPV after being fed a diet containing live virus (Mengeling and Matthews, 1990).

Antibodies to left ORF proteins react with the non-structural proteins of multiple parvoviruses. Antibodies directed against bacterial fusion proteins encoding amino-acid sequences from a highly conserved region of the NS1 proteins of MVM and of B19 both gave specific nuclear fluorescence with BPV-infected cells (Lederman *et al.*, 1987).

## BPV IN THE ENVIRONMENT

Since they are so resistant to thermal and chemical inactivation, there is concern about the persistence of parvoviruses in the environment. The presence of B19 might directly contaminate therapeutic biologicals and BPV might do so indirectly, through its presence in calf serum used in cell cultures that produce molecules of medical importance. Secondarily, the presence of anti-BPV antibodies in calf serum may complicate biological assays. On the other hand, because of their stability, BPV (along with canine parvovirus (CPV) and porcine parvovirus (PPV)) are becoming model organisms for testing methods designed to ensure the safety of biologicals and methods for inactivation of sludge and agricultural waste.

A major cause for concern is the presence of parvoviruses in calf sera used to prepare recombinant therapeutic proteins, such as clotting factors (Ragni, 2001). This contamination was noted a quarter of a century ago by Nettleton and Rweyemamu (1980); an infectious agent with the characteristics of a parvovirus (typical chemical and heat resistance, S-value, density in cesium chloride ( $\text{CsCl}$ ), and number of capsid proteins) was isolated after killing rapidly dividing baby hamster kidney (BHK) cells in suspension culture. The source of the virus was found to be two batches of serum. Since the virus was serologically

related to minute virus of mice (MVM), it is not likely to have been BPV (HADEN) but another bovine parvovirus, perhaps similar to those whose genomes have recently been found in serum (Allander *et al.*, 2001).

A variety of treatments to inactivate BPV in serum-based biologicals have been tested. The similarity of the thermal resistance between BPV and hepatitis B virus is the basis for BPV being a proxy for inactivation of this severe human pathogen. Standard pasteurization ( $70^\circ\text{C}$  for 30 min) is ineffective (Brauniger *et al.*, 1994). The virus is resistant to dry heat treatment at  $80^\circ\text{C}$  for 72 hours (Roberts and Hart, 2000) or  $100^\circ\text{C}$  (Brauniger *et al.*, 2000) and to freeze-drying (Roberts and Hart, 2000). However, exposure to ultraviolet C radiation will clear BPV without affecting the ability of the serum to support growth (Kurth *et al.*, 1999).

Currently employed solvent-detergent methods of decontamination of pooled blood products such as immunoglobulin do not remove naked viruses (Schmidt *et al.*, 2002). Nanofiltration has been studied as a method for removing viruses from clinical immunoglobulin preparations. Human intravenous immunoglobulin (IVIG) solutions were spiked with CPV and BPV and passed first through a 75 nm filter followed by two 35 nm filters. CPV was partially removed by this protocol but the removal of BPV could not be assessed. The IVIG itself interfered with neutralization and plaque formation, probably due to anti-BPV antibodies in the serum (Troccoli *et al.*, 1998). Omar and Kempf (2002) took advantage of this interaction, finding that BPV with the antibody 'halo' was retained efficiently by 20 nm filters, while MVM, which was unaffected by IgG, was detected in the filtrate.

The bound antibody might also protect BPV from inactivation by physicochemical reagents. For example, Pruess *et al.* (1997) compared the effectiveness of ethyleneimine and electron beam radiation in inactivating PPV as a model for parvoviruses. Using radiation, a straightforward  $6 \log^{10}$  reduction in infectivity was obtained with a linear dose response. However, a biphasic reaction over time was seen after ethyleneimine treatment and the investigators attribute this tailing, representing incomplete inactivation, to masking of the virus by other proteins in the serum. Therefore, selecting which viruses to use as models should take into account the physical and chemical state of the virus in the medium to be sterilized.

Organs intended for transplantation must also be free of viral pathogens. Testing of procedures to prevent transmission during bone grafting used BPV as a model for B19. BPV and five other naked and enveloped viruses were placed in contact with small cylinders of human bone located within 'femoral heads,' spheres of bone  $\sim 56$  mm in diameter. These models for bone implants were held at  $82.5^\circ\text{C}$  for at least 15 minutes in an apparatus, the 'Lobator sd-1,' approved in Germany for bone sterilization. Under these conditions, BPV and the other viruses, HIV-2, BVDV, and polio among them, were inactivated by at least a factor of  $4 \log^{10}$  (Pruss *et al.*, 2003). A similar level of inactivation of BPV was seen

with a dose of  $34 \times 10^5$  rad of gamma radiation, the highest level required for inactivation of the six viruses after inoculating the shafts of femoral bones (Pruss *et al.*, 2002). Xenotransplantation from non-human primates to humans carries the risk of transmission of pathogenic parvoviruses; these viruses may be related to B19, and may cause anemia in immunocompromised individuals (Green *et al.*, 2000). Further, viral persistence in donor tissue within the host milieu may allow viral adaptation to the host and consequent cross-species adaptation.

BPV is also used as a marker for effective treatment of sewage sludge and cattle manure. In sludge, the most effective treatment for eliminating BPV is heating to 60°C followed by anaerobic mesophilic (35°C) digestion (Spillman *et al.*, 1987) or anaerobic mesophilic digestion followed by pasteurization (Wekerle *et al.*, 1987). The recommended method for viral inactivation in single-cell protein (the solids recovered after centrifugation of liquid manure that are refed to livestock) is anaerobic thermophilic digestion (Monteith *et al.*, 1986).

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# Shrimp parvoviruses

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TIMOTHY W. FLEGEL

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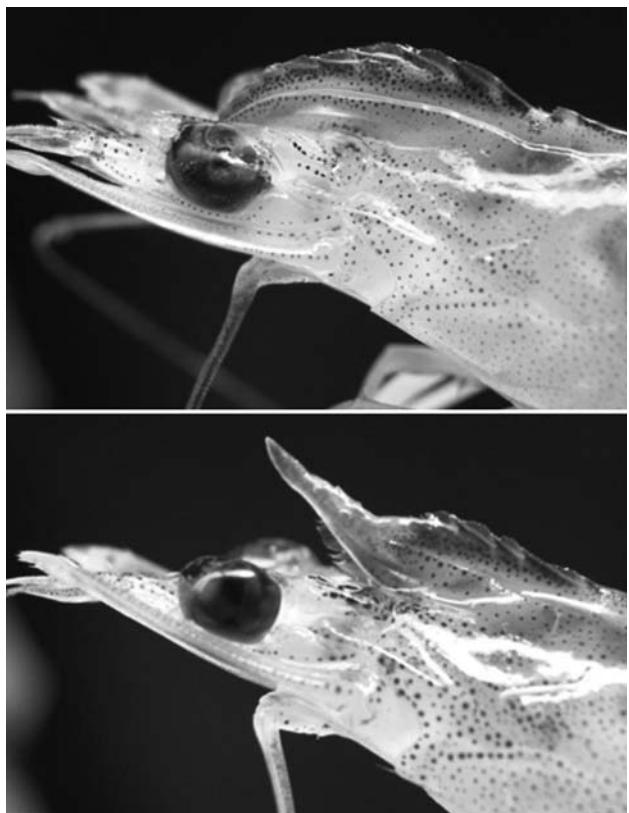
Three parvoviruses from shrimp species have been partially characterized. In order of discovery, these have been called infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Lightner *et al.*, 1983b; Bell and Lightner, 1984), hepatopancreatic parvovirus (HePV) (Chong and Loh, 1984; Lightner and Redman, 1985) and spawner isolated mortality virus (SMV) (Fraser and Owens, 1996). All should probably be grouped within the subfamily Densovirinae together with the densonucleosis viruses (DNV) or densovirus of insects (Tijssen and Bergoin, 1995; Bern, 1996). Information on IHHNV and HePV has been included in several general reviews on shrimp viruses (Flegel, 1997; Lightner, 1988, 1993, 1996b; Lightner *et al.*, 1990; Loh *et al.*, 1997; Lightner and Redman, 1998; Alday de Graindorge and Flegel, 1999). Hepatopancreatic parvovirus of shrimp is usually abbreviated to HPV, as is human papillomavirus. To avoid confusion in this volume, we use HePV for shrimp virus, but readers should be aware that this designation is not customary in the aquaculture literature.

A phylogenetic analysis of DNA and putative protein sequences of shrimp and insect densoviruses (Roekring *et al.*, 2002) revealed that the shrimp densoviruses for which DNA sequence information is currently available (i.e. GenBank records for IHHNV AF218266, HPV AF456476 and AY008257, SMV AF499102) did not cluster as a clade separate from the insect densoviruses but interdigitated with them. This suggested the possibility that densoviruses may shuttle between shrimp and adult or larval insects. If so, it may be worthwhile examining aquatic insects as possible vectors for parvoviral diseases of cultivated shrimp. Should this turn out to be the case, it is likely that the shuttle

virus would be able to replicate in insect cell lines. This would open the way for some types of experiments not currently possible owing to the lack of continuous cell lines for shrimp or any other crustacean species (Crane and Benzie, 2000).

## INFECTIOUS HYPODERMAL AND HEMATOPOIETIC NECROSIS VIRUS

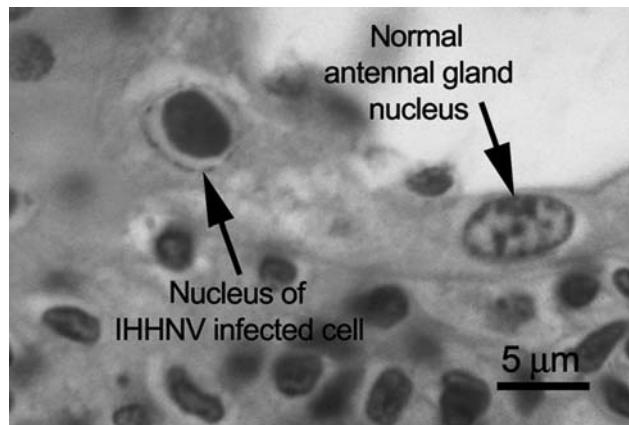
IHHNV was first discovered in blue shrimp *Penaeus stylirostris* and white shrimp *P. vannamei* (also called *Litopenaeus stylirostris* and *L. vannamei*) in the Americas in the early 1980s (Lightner *et al.*, 1983a,b), where it was believed to have been introduced by importation of live experimental stocks of the black tiger shrimp *P. monodon* from Asia (Lightner and Redman, 1991; Lightner, 1996a, 1999). It is a typical densovirus with non-enveloped icosahedral virions averaging 22–23 nm in diameter and containing linear single-stranded DNA (ssDNA) of 4.1 kb (Bonami *et al.*, 1990; Bonami and Lightner, 1991; Lightner, 1996a). Although IHHNV has been reported to occur in several species of wild and cultured penaeid shrimp throughout the world (Lightner, 1996a), it has been reported to cause acute epizootics and mass mortality only in *P. stylirostris*, especially in juveniles and subadults (Lightner, 1996a). By contrast, it does not cause mortality in *P. vannamei* but rather reduced, irregular growth and cuticular deformities, gross signs collectively referred to as ‘runt-deformity syndrome’ (RDS) (Figure 35.1) (Bell and Lightner, 1984, 1987; Kalagayan *et al.*, 1991; Brock and Main, 1994). In spite of



**Figure 35.1** Runt deformity syndrome in *Penaeus vannamei*. Two shrimp specimens showing deformed rostra – one curved down and the other up and both shorter than normal. See also Color Plate 35.1.

no mortality, commercial losses from RDS can be high (Carpenter and Brock, 1992). *P. stylirostris* and *P. vannamei* that survive IHHNV epizootics may carry the virus for life and pass it on by vertical and horizontal transmission (Lightner, 1996a). Infected adults seldom show signs of the disease or mortalities. Vertically infected larvae and early postlarvae of *P. stylirostris* do not contract disease, but massive mortalities may occur in juveniles at approximately 35 days or more. *P. monodon* appears to be relatively unaffected by IHHNV while *P. indicus* and *P. merguiensis* (also called *Fenneropenaeus indicus* and *F. merguiensis*) appear to be refractory to infection (Lightner, 1993, 1996a; Brock and Main, 1994).

Histologically, IHHNV can be provisionally diagnosed by the presence of prominent intranuclear, Cowdry type A inclusions (Figure 35.2). With H&E staining of Davidson's fixed tissues of ectodermal and mesodermal origin, these occur in enlarged nuclei as eosinophilic, often haloed inclusions surrounded by marginated chromatin (Lightner, 1996a; Alday de Graindorge and Flegel, 1999). Even with heavy infections, there is an absence of a visible host inflammatory response. *In situ* DNA hybridization assays with a specific IHHNV probe can be used to give a definitive diagnosis of IHHNV infection with very lightly infected shrimp and they can also be used to distinguish IHHNV



**Figure 35.2** Cowdry type A inclusion of infectious hypodermal and hematopoietic necrosis virus (IHHNV) in a nucleus of an antennal gland cell of *Penaeus monodon* from Thailand. Note the eosinophilic central inclusion separated from the marginated chromatin by a lightly stained zone that occurs as an artifact in tissues fixed with Davidson's fixative (Lightner, 1996b). See also Color Plate 35.2.

unambiguously from white spot syndrome virus (WSSV) (Lightner *et al.*, 1992; Lightner, 1993, 1996a; Lightner and Redman, 1998). Also available is a polymerase chain reaction (PCR) assay described in the OIE manuals on aquatic animal health (Anonymous, 2000, 2003). This gives a 356 bp IHHNV specific fragment using the primers 5'-ATC GGT GCA CTA CTC GGA 3' and 5' TCG TAC TGG CTG TTC ATC 3'. Other detection techniques used range from digital color correlation (Alvarez-Borrego and Chavez-Sanchez, 2001) to real-time PCR in either single (Tang and Lightner, 2001) or duplex assays (Dhar *et al.*, 2001).

Application of PCR primers (IHHNV3065F 5' GAC GAC GAA GAA TGG ACA GA 3' and IHHNV3065R 5' TGC CTG GGT AGC TGG TAT GTA TA 3') to amplify and sequence 3 kb fragments (i.e. approximately 75 percent of the genome) from geographical specimens of IHHNV-infected shrimp (Tang *et al.*, 2003b) revealed that IHHNV of Hawaii was virtually identical to that of the Philippines but that it differed from IHHNV of Thailand and Taiwan by approximately 4 percent and from that of Tanzania and Madagascar by 8 percent and 14 percent, respectively. Crude viral extracts of IHHNV-positive shrimp from Madagascar did not cause infections in experimentally infected *P. vannamei* while similar extracts from Thailand did (Tang *et al.*, 2003b). The study was based mostly on one sample from each country, so we still have little idea of the regional and global variation in what might be called the IHHNV complex. Clearly, histopathological analysis alone is not sufficient to characterize viruses in the IHHNV complex. For example, IHHNV-like lesions described in shrimp from Australia (Owens *et al.*, 1992) did not react with a DNA probe developed from Hawaiian IHHNV, suggesting that these two types might also differ by 10 percent or more in DNA sequence (Owens,

1997). Indeed, more recent work has shown that primer modification is required to detect Australian IHHNV and that it resembles most closely the type described from Madagascar (Krabsetsve *et al.*, in press). Obviously, more analysis is needed to determine the range of variation and any potential impact on the shrimp industry.

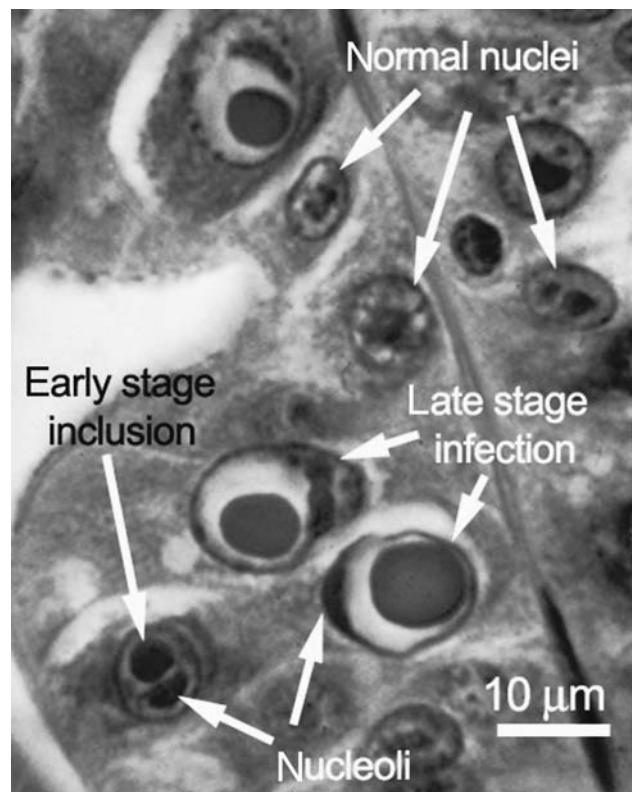
A recent publication (Tang *et al.*, 2003a) has shown that *P. stylirostris* pre-infected heavily with IHHNV is markedly protected from mortality upon subsequent challenge with WSSV, the most serious threat to the global shrimp farming industry today (Flegel and Alday-Sanz, 1998). Similar tests with *P. vannamei* were not protective, in spite of heavy infections. It has also been shown that selected strains of *P. stylirostris* are refractory to IHHNV infection (Tang *et al.*, 2000). Understanding the mechanism behind these phenomena could lead to new methods for control of shrimp viral diseases. It has been suggested in earlier publications (Flegel and Pasharawipas, 1998; Flegel, 2001) that shrimp and perhaps all arthropods may have the capability to accommodate viral pathogens in an adaptive manner. Certainly, IHHNV can often occur as heavy infections in *P. monodon* with few lesions, no inflammatory response, and no measurable negative impact (Flegel, 1997).

## HEPATOPANCREATIC PARVOVIRUS (HePV)

HePV was first described from farmed marine shrimp in Singapore (Chong and Loh, 1984). A second virus causing similar histopathology was later described by Lightner and Redman (1985). Like IHHNV, HePV is a typical densovirus with non-enveloped icosahedral virions averaging 22–23 nm in diameter and containing linear ssDNA. Two types have been characterized at the molecular level. One originated from *P. chinensis* from Korea (Bonami *et al.*, 1995) and the other from *P. monodon* in Thailand (Sukhumsirichart *et al.*, 2002), and these differed in total genome length (approximately 4 and 5 kb, respectively).

According to Lightner (Lightner, 1996b), HePV may have been of Indo-Pacific origin but later spread to wild shrimp in the Americas via importation of live Asian shrimp for aquaculture. It is now considered worldwide in distribution. Although HePV does not appear to be highly lethal, it has been associated with retarded growth and this can cause considerable economic loss for shrimp farmers (Flegel *et al.*, 1999, 2004). Like IHHNV, it is curious that even heavy infections result in no visible inflammatory response (Flegel, 2001).

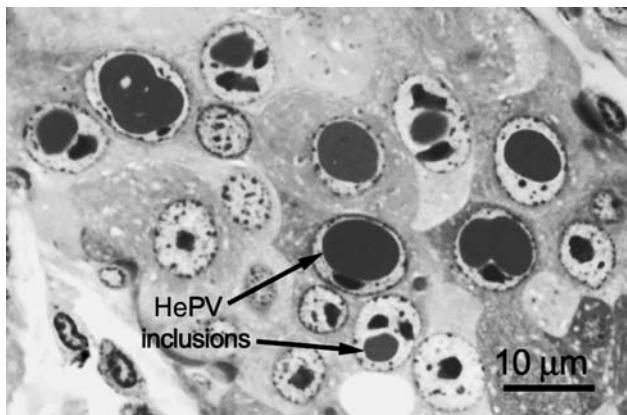
Previously, experimental work on HePV was hampered by the lack of an experimental transmission model. However, successful experimental infections by oral challenge in post-larvae of the black tiger shrimp *P. monodon* have recently been reported (Catap *et al.*, 2003) and this should open the way for precise analytical tests on the effects of HePV infection in shrimp.



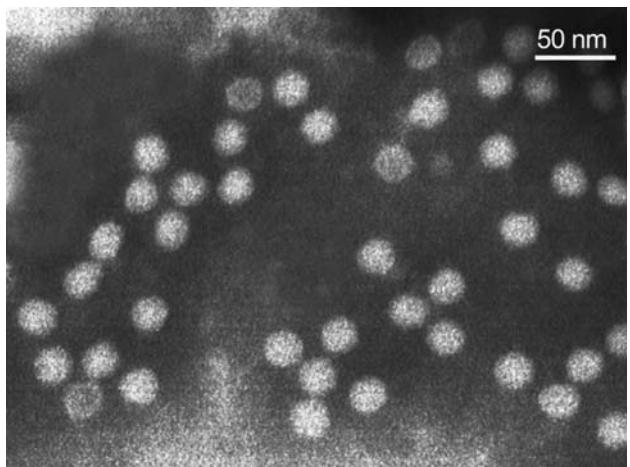
**Figure 35.3** Histopathology of hepatopancreatic parvovirus (HPV) in hepatopancreatic tubule epithelial cells of *Penaeus monodon* from Thailand. The basophilic intranuclear inclusions accompanied by a marinated nucleolus and chromatin are typical for all types of HePV reported from shrimp. Note that the early stage inclusion is eosinophilic rather than basophilic. See also Color Plate 35.3.

Lightner (Lightner, 1996b) reported that F1 progeny of *P. chinensis* (also called *Fenneropenaeus chinensis* or *P. orientalis*) held in quarantine in Hawaii developed HePV infections, suggesting that transmission could be vertical from parental broodstock. Recent reports of HPV infections in hatchery larvae in India (Manivannan *et al.*, 2002; Umesha *et al.*, 2003) support this contention, but do not correspond to the experience in Thailand where infections have been reported only from post-larvae moved to outdoor nurseries (Flegel *et al.*, 1999). In any case, the recently successful experimental transmission of HePV by feeding uninfected with infected post-larvae suggests that horizontal transmission by cannibalism will likely turn out to be the most serious problem for shrimp farmers.

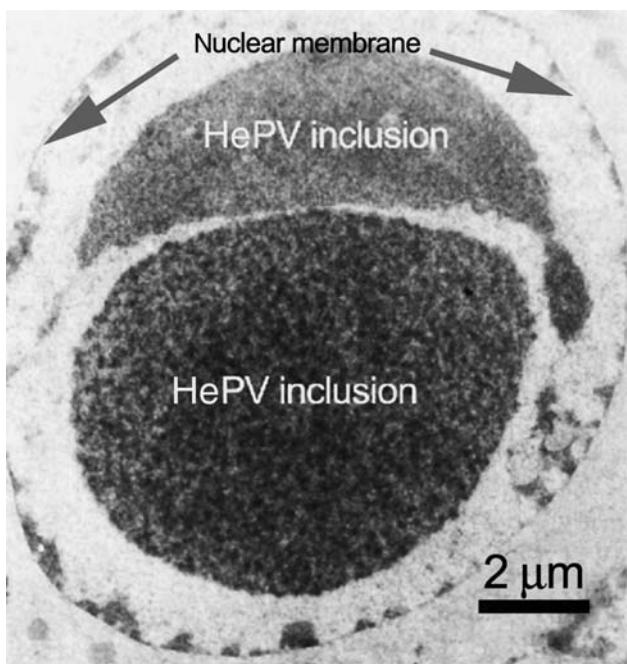
Since gross signs of HePV infection are not distinctive, diagnosis is based primarily on the presence of pathognomonic lesions that show basophilic inclusions contained within enlarged nuclei of hepatopancreatic tubule epithelial cells (Figures 35.3 and 35.4) and sometimes cells in the adjacent mid-gut (Lightner, 1996b), i.e. only cells of endodermal origin. The highest density of lesions occurs in actively dividing cells (E-cells) at the distal ends of hepatopancreatic tubules. Examination with the electron microscope reveals



**Figure 35.4** Semi thin section of hepatopancreatic tissue showing hepatopancreatic parvovirus (HePV) histopathology in *Penaeus monodon* from Thailand. Note that some nuclei have two nucleoli or what appear to be two or more HePV inclusions that have fused together. See also Color Plate 35.4.



**Figure 35.6** High magnification of semi-purified hepatopancreatic parvovirus (HePV) virions from *Penaeus monodon* in Thailand.



**Figure 35.5** Low magnification TEM of an hepatopancreatic parvovirus (HePV) infected nucleus from hepatopancreatic tissue of *Penaeus monodon* from Thailand showing two HePV inclusions with virions oriented at different angles to the electron beam.

the presence of unenveloped, icosahedral viral particles measuring 22–24 nm in diameter (Figures 35.5 and 35.6). Based on these types of analyses, many penaeid shrimp species have been reported to be infected with HePV (Lightner, 1996b). A virus with similar histopathology has also been described from *Macrobrachium rosenbergii* (Lightner *et al.*, 1994).

Because histopathological analysis by normal methods (Lightner, 1996b), rapid methods (Lightner *et al.*, 1993), or transmission electron microscopy (TEM) (Pantoja and Lightner, 2001) is a destructive process, several rapid molecular tools have been developed to allow PCR testing of broodstock specimens and pond-reared shrimp using only small tissue or fecal samples that leave the shrimp unharmed (Sukhumsirichart *et al.*, 1999, 2002; Pantoja and Lightner, 2000; Phromjai *et al.*, 2002). These methods also make it easier to screen a wide range of potential hosts and life stages for the presence of the virus even at low levels of infection. Some of these may turn out to be important reservoirs of concern to shrimp farmers.

Despite the similarity in histopathology and ultrastructural morphology, use of molecular probes and DNA sequence analysis has shown that there is significant variation in geographical isolates of HePV. For example, the genome size of HePV from *P. chinensis* (HePVchin) in Korea and HPV from *P. monodon* (HePVmon) in Thailand differ by approximately 1 kb (Sukhumsirichart *et al.*, 1999) and the DNA sequence identity is only 70 percent, despite the fact that both give positive *in situ* hybridization reactions with a commercial probe designed from HePVchin (Phromjai *et al.*, 2001). Partial sequences for HePVchin (AY008257) and HePVmon (AF456476) are available at GenBank. The DNA probe developed from HePVchin also gives a positive *in situ* hybridization signal for HePV from Australian penaeids (*P. merquiensis* and *P. esculentis*) (Owens, 1997) but reference to the situation with HePVmon suggests caution in inferring high genomic sequence identity. In contrast to the penaeid shrimp, the HePVchin probe does not give positive *in situ* hybridization results, with *M. rosenbergii* showing HePV-type histopathology (Lightner *et al.*, 1994).

Because of the difference in DNA sequence between HePVchin and HePVmon, it is recommended that different

PCR primers be used for their detection. The commercial primer pair for HePVchin (5' TGG AGG TGA GAC AGC AGG 3' and 5' CCA ACT GTC CTC GCT CTT 3' from Diag-Xotics Co. Ltd, Wilton, CT) gives a 350 basepair amplicon with HePVchin at high sensitivity but a 732 bp amplicon at lower sensitivity with HePVmon (Phromjai *et al.*, 2002). A specifically designed pair for HPVmon (5' GCA TTA CAA GAG CCA AGC AG 3' and 5' ACA CTC AGC CTC TAC CTT GT 3') yields a 441 bp amplicon at high sensitivity (Phromjai *et al.*, 2002) and it was effective with HPV in *P. monodon* from India, while another pair designed for HePVchin (5' GGT GAT GTG GAG GAG AGA 3' and 5' GTA ACT ATC GCC GCC AAC 3') was not (Umesh *et al.*, 2003). This evidence suggests that Indian HePV is more closely related to Thai HePV than to Korean HePV, but the degree of similarity needs to be determined by genome sequence comparisons. In any case, we may conclude as with IHHNV that HePV is likely to occur as a complex of types and that more work is needed to assess the variety and impact on shrimp farming.

## SPAWNER-ISOLATED MORTALITY VIRUS (SMV)

Compared with IHHNV and HePV, much less is known of SMV. Fraser and Owens (1996) discovered this parvovirus in Australia from a disease outbreak that caused high mortality in *P. monodon* broodstock being held in a maturation facility. There was no clearly distinctive pathognomonic histopathology, but there were some enlarged nuclei with marginated chromatin suggestive of IHHNV. On the other hand, there was no hybridization with commercial probes for IHHNV (Owens, 1997) and the reason for this was eventually apparent from the low comparative sequence identity with the partial SMV genome sequence (GenBank AF499102). Subsequent TEM work revealed the presence of 20 nm viral particles around the nuclei of midgut cells. A similar virus was later found associated with mid-crop losses in farmed *P. monodon*, but often in the presence of other viruses, so that direct causative effects were difficult to assess (Owens, 1997). Because of the lack of distinctive pathology, molecular diagnostic tools or TEM were required to confirm infections (Owens *et al.*, 1998). Owens and McElnea (2000) have also detected SMV in Australian crayfish *Cherax quadricarinatus* using molecular methods, and they have suggested that further work is needed to determine whether the virus moved from crayfish to shrimp or *vice versa* or whether it has always been present in both. Because of uncertainty regarding its carrier status for SMV and other viruses, they cautioned against translocations of live crayfish until this could be confirmed. Indeed, the propensity to carry dual and multiple viral infections without gross signs of disease suggests that the precaution should apply to translocations of all live crustaceans (Flegel and Fegan, 2002).

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PART  
**4**

# Use of Parvoviruses as Gene Therapy and Vaccine Delivery Vectors

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# Adeno-associated virus vectors and biology of gene delivery

BARRIE J. CARTER

The chapters in this section of the book describe a remarkable expansion in studies of adeno-associated virus (AAV). From early work mostly with AAV2, there has been an extraordinary expansion of work driven by two major themes: one focused on the biology and molecular biology of AAV in its own right and a second theme driven by the development of AAV vectors. This second track in particular, given the initial evidence of gene expression and safety with AAV vectors, has drawn a large crowd of investigators. The development of vectors, especially for therapeutic applications, has both necessitated greatly expanded studies of AAV biology and in turn provided more sophisticated tools to enhance these investigations. The two themes of studies of AAV vectors and AAV biology per se are inextricably intertwined and have combined to generate a highly sophisticated and growing field of basic science of AAV and applied technology – this is expanded upon more fully in the following chapters.

The basic concept of treating disease by gene delivery is to deliver a nucleotide sequence to a target cell in order to modify the function of that cell directly or to express a secreted protein that will correct the aberrant behavior of other cells. The design and development of all gene delivery systems, including AAV vectors, must be guided and informed by the biology of the disease, the biology of the target cell and the biology of the delivery system. Considering these in the reverse order, the most relevant biology of AAV in the context of vector development is the ability of AAV genomes or vector genomes to persist in cells, most readily as an episome, essentially for the lifetime of the cell. Because of the ability for the vector genome to persist in an unintegrated state, the ideal cellular targets for AAV vectors are cells that are terminally differentiated and non-dividing or turning over only slowly. Consequently, AAV vectors may be particularly suited to potential treatments of chronic diseases in which gene expression will probably be required for a prolonged period and, ideally, delivery should be infrequent. Thus, the current applications to which AAV vectors

are being directed in clinical trials reflect these considerations (see Chapter 37).

Some ideal cellular targets for AAV vector delivery in which cells are terminally differentiated but long lived include muscle, liver (see Chapter 42), brain and retinal pigment and photoreceptors in the eye (see Chapter 43). In addition, airway epithelial cells, which turn over relatively slowly and are easily accessible, are being targeted in the context of cystic fibrosis (see Chapter 37). In some cases, the target tissue is the actual site of the disease, such as lung for cystic fibrosis and brain for various central nervous system (CNS) diseases, but in others it is simply a convenient location to express a secreted protein. Examples of the latter such applications that are currently in clinical trials include treatment of hemophilia B by secretion of factor IX from either muscle or liver, and treatment of hereditary emphysema by secretion of alpha-1-antitrypsin from muscle. In addition, persistent expression of HIV antigens from muscle is being examined for development of vaccines.

Development of AAV vector biology has been of critical importance to the therapeutic development of AAV vectors. There have been many new insights into the mechanism of transduction by AAV vectors and into metabolism of the vector genomes once they are in the cell nucleus. The mechanism of transduction involves binding to receptors on target cells, cellular entry, and trafficking and conversion of the single-strand vector genome to a duplex molecule that can function as a transcription template. All of these issues may play important roles in the ultimate efficiency of transduction by AAV vectors. Studies in this area have provided important insights into specific receptors and co-receptors used by AAV and to important interactions with the cytoplasmic ubiquitin-proteosome pathway for protein modification. Also, there is accumulating evidence that nuclear entry and unpackaging of the genome may involve rate-limiting steps. Our knowledge of the mechanism of transduction by AAV vectors is discussed in Chapter 38. Another highly important observation is that the infecting single-strand

genome is metabolized to generate duplex, circular concatameric molecules that generally persist as episomes, rather than being integrated into the host cell genome. The metabolism of AAV vector genomes and studies on persistence and integration are discussed in Chapter 16 in Section 1.

The advances in understanding the mechanism of transduction by AAV vectors and the metabolism of vector genomes are collectively leading to increased sophistication in the development of AAV vectors. Thus these studies of AAV vector biology are providing opportunities for more judicious design of AAV vectors having potentially increased transduction efficiency, increased specificity in cellular targeting, and even an increased payload capacity.

The AAV capsid plays an important role in determining the transduction efficiency of AAV vectors. Many studies have now shown that capsids of different AAV serotypes can mediate transduction of various cell types with very different efficiencies. Some of these differences reflect those in cellular trafficking as well as differential usage of cellular surface receptors and co-receptors among serotypes. As we accumulate information on the atomic structures of AAV capsid and knowledge of the various receptor interactions, it may become feasible to target specific cell types with a greater degree of precision. In addition, increasing knowledge and understanding of the arrangement of the capsid is enabling modification of the capsid sequence by mutation, or by insertion of new ligands, to modify, or even retarget, AAV capsids to recognize different receptors and target cells. The properties of AAV serotypes are discussed in Chapter 13 and progress in the tailoring of AAV capsids is described in Chapter 40.

The structure of the AAV capsid plays one important limiting role in the design of the payload for AAV vectors. However, in Chapter 38, Engelhardt describes how the formation of multimeric concatamers by AAV vector genomes may be used to increase the payload capacity of AAV vectors through the use of a dual vector strategy. The payload capacity of a single AAV particle is about 4.5 kb of transgene sequence, but this can effectively be doubled if a gene sequence, cDNA or expression cassette is divided between two AAV vectors. Mixed infection with both vectors then allows the complete sequence to be reassembled via concatamer formation in the host cell nucleus.

The cellular targeting and trafficking depends primarily on the sequence and conformation of AAV capsid and the

size of the payload is limited by the capsid. However, the packaging of vector DNA into AAV capsids relies on interaction with nucleotide sequences in the AAV inverted terminal repeat (ITRs).

An emerging field for AAV vectors, but one of potentially general importance for gene therapy is the concept of using gene delivery to directly correct a mutated gene as opposed to the more indirect method of supplying a correct version of the gene. This may be important for obtaining high precision in targeting and regulating some gene therapies. Also, it may be an important approach for some genetic disease caused by dominant mutations. Because the incoming AAV vector genome is linear and single stranded, it may offer some unique recombinogenic properties for application in targeting for direct gene correction and this is discussed in Chapter 41.

The ultimate success of therapeutic applications of AAV vectors will depend on the appropriate combination of AAV biology, target cell biology and disease biology, and the demonstration of safety and efficacy in clinical trials. However, licensing of any AAV vector as a therapeutic product will require manufacturing of such a product under very stringent conditions. Not only must any manufacturing process be scalable, reproducible, and commercially viable in terms of cost of goods, but it must be carried out under very stringent conditions of cGMP (current good manufacturing practice) as defined by the US Food and Drug Administration or similar such regulatory bodies in other countries. These requirements are to ensure safe and reproducible manufacture and quality control for useful therapeutic products and commercially scalable systems are now becoming available for AAV vector production. The fundamental process for AAV vector production was provided by the molecular cloning of infectious AAV genomes and the provision of a genetic complementation system for vector production. From this initial work, a variety of strategies for vector production are possible.

Overall, the development of AAV vectors is proceeding at an ever-increasing pace. This rapid increase in the basic science studies and the expanding clinical development of AAV vectors provide an exciting environment to expand basic science and to apply this technology to the goal of providing therapeutic solutions to unmet medical needs.

# Clinical development with adeno-associated virus vectors

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Adeno-associated virus (AAV) vectors have a number of advantageous properties as gene delivery vehicles. The parental virus does not cause disease. AAV vectors are the smallest and most chemically defined particulate gene delivery system and potentially could be classified as well characterized biologics for therapeutic applications. AAV vectors contain no viral genes that could elicit undesirable cellular immune responses and appear not to induce inflammatory responses. The primary host response that might impact use of AAV vectors is a neutralizing antibody response. The vectors readily transduce dividing or non-dividing cells and can persist essentially for the lifetime of the cell. Thus, AAV vectors can mediate impressive long-term gene expression when administered *in vivo* particularly in terminally differentiated cells, which may be the preferred cellular targets.

Consequently, these vectors may be well suited for clinical applications where the vector is delivered infrequently and where any potential host antibody response to the AAV capsid protein may be less inhibitory. However, potential humoral immune responses against the viral capsid, either pre-existing in the human population or induced by vector administration, must be considered (Blacklow, 1988). Re-infection of humans by AAV is not prevented by serum neutralizing antibodies (Blacklow, 1988) but more extensive studies will be required to assess the extent to which induction of neutralizing antibody responses may pose limitations to AAV vectors as therapeutics (Carter *et al.*, 2004). In this respect, the route of delivery of the vector may play an important role. For instance, immune responses are greatly reduced following airway administration of AAV (Hernandez *et al.*, 1999) and vector transduction could be

seen after several repeated administrations to the lungs of rabbits and rhesus macaques (Beck *et al.*, 1999; Fischer *et al.*, 2003). It remains to be determined how neutralizing antibody responses to AAV vector capsids might impact applications of AAV vectors. This will most likely require studies in humans to determine if the various animal models such as rodents or rabbits are predictive for the immune response to AAV vectors in humans and whether such immune responses will pose any limitations to their therapeutic application.

The lack of good production systems that could generate high titer vectors was an early obstacle to development of AAV vectors, but this has been overcome through significant advances in both upstream production and downstream purification of AAV vectors. In concert with the advances in AAV vector production, clinical development of AAV vectors has progressed significantly and studies of an AAV vector in cystic fibrosis patients (Wagner *et al.*, 1999a, 2002; Aitken *et al.*, 2001; Flotte *et al.*, 2003) have been extended to Phase II trials (Moss *et al.*, 2004). A series of other AAV vectors have now entered phase I clinical trials for at least eight other clinical indications including genetic, neurologic, and inflammatory diseases as well as cancer, and as a vaccine for an infectious disease, HIV.

This chapter will discuss only AAV vectors that have actually entered into clinical trials, although several more will likely begin in clinical trials in the near future. All of the examples to be discussed are chronic diseases that affect various organ systems and require different routes of delivery. These examples serve to illustrate the potential wide applicability of AAV vectors particularly for persistent gene expression in non-dividing or slowly dividing cells. With

one exception, all of the clinical trials currently use AAV vectors of serotype 2. AAV2 vectors were the first type to be studied and thus have reached a more advanced stage of development. It is likely, as the comparative biology of other AAV serotypes is extended, that vectors of other serotypes will be advanced into clinical development.

## GENETIC DISEASE

Two genetic diseases in which loss of pulmonary function is a major component have been developed as targets for gene therapy. First, cystic fibrosis (CF) was the first clinical indication for which any AAV vector entered into clinical trials and there is now extensive experience with delivery of a gene therapy for this disease by the pulmonary route. Very recently, a phase I clinical trial has been initiated to target pulmonary disease from a deficiency of alpha-1-antitrypsin deficiency, but in this case the gene therapy is delivered by an intramuscular route.

### Cystic fibrosis

CF is a lethal autosomal recessive disease that is caused by a mutation in the cystic fibrosis transmembrane regulator (CFTR) gene. The defect in the chloride ion channel expressed from the CFTR gene in epithelial cells causes biochemical changes in several organs, especially lung and the exocrine pancreas. The defect in the exocrine pancreas can be managed via current therapy with enzyme supplements. Morbidity and mortality in CF patients primarily results from progressive loss of lung function. This is caused by a complex cascade of events that include decreased mucociliary clearance, increased bacterial colonization and a chronic neutrophil-dominated inflammation that leads to progressive destruction of tissue in the conducting airways. The current treatments for the pulmonary aspect of the disease are directed at treating the symptoms including physiotherapy for clearance of mucus, Pulmozyme (DNase) to degrade the DNA component of the mucus, and antibiotics such as TOBI (inhaled tobramycin) for treatment of infections. Whereas these treatments address the symptoms of the airway disease, a gene therapy offers the possibility of delivering a functional copy of the CFTR gene and thus addressing the underlying cause of the disease in the lung. CF is the first disease indication for which an AAV vector was introduced into the clinic and is the most advanced clinical development program with any AAV vector. Over 170 CF patients have been enrolled in clinical trials of an AAV-CF vector, a number that is significantly more than all the patients treated in the clinical trials of the other eight AAV vectors. Because CF was the first clinical target for an AAV vector, the development program has been reflective of this in the very cautious and

careful collection of safety data in early trials using a variety of target organs in addition to lung.

CF is an attractive target for a gene therapy. There is a clear unmet medical need for an effective treatment of the lung disease and the lung is a directly accessible organ to which therapeutics may be delivered by non-invasive means such as an inhaled aerosol. Consequently, the goal of gene therapy for CF is to deliver a CFTR cDNA to the epithelial cells that line the lumen of the conducting airways of the lung. These cells are terminally differentiated and thus are a good target for an AAV vector but they do turn over with a half-life of several months. This means that an AAV gene therapy may be delivered at relatively infrequent intervals but it will require repeated delivery. Thus, one important goal of the clinical trials with an AAV-CF vector has been to determine the safety of repeated delivery.

There was an important technical challenge in designing an AAV vector to express CFTR, which is a protein of about 170 000 in molecular weight. Expression of the CFTR protein requires a minimum coding region of 4400 nucleotides and, together with the 300 nucleotides required for the two ITRs, this is equal to the 4.7 kb thus leaving very little space for a transcription promoter. In order to package this cDNA into a single AAV vector, advantage was taken of the serendipitous discovery of the transcription promoter properties of the AAV inverted terminal repeat (ITR) (Flotte *et al.*, 1993a) and this provides the transcription promoter for the AAV-CF vector currently in clinical trials. This vector, tgAAV-CF, has the 4.4 kb CFTR cDNA inserted between the AAV2 ITRs and is packaged in an AAV2 capsid (Flotte *et al.*, 2003a). The strength of the AAV2 ITR as a transcription promoter is modest but this may suffice because the steady state amount of CFTR mRNA in human airway epithelial cells is about one copy per cell.

An alternative possibility to solve the vector design challenge would be to delete part of the CFTR gene and thus provide space for a larger and presumably stronger transcription promoter. However, this has been deemed inadvisable for clinical applications because the CFTR protein performs a complex role, and in addition to functioning as a chloride channel, it closely regulates a second chloride channel, the outward rectifier channel, and also regulates the epithelial sodium channel (ENaC). Thus it is still not precisely known which parts of the protein might be deleted while still retaining the potential to correct the pathophysiology of CF disease.

The toxicity of the tgAAV-CF vector was examined following delivery directly to the lungs of rabbits and rhesus macaques. In both species, the vector persisted and expressed for up to 6 months but neither short-term nor long-term toxicity was observed after gross morphological and histopathological examination of organ tissues, and there was no indication of T-cell infiltration or inflammatory responses (Flotte *et al.*, 1993b; Conrad *et al.*, 1996). In rhesus macaques (Conrad *et al.*, 1996), no toxicities were

observed in many additional tests including pulmonary function, radiological examination, blood gas analysis, and cell count and differential in bronchoalveolar lavage fluid. Analysis of biodistribution of the tgAAV-CF vector following pulmonary delivery in rabbits and macaques showed that there was minimal spread of vector to organs outside of the lung including the gonads (Conrad *et al.*, 1996). However, use of different delivery routes for AAV vectors may lead to more extensive biodistribution as described below.

The potential of the vector to be shed or mobilized from a treated individual was examined in rhesus macaques (Afione *et al.*, 1996). tgAAV-CF particles were delivered to the lower right lobe of the lung and 10 weeks later a high dose of both adenovirus and wild-type AAV (wtAAV) particles was administered to the nose of the animals. In an even more stringent test, the wtAAV vector was instilled directly in the lung 4 weeks prior to instillation of the vector followed by the intranasal application of adenovirus 10 weeks later. Little or no mobilization of the vector was observed, which suggested that the probability of vector shedding and transmission to others is likely to be low. Interestingly, this study showed that the AAV vector persisted at 3 months in the rhesus lungs as episomal dimeric molecules and was the first *in vivo* observation that AAV vectors may persist as unintegrated concatameric episomes. The favorable safety profile of the tgAAV-CF vector observed in these preclinical studies has been predictive of the safety profile observed in clinical trials of this vector in CF patients.

The initial clinical trial of the AAV-CF vector in CF patients employed a bronchoscope to deliver doses of the vector to a single lobe of the lung (Flotte *et al.*, 2003). The trial was designed as an open-label, dose-escalation study beginning at extremely low doses of  $6 \times 10^4$  DNase-resistant particles (DRP) and progressing cautiously through a series of half-log increments. This caution reflected the fact this was the first time an AAV vector had been introduced into clinical trials. The doses were administered to the superior segment of the lower lobe of the right lung and to one side of the nose. In a total of 25 patients treated with doses of up to  $10^{12}$  particles the administration of the vector was safe and well tolerated. In the early dose cohorts the patients were all adults aged 18 years or older but in the second part of the trial, based upon the good safety results, patients aged as young as 15 years were enrolled. At the highest doses there was some neutralizing antibody response to the AAV capsid that was detected in serum. Because of the concern by regulatory agencies for possibly high levels of shedding of vector from patients, the earliest subjects were treated as inpatients and retained in relative isolation for a period. This requirement was eliminated after it was shown that vector shedding in body fluids, including sputum, broncholaveolar lavage, nasal washes, urine, and stool, was minimal. Vector genomes were detected by DNA polymerase chain reaction (PCR) from airway cells, obtained by inserting a small brush through a bronchoscope, or from nasal cells

obtained by scraping the nasal epithelium. After administration to the nasal epithelium an attempt was made to observe functional effects of the vector by measurement of nasal transmembrane epithelial potential difference (TEPD) because this is a direct measure of the CFTR chloride channel function. However, this was not useful in assessing function of the vector because the nasal epithelium is relatively resistant to infection by viruses, including AAV2, as was borne out by the DNA PCR analysis.

Although the lung is the target for a therapeutic gene therapy for CF, delivery to the maxillary sinus was undertaken as a novel attempt to obtain an early indication of the potential of the AAV CFTR vector. The maxillary sinuses of CF patients also exhibit chronic inflammation and bacterial colonization and the sinus epithelium is more similar to the small airway epithelium in the lung than is the nasal epithelium. In CF patients who have undergone surgical bilateral antrostomy, the maxillary sinus is accessible to direct instillation of vectors and for sampling and biopsy. This also permits instillation of electrodes in order to measure TEPD in the sinus. An initial phase I, open label, dose escalation trial of tgAAV-CF was conducted in the 15 sinuses of CF patients (Wagner *et al.*, 1999a). DNA PCR analysis of sinus biopsies showed dose-dependent delivery of the vector genome that persisted in the sinus for at least 70 days after instillation. Similarly to the nasal epithelium, in the sinus epithelial cell surfaces of CF patients, the transmembrane potential is hyperpolarized compared with normal patients because of the absence of a functional CFTR chloride channel. In the treated sinuses at the higher doses of vector there was some reversal of the electrophysiological defect, which provided suggestive evidence for expression of the CFTR protein from the delivered vector.

A follow-up, double-blinded, randomized, placebo-controlled Phase II study was then performed in 23 adult CF patients to whom vector was administered in one maxillary sinus, while the contralateral sinus received a placebo treatment (Wagner *et al.*, 2002). This study confirmed that delivery of tgAAV-CF to the sinus was safe and well tolerated. An important observation in these trials was that the anti-inflammatory cytokine interleukin-10 showed a significant difference between vector- and placebo-treated sinuses over a 90-day period and there was also a decrease in the pro-inflammatory cytokine, IL-8 (Wagner *et al.*, 1999b). Inflammation in the CF lung is characterized by elevated levels of IL-8 and decreased amounts of IL-10 so the results suggested that gene transfer could modulate levels of cytokines and this may provide a useful surrogate marker for additional trials. Several patients participated both in the previous phase I trial and the phase II study, which further suggested that the vector remained safe after multiple administrations to the sinus without induction of serum neutralizing antibodies (Wagner *et al.*, 2002). The absence of serum neutralizing antibody responses may also have reflected the lower doses than in the pulmonary delivery.

On the basis of these preliminary trials, a phase I, single-administration, dose-escalation trial designed to assess safety and delivery of AAV-CFTR by inhaled aerosol to the lung was carried out in 12 adult CF patients exhibiting mild lung disease. The vector was well tolerated and no apparent safety concerns were demonstrated in the study. Vector administration at the highest dose of  $10^{13}$  DRP resulted in significant gene transfer to the airway as measured by DNA PCR analysis of DNA extracted from airway cells recovered by endoscopic brushing. A clear dose-response relationship was observed in vector gene transfer over 30 days, although the vector DNA declined over 90 days (Aitken *et al.*, 2001). Again at the highest dose there was some neutralizing antibody response to AAV2 capsid detected in the serum but not in bronchial wash fluid. The level of serum antibody was not correlated with the degree of gene transfer, adverse events, or any other endpoints that were measured. In this trial no useful measurements of pulmonary function, inflammation, or microbiology could be obtained because these parameters are significantly perturbed by three or four invasive bronchoscopies that all the subjects underwent over a 3-month period to obtain samples for analysis. Nevertheless, this trial did establish a safe dose of up to  $10^{13}$  DRP, administered by aerosol, at which gene transfer could readily be measured and this was used as the basis for a phase II trial of repeated administration.

To extend the safety analysis of tgAAV-CF, a multidose, double-blinded, placebo-controlled and randomized Phase II trial was conducted in 37 CF patients with mild CF disease defined as a forced expired volume (FEV<sub>1</sub>) of  $\geq 60$  percent predicted. Patients received three doses ( $10^{13}$  DRP per dose) of the vector or matching placebo at monthly intervals, administered by inhaled aerosol, and were followed for a total of 150 days (Moss *et al.*, 2004). The trial was also conducted under the periodic oversight of an independent data safety monitoring board (DSMB), which enabled enrollment of patients as young as 12 years. This is important because the eventual target population to treat CF is likely to be younger patients. The primary endpoint of this trial was the safety of repeat delivery and this was clearly met (Moss *et al.*, 2004) 20 subjects received at least one dose of tgAAV-CF and 17 received placebo and no differences were noted in the pattern of adverse events or laboratory abnormalities between the two treatment groups. As well as the good safety and tolerability profile, there were suggestions of clinical effects in the short term based on surrogate measures of pulmonary function and the sputum inflammatory cytokine IL-8. This study was not powered for efficacy but various established and exploratory outcome measurements were employed.

The traditional efficacy outcome measure for new CF therapies is spirometry to measure pulmonary function and in particular the absolute or percent predicted values of FEV<sub>1</sub> (the forced expired volume of air in the first 1 second of an expiration cycle). In the phase II trial (Moss *et al.*, 2004), a significant improvement in FEV<sub>1</sub> was observed

in subjects receiving tgAAV-CF compared with placebo at 30 days ( $P = 0.04$ ) and generally higher spirometric values were observed through day 90 but these did not reach statistical significance, perhaps owing to lack of statistical power. At day 30, five patients (25 percent) had FEV<sub>1</sub> increases between 10 percent and 20 percent. Also, subjects receiving tgAAV-CF showed a significant decrease in the level of IL-8 in sputum compared with placebo that may have persisted, but the trial lacked power to show this at the later time points. This trial was likely successful in detecting these changes because it was designed to avoid any invasive bronchoscopy until at least day 90 after all the other measurements had been made and only a subset of patients (six treated and 2 placebo) were then subjected to this procedure. Robust gene transfer as measured by detection of DNA by PCR from brushed cells, at a level of 30–100 vector genomes per cell, but gene expression measured by RNA PCR was not successfully detected. Five of these six treated subjects had antibody titers in their bronchoalveolar fluid, and all subjects who received three doses of vector had at least a 4-fold increase in serum neutralizing antibody. *Post-hoc* subgroup analysis did not reveal any differences between male and female subjects, frequency of homozygosity for the common deltaF508 mutation in CFTR, or any relationship between levels of AAV capsid antibody titer either pre- or post-treatment, although the study lacked statistical power for such analyses. The results of this trial (Moss *et al.*, 2004) are considered promising and a further phase II trial has been initiated that is adequately powered to detect the pulmonary function effects observed in this trial. This confirmatory trial now underway is designed to enroll up to 100 CF patients and has, as of mid-2004, enrolled half of these patients and enrollment has been continued at the recommendation of an independent DSMB.

The phase II repeat administration trial (Moss *et al.*, 2004) is the first trial in CF patients to show any indication that gene delivery may positively affect a pulmonary function measure. Other clinical trials in CF gene therapy have generally focused on measurement of molecular endpoints. However, molecular endpoint measures such as DNA PCR for gene transfer, RNA PCR, or protein assay for gene expression may be useful but it may be difficult to develop validated assays, and even more difficult to perform them in clinical trials than in animal models. For CF clinical trials, molecular endpoint measures have proven particularly challenging. There is no good assay for CFTR protein and, although DNA and RNA PCR can be conducted on airway cells that are obtained via a brush inserted through a bronchoscope, the samples are often of poor quality or contain very few cells. DNA PCR data has been obtained but it has proven extremely difficult to obtain reliable RNA samples. In the clinical trials of tgAAV-CF in lungs of CF patients, data on RNA expression have not been obtained. Another problematic issue as noted above is that the invasive bronchoscopy procedure disrupts other measures of pulmonary function and inflammation. Furthermore, the bronchoscopic

procedure can only provide samples from the upper airways whereas the vector needs to function in the lower airways that are the primary sites of the disease. In view of these considerations, measurement of pulmonary function by spirometry and analysis of inflammatory cytokines in sputum may be less invasive ways to obtain surrogate marker information and have the added advantage of reflecting events in the lower airways.

## Hemophilia

Hemophilia is a severe X-linked recessive disease that results from mutations in the gene for either blood coagulation factor VIII (FVIII) in hemophilia A or IX (FIX) in hemophilia B. The absence of either factor leads to severe bleeding diathesis with severe disease resulting from levels <1 percent of normal, and levels >5 percent of normal can provide normal function. Levels between 1 and 5 percent cause much milder disease and prophylactic delivery of the clotting factors at these levels (2–10 ng/ml and 50–250 ng/ml for FVIII and FIX, respectively) can decrease the risk of spontaneous bleeding into joints and soft tissues and lower the risk of fatal intracranial bleeding. However, the FVIII and FIX proteins have short half-lives and there is interest in developing gene therapy approaches in which the clotting factors may be produced more persistently. AAV vectors for hemophilia A have not entered the clinic because of the large size of the FVIII protein, but clinical trials have been conducted with an AAV2 vector expressing human FIX. FIX is made and secreted predominantly in the liver, but can be secreted from muscle in animal models and both routes have been tested clinically. For hemophilia clinical trials, in contrast to the CF trials, a molecular endpoint is readily available because the level of the FIX in serum can be directly measured and this will likely be related to the degree of clinical benefit. DNA or RNA measures again would require invasive biopsy, especially following portal vein delivery to the liver.

Preclinical studies showed that FIX protein can be expressed for prolonged periods, in both murine and canine models, after delivery of AAV2-human FIX vectors either by portal vein injection to target the liver or by intramuscular injection (Herzog *et al.*, 1997, 1999; Snyder *et al.*, 1999). As measured by the whole blood clotting time (WBCT) assay, a partial correction of the defect in both the murine and canine disease models was achieved. Delivery by portal vein injection into the liver gave prolonged expression of factor IX at serum levels about one-fifth of the normal human level in mice and at ~1 percent of normal canine levels in hemophiliac dogs and sustained partial correction of WBCT for at least 8 months. Intramuscular injection of an AAV human FIX vector gave prolonged expression for at least 6 months in, and at high doses of vector in hemophiliac dogs, expression persisted for over 17 months and with a stable, dose-dependent partial

correction of the WBCT and also a partial correction of the activated partial thromboplastin time (APT).

A clinical study of intramuscular injection of an AAV vector expressing human FIX in adults with severe hemophilia B was recently concluded (Kay *et al.*, 2000; Manno *et al.*, 2003). This was a phase I open-label dose-escalation trial in a total of eight adult men with severe hemophilia B from a missense mutation in FIX and with serum levels of FIX < 1 percent of normal. Three dose levels of vector,  $2 \times 10^{11}/\text{kg}$  ( $n = 3$ ),  $6 \times 10^{11}/\text{kg}$  ( $n = 3$ ) and  $1.8 \times 10^{12}/\text{kg}$  ( $n = 2$ ) were administered, under ultrasound guidance to avoid puncture of large blood vessels, by injection at multiple sites in muscles of one or two legs. The treatment was generally well tolerated and there were essentially no vector-related toxicities in any of the three dose cohorts. Analysis of biodistribution in body fluids showed very little extramuscular distribution of vector and importantly no vector was detected in any semen sample over 24 weeks of the study. Analysis of muscle biopsies detected both the vector DNA and vector-specific mRNA as well as accumulation of FIX at the injection sites. Although all treated patients showed elevation of anticapsid antibody levels in serum, the levels of gene transfer and expression in muscle did not correlate with either pre- or post-treatment levels of AAV capsid antibody. Importantly, there was no formation of inhibitory antibodies to FIX. However, the levels of circulating FIX in serum were disappointing and only four of eight patients showed any detectable level at any time point over the 24 week period, and these were generally at the level of 1–1.4 percent. There was no dose response. There were no significant changes in clinical endpoints, although the first patient treated at the lowest dose reported a 50 percent decrease in the frequency of self-administration of FIX protein. The intramuscular route may not be preferred for several reasons. Although the muscle is more accessible and may be a safer route in hemophilia patients, who generally have compromised livers due to viral infection, the dose required by the intramuscular route appears to be extremely high, above that used in this trial, and requires injection at multiple sites (up to 100), which is impractical. In addition, for full biological activity, FIX protein must be modified post-translationally by  $\gamma$ -carboxylation and this does not occur as efficiently in muscle as in liver where it is normally synthesized.

A second Phase I clinical trial with the AAV2-FIX vector was initiated in patients with severe hemophilia B, but in this trial the vector was administered via hepatic artery injection to target the liver, which may be the natural source of FIX production. This was based on preclinical studies in hemophiliac dogs in which therapeutic levels of FIX were sustained over prolonged period with a dose of vector at  $10^{12}/\text{kg}$ . This trial has enrolled about six patients at three dose levels of  $1 \times 10^{11}/\text{kg}$ ,  $10^{12}/\text{kg}$ , and  $2 \times 10^{12}/\text{kg}$  and there have been several observations of significance some of which were unanticipated. In this trial, following intravenous hepatic administration of the AAV-FIX vector

at the lowest doses, patient semen samples were positive for the vector genome for several weeks (Kay and High, 2002). This suggested that vector genomes may have been distributed to gonads and caused a halt in the trial. However, further investigation indicated that the vector was not present in motile sperm and more extensive study of AAV-FIX vector delivery by both intramuscular and intravenous routes with a sensitive DNA-PCR assay showed that there was a dose-dependent detection of vector genome sequences in the gonads of males of several animal species including mice, rats, rabbits, and dogs (Arruda *et al.*, 2001). However, although testis tissue of these species was positive for vector for a short period after delivery, in both rabbits and dogs, semen and sperm were negative for vector sequences, suggesting that the risk of low inadvertent germline transmission of vector sequences after intramuscular or intravenous delivery is extremely low (Arruda *et al.*, 2001). Thus, the hepatic artery delivery trial was continued and two additional patients were treated at the intermediate dose.

Overall in the trial via the hepatic artery route, administration of the two lower doses of vector was safe and well tolerated and no toxicities were noted, but there was also no significant therapeutic level of FIX protein produced. However, administration of the highest dose of  $2 \times 10^{12}$  to two subjects resulted in several observations (High *et al.*, 2004). Both subjects showed detectable levels of FIX expression, but expression was transient and peaked at 3 percent in one and 11 percent in the other at 4 and 10 weeks post-administration, respectively. In the latter subject, the loss of expression was accompanied by a transient rise in serum transaminase levels. These results are suggestive of the occurrence of an immune response, perhaps a cellular immune response mediated by CTLs. The mechanism of this remains to be resolved but preliminary analysis of peripheral blood mononuclear cells from the patients with peptides from FIX and AAV2 capsids for IFN- $\gamma$  secretion in ELISpot assays compared with normal controls indicated a 10-fold increase for the FIX peptide pool and 2-fold increase for one AAV peptide. Whether this will prove to be limiting for FIX gene therapy remains to be determined and it is unclear if this is a specific observation related to hemophilia patients, FIX protein, or the particular vector used. However, it is important that the higher dose of vector did initially produce therapeutic levels of FIX protein and this was accurately predicted from the animal models in contrast to the intramuscular delivery models.

## Hereditary emphysema: alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin (AAT or SERPINA1) is a 52 KDa protein that is synthesized in hepatocytes, but secreted into serum and circulated to the lung where it functions to protect elastin fibers and other tissue components of the alveolar wall from degradation by neutrophil elastase. AAT functions as

a protease to inactivate the neutrophil elastase. AAT deficiency (hereditary emphysema), which leads to chronic obstructive lung disease, is the second most common monogenic lung disease with a carrier frequency of 5 percent in North America and accounts for about 3 percent of early deaths from pulmonary obstruction. The most common clinical deficiency of AAT is due to a missense mutation Glu342Lys ( $\text{PI}^*\text{Z}$ ) and most afflicted individuals possess the homozygous mutation  $\text{PI}^*\text{ZZ}$ . Current therapy for AAT deficiency includes avoidance of exposure to cigarette smoke and protein replacement therapy by weekly intravenous infusion of human AAT (Prolastin) derived from pooled donor plasma. Recombinant sources of the protein are being tested but the production and supply is severely limited and the infused protein has a half-life of  $<1$  week. A gene therapy, particularly from an AAV vector would theoretically be able to deliver a constant level of the protein for a prolonged period of time. One important challenge is that the steady state level of AAT in serum of humans is about 800  $\mu\text{g}/\text{ml}$  and thus high level expression will be required for any gene therapy approach.

Song *et al.* (1998) examined intramuscular administration of AAV2 vectors expressing the human AAT cDNA from either a CMV promoter or a human elongation factor promoter in several strains of mice including C57Bl/6, Balb/c, and SCID. The human elongation factor promoter gave 10-fold higher expression than the CMV promoter. They obtained sustained steady levels in the serum that reached 800  $\mu\text{g}/\text{ml}$  in severe combined immunodeficient (SCID) mice and 400  $\mu\text{g}/\text{ml}$  in C57Bl/6 mice. Interestingly, the C57Bl/6 mice did not mount an antibody response to the human AAT whereas Balb/c mice did. In addition, in the C57Bl/6 mice there was no evidence for a CTL response against either the human AAT or the AAV2 capsid protein. Thus, levels that may be expected to be therapeutic for humans were obtained but the dose of vector required was up to  $1.4 \times 10^{13}$  particles per mouse, which may require scaling to very high doses for human application. AAV2-hAAT vectors also gave similar stable levels of expression of AAT in the therapeutic range in serum after delivery to mice by injection via the portal vein in order to directly target the liver (Song *et al.*, 2001).

The safety of AAV2-AAT vectors was examined more extensively in a non-human primate using baboons (Song *et al.*, 2002). Initial tests with the AAV2 vector expressing the human AAT from a cytomegalovirus (CMV) promoter indicated that four of four baboons mounted a circulating serum antihuman AAT antibody response. To avoid this confounding variable in the preclinical studies, the subsequent studies in four additional baboons were conducted using an AAV2 vector expressing the baboon AAT with a 10 peptide tag from a promoter (CB) comprised of the CMV enhancer and the chicken  $\beta$ -actin promoter. Expression of the tagged protein could be detected in the muscle by histochemical staining at 4 months after injection, and there were no clinically significant findings indicating toxicity.

Importantly, biodistribution of the vector to gonads was not detected. In this study, doses up to  $5 \times 10^{12}$  particles/kg (a total dose of  $5 \times 10^{13}$  per animal) were investigated.

A phase I clinical study of an AAV2 vector expressing human AAT from the CB promoter has now begun (Flotte *et al.*, 2004). This is a phase I open label trial to test four dose levels of vector (total dose from  $2.1 \times 10^{12}$  to  $7 \times 10^{13}$  particles) administered by intramuscular injection to 12 adults with AAT deficiency. The primary goal will be to assess safety and to measure serum levels of AAT. Thus far, the first cohort of three patients at the lowest dose has been enrolled in this trial in 2004 (Flotte, personal communication). However, AAV serotype 2 may not be the optimal vector for administration via the intramuscular route and AAV1 vectors are known to generally give much more efficient transduction. Consequently, the same group of investigators is planning to begin a directly comparable phase I clinical trial in AAT-deficient patients to test an analogous AAV-AAT vector having an AAV1 capsid.

## Muscular dystrophy

The muscular dystrophies are a heterogeneous group of diseases involving degeneration of myofibers leading to progressive muscle wasting and weakness. They are caused by mutations in several types of genes that encode extracellular matrix and integral membrane proteins, cytoskeletal proteins, and various proteases, nuclear proteins, and signaling molecules (Emery, 2002).

The most common myopathy in children is Duchenne muscular dystrophy. This is an X-linked disease caused by mutations in the gene for dystrophin for which the cDNA is too large to fit in an AAV-B vector. Consequently AAV vectors have not been used clinically for this disease but generation of minidystrophin genes may soon permit this. In contrast, the limb girdle muscular dystrophies (LGMD) are inherited, autosomal recessive neuromuscular diseases that may be caused by mutations in one of the four muscle sarcoglycan genes ( $\alpha$ ,  $\beta$ ,  $\gamma$ ,  $\delta$ ) expressed predominantly in striated muscle. Each of these proteins is expressed from a cDNA that is  $<2$  kb and therefore is well suited for use in AAV vectors. The sarcoglycan transmembrane glycoproteins associate with each other in equal stoichiometry to form the sarcoglycan complex, and a deficiency of one component typically leads to partial or complete absence of all the other sarcoglycan proteins on the sarcolemma. The Bio 14.6 cardiomyopathic hamster is a naturally occurring LGMD model due to a deletion in the  $\delta$ -sarcoglycan gene. In these animals, correction of disease in the muscle was obtained after administration of AAV2  $\delta$ -sarcoglycan vectors by intramuscular or intravascular routes. Genetic, biochemical, histological, and functional rescue of relatively large regions of muscle was observed (Greelish *et al.*, 1999). On this basis, a Phase I clinical trial of AAV to deliver sarcoglycan genes via intramuscular injection was initiated

but only one patient was enrolled and treated (Stedman *et al.*, 2000). The trial has not yet been continued.

## NEUROLOGIC DISEASE

### Canavan disease

A clinical trial to treat Canavan disease (CD), a childhood leukodystrophy, has been initiated (Janson *et al.*, 2002). This severe childhood leukodystrophy disease results from an autosomal recessive mutation in the gene for aspartoacylase (ASPA) that causes a toxic accumulation of the metabolite N-acetyl-aspartate (NAA) in the central nervous system. This leads to disease characterized by impaired myelination and spongiform degeneration of the brain. There is no effective treatment for this disease. An initial attempt to deliver the ASPA gene directly to the brain of affected children by neurosurgical injection using a plasmid based, non-viral delivery system showed regional lowering of NAA levels as measured by proton magnetic resonance spectroscopy, but in most of the treated subjects but there were only mild neurological and radiological improvements (Leone *et al.*, 2000). It was suggested that the modest and transient nature of the changes in clinical parameters probably reflected inadequacies of the vector.

In a mouse knockout model of CD (Matalon *et al.*, 2003) and a naturally occurring rat model of CD (Kitada *et al.*, 2000), which show similar pathology to that seen in children with CD, injection into the brain of AAV2 vectors expressing ASPA shows lowering of NAA and abolition of the pathological changes in the areas that were injected. These changes were maintained over at least 3–5 months and suggest that AAV vectors may have improved potential for treatment of CD. A trial of an AAV2-ASPA vector in children with CD is now underway (Janson *et al.*, 2002). This trial is testing an AAV2 vector in which ASPA is expressed from a human neuron-specific enolase transcription promoter. Up to 21 CD patients, aged 2–6 years will be enrolled in an open-label study and treated by intraparenchymal injection in the brain with a dose of  $9 \times 10^{11}$  particles ( $10^{10}$  infectious units) of the vector. To maximize the distribution of the vector to the affected regions of the brain, the vector dose will be fractionated and administered in up to six sites in the frontal, parietal, and occipital regions of the brain. Thus far, a total of 10 patients have been treated ranging from age 2 to 4.5 years; the vector has been well tolerated and there is some indication of neurological improvement (Leone, personal communication).

### Parkinson disease

Parkinson disease (PD) is a common progressive neurodegenerative movement disorder that affects 5 percent of the

population over 65 years of age. This disease is characterized by loss of dopaminergic neurons, mainly in the substantia nigra, that leads to a deficiency of the neurotransmitter dopamine in the striatum. Clinical symptoms usually appear once 60–80 percent of the dopaminergic neurons have been lost and this is correlated with the dopamine deficiency. Dopamine is synthesized from tyrosine which is converted to L-dihydroxyphenylalanine (L-DOPA) by tyrosine hydroxylase and then to dopamine by aromatic amino acid decarboxylase (AADC). L-DOPA therapy is effective but the response declines as the disease progresses and is complicated by adverse side effects. Thus, the different enzymes involved in dopamine synthesis have been targeted for gene transfer replacement therapy in order to restore dopaminergic stimulation of the striatum and a clinical trial of an AAV2 vector expressing AADC has been proposed but not yet initiated. An alternative approach is to express growth factors in an attempt to boost survival of neurons, and at least one clinical trial of an AAV vector expressing a growth factor is also being considered.

However, one clinical trial with AAV vectors has begun on the basis of quite a different hypothesis as an approach to treat the motor symptoms of PD (During *et al.*, 2001). In this clinical study, PD patients will be administered two different AAV2 vectors each expressing, respectively, the gene for one of the two isoforms of the enzyme glutamic acid decarboxylase (GAD-65 and GAD-67). These vectors will be co-injected into the subthalamic nucleus (STN) region of the brain. The STN has a central role in the region of the brain responsible for regulation of movement, and becomes disinhibited in PD. This disinhibition is believed to be responsible for the motor activity symptoms, including tremor, rigidity, bradykinesia, and gait disturbance. Electrical silencing of the STN by deep brain stimulation (DBS) has shown significant improvement in the motor dysfunction symptoms in PD patients. The hypothesis to be tested is that gene transfer will palliate these symptoms by inhibiting STN activity, as would DBS, and also will result in production of  $\gamma$ -aminobutyric acid (GABA). This may cause the STN to become a GABA producer and thus convert excitatory STN projections into inhibitory projections. Should there be any untoward effects then the STN will be ablated by the conventional DBS procedure. The clinical trial is an open-label, dose-escalation that will enroll six patients who are candidates for DBS. Patients must have asymmetrical disease and the vector will be administered only unilaterally. This trial began in 2004 and several patients have been treated.

## INFLAMMATORY DISEASE

### Rheumatoid arthritis

Rheumatoid arthritis (RA), the most common inflammatory joint disease, is a chronic autoimmune disorder that

affects approximately 1 percent of the population and causes significant disability (Firestein, 2003). The etiology of RA is largely unknown but appears to involve both environmental and genetic components. The chronic inflammation that occurs in arthritic joints involves recruitment of immune cells, including lymphocytes, macrophages, and plasma cells that leads to massive thickening of the synovium and formation of pannus tissue. This is accompanied by release of pro-inflammatory mediators and eventual invasion and destruction of articular cartilage and bone. At the molecular level, chronic inflammatory arthritis is characterized by involvement of T cells and an abundance of cytokines and growth factors such as interleukin-6 (IL-6), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) produced by macrophages and synovial fibroblasts. Two cytokines play key roles. TNF- $\alpha$  is a major cytokine involved in the joint inflammation and probably the subsequent joint destruction, and IL-1 $\beta$  also is a key cytokine that induces cartilage degradation. These cytokines, and particularly TNF- $\alpha$ , represent validated therapeutic targets.

Conventional treatments to manage arthritis symptoms involve general anti-inflammatory agents, including both steroidal and non-steroidal drugs, as well as disease-modifying drugs such as methotrexate, but none of these pharmacologic agents have proven effective in halting the progression of disease. However, the recently introduced biologic anti-TNF- $\alpha$  agents have proven much more effective in not only ameliorating arthritis symptoms but also in halting the progression of disease (Feldmann and Maini, 2003). Two classes of inhibitors of TNF- $\alpha$  that are either monoclonal antibodies (infliximab and humira) or a soluble receptor (entanercept, a soluble TNF receptor-Fc fusion protein, TNFR:Fc) that sequester TNF- $\alpha$  have been approved and are being widely used to provide therapeutic benefit. In addition, an inhibitor of IL-1 $\beta$ , the IL-1 receptor antagonist protein (IL-1Ra), has been approved for therapeutic use.

The use of biologics for arthritis therapy presents several challenges. These proteins have a relatively short half-life and may need fairly frequent dosing, perhaps at weekly intervals, and effective levels of the therapeutic protein may not be maintained for extended periods. In addition, the therapeutic proteins are administered systemically, either by injection for entanercept or by intravenous infusion for infliximab, and may have reduced bioavailability in some affected joints. Thus, although the overall therapeutic result is often impressive in many cases, individual joints respond poorly. Gene transfer may be a more efficient means of delivery of these biological agents. Persistent transgene expression may enable attainment of steady-state levels of the inhibitor as opposed to the peaks and troughs associated with intermittent protein administration. This might also facilitate very infrequent administration and circumvent the need for frequent repeat dosing. Furthermore, if the limited response of some joints is due to limited availability of systemically administered protein to reach these joints, then expression directly in the joint may overcome this limitation.

AAV2 vectors encoding genes that express TNF- $\alpha$  or IL-1 inhibitors have been tested in rodent models of inflammatory arthritis. An AAV2 vector encoding an IL-1Ra cDNA from a CMV transcription promoter was evaluated in a lipopolysaccharide (LPS)-induced arthritis model in rats using several therapeutic, recurrent, and preventative protocols (Pan *et al.*, 2000). IL-1Ra expression was up-regulated by LPS-induced joint inflammation and showed good efficacy in all the protocols. Importantly, the IL-1Ra transgene persisted for >3 months and could be induced to express therapeutic levels of soluble IL-1Ra upon re-administration of LPS. This resulted in suppression of inflammation and IL-1 $\alpha$  production in the treated knee joints.

TNF- $\alpha$  inhibitors have been tested in two different rodent models of arthritis and also have shown excellent efficacy. One model is the TNF- $\alpha$  transgenic mouse in which intra-articular injection of an AAV2 vector encoding a soluble TNF receptor type I (TNFR I) significantly decreased synovial hyperplasia, and cartilage and the destruction of cartilage and bone (Zhang *et al.*, 2000a). An alternate model is the rat induced-arthritis model in which female Lewis rats are injected intraperitoneally with a streptococcal cell wall (SCW) preparation. This leads to establishment of a chronic inflammation in the hind ankles that is mediated largely by T cells and macrophages, and shows both inflammation and joint destruction that is characteristic of the joint disease in humans with RA.

In the SCW-induced arthritis rat model, administration of an AAV2 vector encoding the rat soluble TNFR:Fc fusion gene expressed from a CMV early gene transcription promoter, either systemically (intramuscular), or locally (intra-articular), resulted in profound suppression of arthritis. This was reflected in decreased inflammatory cell infiltration, pannus formation, cartilage and bone destruction, and mRNA expression of pro-inflammatory cytokines in the joint (Chan *et al.*, 2002). Administration of a similar dose of vector by either the intramuscular or intra-articular routes was equally effective in ameliorating disease in the joints. However, whereas intramuscular delivery resulted in significant levels of soluble TNFR:Fc protein in the circulation, intra-articular delivery resulted in little or no detectable soluble protein in serum. Moreover, administration of the vector by the intra-articular route but to only one hind joint suppressed arthritis in the contralateral joint equally as well as the ipsilateral joint. The mechanism of the contralateral suppression is not clear but has been seen in other animal models of gene therapy for inflammatory joint disease. The efficacy of treating joint disease by intra-articular delivery in the absence of measurable levels of circulating TNFR:Fc protein is important for clinical applications:

- The ability to express the protein directly in an infected joint may allow improved efficacy over systemic delivery of the soluble protein in which failure of some joints to respond may be due to insufficient protein reaching the joint.

- The contralateral effect may enhance the likelihood of other affected joints responding as well without the necessity of injecting all affected joints.
- The ability to express the soluble protein in the joints in the absence of significant circulating levels enhanced the likely safety profile. Significant levels of constitutively and persistently expressed and circulating protein may be undesirable in the event of the need to shut off protein in response to infection in which TNF- $\alpha$  plays an important role.

The studies in the SCW-induced rat arthritis model and additional extensive safety, toxicity, and biodistribution studies formed the basis to proceed with a clinical trial of an AAV vector expressing the TNFR:Fc protein in RA patients. This trial is testing a vector, tgAAC094, comprised of an AAV2 capsid and a genome that contains the human TNFR:Fc gene expressed from a CMV promoter. The vector is being tested in a phase I trial in patients with RA having only one or several afflicted joints and who are not yet candidates for systemic treatment with anti-TNF- $\alpha$  inhibitors. The trial is a placebo-controlled, double-blinded, dose-escalation study that will test at least three dose levels of the vector. At each dose level, eight subjects will be enrolled and randomized 3:1 for treatment with a single dose of the vector tgAAC094 or a placebo, administered by intra-articular injection to one of several types of joints including the knee, ankle, or wrist. The primary objective of this trial is to evaluate the safety of intra-articular injection of tgAAC094. Secondary objectives will include evaluation of a number of clinical parameters including pain and swelling as well as biochemical endpoints including the level of TNFR:Fc protein in joint fluid and serum and serum levels of any antibody response to the AAV2 capsid. This trial was initiated in 2004 and patient enrollment is underway but results are not yet available.

## VACCINES

For most applications of gene therapy aimed at correcting disease an immune response against the expressed transgene will be highly undesirable. However, there are two types of applications where a transgene is expressed either to stimulate an immune response or to generate an immune response directly against the expressed gene:

- In the former type of application the expressed gene is aimed at increasing immune system recognition of tumor cells to generate potential therapeutic vaccines for cancer.
- A second class of vaccines is aimed at prophylactic use against infectious disease and, in this case, the expressed transgene will contain antigens of the infectious agent to which a direct immune response is desired.

AAV vectors have entered clinical trials for both applications including a potential therapeutic vaccine against malignant melanoma and a prophylactic vaccine against HIV.

## Cancer

One hypothesis for the mechanism of cancer is that there are neo-antigens expressed on tumor cells but these are poorly recognized by the immune system. Certainly, in many tumor cells the expression of the immune costimulatory molecules B7-1 and B7-2 on the cell's surface is suppressed and it is believed that this results in poor immune recognition of tumor cells particularly by the cellular arm of the immune system. Furthermore, expression of these molecules on tumor cells can, in many tumor models, enhance the costimulatory effect of the modified cells and induce a more exuberant T-cell proliferative response and an enhanced cytolytic T-cell response. The costimulation by B7 molecules occurs via interaction with the T-cell surface molecule CD28 and this interaction is enhanced by a synergistic action of the cytokine IL-12. IL-12 is a heterodimer of two subunits, p35 and p40, which must be expressed in equal amounts. AAV2 vectors expressing B7 and IL-12 genes have been tested in tumor cells and tumor models and shown potential to enhance co-stimulation and induce cytolytic cell responses.

Multiple myeloma cells were efficiently transduced by rAAV vectors expressing a neomycin resistance and either of the B7-1 or B7-2 genes and a cell line was generated with each virus. When the two cell lines were irradiated and exposed to human allogeneic T cells there was a 10-fold increase in T-cell proliferation, increased secretion of IFN- $\gamma$  and IL-12 by the T cells and an increased cytolytic T-cell (CTL) response (Wendtner *et al.*, 1997). Additional experiments showed that freshly isolated tumor cells from patients with malignant melanoma could be simultaneously transduced with three AAV vectors expressing respectively B7-2, the IL12 p35 subunit or the IL12 p40 subunit and resulted in high level expression of B7-2 on the cell surface and secretion of IL12 (Maass *et al.*, 1998). Based upon these studies a phase I/II clinical trial began in Europe in 2001 to examine use of rAAV vectors expressing immunostimulatory genes as a cancer vaccine in patients with malignant melanoma. The patient's tumor cells are excised, transduced *ex vivo* with the vectors and then irradiated and injected back into the patient (Hunt, 2001). Thus far there has been no public report of the outcome of this trial.

## Infectious disease

An AAV vector is being tested in clinical trials as a potential prophylactic vaccine for AIDS. The underlying hypothesis of this approach is that intramuscular administration of AAV vectors is known to lead to prolonged persistence as

an episome. It is hoped that in a vaccine setting such prolonged expression of an antigen may be advantageous in developing more robust immune responses, both cellular and humoral. It has been argued that the most likely approach to generation of a successful AIDS vaccine would be to use a live attenuated human immunodeficiency virus (HIV) but safety considerations preclude this directly. In the context of an AIDS vaccine, an AAV vector expressing HIV antigens may function as a surrogate for a live attenuated vaccine. The mechanism of antigen presentation following expression from AAV vectors administered to the muscle is unknown but may involve cross-presentation by antigen-presenting cells, rather than by direct transduction of dendritic cells, leading to activation of T cells in the draining lymph nodes (Zhang *et al.*, 2000b).

Generation of an anti-HIV-1 immune response using an AAV vector has been tested in mice. A single, intramuscular injection of an AAV2 vector encoding the HIV-1 *env*, *tat*, and *rev* genes (AAV-HIV) induced robust, long-term production of HIV-1-specific serum IgG and MHC class I-restricted CTL activity (Xin *et al.*, 2001). In rhesus macaques, a single intramuscular administration of an AAV2 vector expressing the simian immunodeficiency virus (SIV) major structural genes resulted in long-term CD8 $^{+}$ , antigen-specific CTL responses against multiple SIV protein epitopes that were similar to responses observed in monkeys directly infected with pathogenic SIV (Johnson *et al.*, 2001). There were also robust neutralizing antibody responses that persisted for >1 year. Furthermore, in studies to test the efficacy of AAV2-SIV vaccines in an SIV-macaque challenge model, immunized macaques had a significantly lower virus burden at peak (2 weeks) and set-point (10 weeks) after an intravenous challenge with SIV than did mock-vaccinated control animals.

A clinical trial of an AAV2 vector expressing HIV-1 clade C antigens, tgAAC09, has been initiated in Europe. This is a dose-escalation, placebo-controlled, double-blind, and randomized phase I trial being conducted in healthy HIV seronegative volunteers. Each subject is administered one dose of the vector or placebo by intramuscular injection. The objectives of the trial are to measure safety and local tolerability, and to analyze immune responses to the HIV antigens. The vector expresses clade C HIV antigens because this potential vaccine is designed for use in the developing world. The potential for use of AAV vector as vaccines is discussed more extensively elsewhere in this volume but there are two points worthy of note with regard to the current AAV-HIV vaccine trial that required particular pre-clinical experiments.

The AAV-HIV vaccine currently being tested in clinical trials is highly purified but was manufactured in a cell line, HeLa, originally derived from a human tumor. Because such cells generally have not been used as cell substrates for preparation of vaccines, there was a requirement for additional testing of the cell line. An extensive analysis of *in vivo* tumorigenicity of the cells and the oncogenicity of high

molecular weight DNA derived from the cells was performed as well as characterization of the normality of endogenous prion gene and protein, cell clearance, viral clearance, and residual host cell DNA levels (P. Anklesaria, personal communication). Also, an extensive analysis was performed to determine the likelihood for integration of the persisting vector genome into host cell chromosomes. Studies performed in several animal species, including macaques, rabbits, and mice (Schnepp *et al.*, 2003) indicated that the integration frequency of the vector genome after intramuscular injection was at least several hundred-fold less than the known rate of spontaneous mutation in human genes which is  $10^{-5}$ .

## SUMMARY

The studies described in this chapter illustrate the good progress being made in developing therapeutic applications for AAV vectors. The early clinical trials of AAV vectors for the treatment of CF were important in establishing the initial regulatory environment for AAV vectors and demonstrating their generally good safety profile. In addition to the significant number of AAV vectors now being tested clinically, it is likely that soon there will be a significant increase in clinical testing of AAV vectors for additional therapeutic applications. The increasing sophistication of AAV vector production systems is enhancing the quantity and quality of vectors that can be produced. Expanding studies of AAV biology to modify the transduction efficiency by judicious choice of serotype for the capsid or by modification of the capsid structure will lead to further expansion of the use and potency of AAV vectors.

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# Mechanism of recombinant adeno-associated virus transduction

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Adeno-associated virus (AAV) is a replication-defective, non-pathogenic parvovirus with an approximately 4.7 kb single-stranded DNA genome. AAV requires the presence of a helper virus, such as adenovirus or herpes simplex virus, to replicate progeny virions in a lytic cycle (Handa and Carter, 1979). In the absence of a helper, a latent infection life cycle is established, and AAV is propagated as an integrated provirus in the chromosomes of host cells (Muzychka, 1992; Berns and Linden, 1995; Berns and Giraud, 1996). In the mid-1960s, AAV was discovered as a small contaminating DNA virus in the adenovirus preparation (Atchison *et al.*, 1966; Hoggan *et al.*, 1966), and the AAV serotype 2 (AAV2) has been the most extensively investigated serotype since the 1970s (Carter *et al.*, 1975; de la Maza and Carter, 1980). In the early 1980s, it was discovered that the prokaryotic clones of wild-type AAV2 were infectious (Samulski *et al.*, 1982; Laughlin *et al.*, 1983). In the presence of adenovirus helper, it was subsequently shown that proviral plasmids encoding the AAV genome could rescue and replicate virus following transfection into cells (Samulski *et al.*, 1983; Senapathy and Carter, 1984). This finding paved the way for the development of recombinant AAV2-based vectors as gene transfer vehicles to transduce mammalian cells (Hermonat and Muzychka, 1984; Tratschin *et al.*, 1984; Samulski *et al.*, 1987, 1989).

Over the last decade, recombinant AAV2 (rAAV2) has become a very promising viral vector for gene delivery (Carter, 1992, 2000; Carter and Samulski, 2000) and has led to a large body of preclinical and clinical investigation on rAAV2 (High, 2001; Kay *et al.*, 2001; Samulski, 2003; Ponnazhagan, 2004). These studies have also extended to other AAV serotypes. At present, at least eight serotypes of

the AAV family have been identified, cloned, and developed as gene transfer vectors (Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997, 1999; Rutledge *et al.*, 1998; Bantel-Schaal *et al.*, 1999; Xiao *et al.*, 1999; Gao *et al.*, 2002). While these various serotypes have very similar genomic organizations, their infectious tropisms vary greatly as a result of evolutionary diversity among capsid proteins. The identification of optimal AAV serotypes for a given tissue target has also been an area of intense interest to gene therapy (Xiao *et al.*, 1999; Chao *et al.*, 2000; Davidson *et al.*, 2000; Zabner *et al.*, 2000; Duan *et al.*, 2001; Halbert *et al.*, 2001; Yan *et al.*, 2002; Yang *et al.*, 2002; Grimm and Kay, 2003; Sarkar *et al.*, 2004). The serotype-dependent differences in rAAV vector transduction have historically been attributed to the serotype-specific receptors that affect viral binding and uptake by the target cells; however, this theory has been challenged as the efficacy of rAAV uptake doesn't always directly correlate with the level of transgene expression. Recently, intracellular barriers, which limit nuclear accumulation of rAAV, have been uncovered (Duan *et al.*, 2000b; Hansen *et al.*, 2000). These barriers may also differ for various AAV serotypes that use distinct receptor entry pathways (Zabner *et al.*, 2000; Yan *et al.*, 2002, 2004). The overall efficiency of rAAV transduction for a given cell type and vector serotype can be attributed to the efficiency of multiple steps characterizing biologic interactions of the virus with its host target cell. In this context, barriers for viral transduction can be encountered at the level of viral receptor and/or co-receptor abundance on the target cell, intracellular trafficking of virus to the nucleus, virion uncoating, and the rate of conversion of single-stranded viral genomes to double-stranded transduction intermediates capable of expressing

an encoded transgene. Furthermore, the genetic fate of these transduction intermediates (i.e. integration into chromosomes or episomal persistence as circular or linear genomes) can affect the stability of transduction. A better understanding of the biology and mechanisms of AAV transduction is a fundamental prerequisite in developing more efficient rAAV vectors. Vectoring approaches have been successfully applied to overcome barriers to receptor abundance (Girod *et al.*, 1999; Wu *et al.*, 2000) and genomic conversion (McCarty *et al.*, 2001; Fu *et al.*, 2003; McCarty *et al.*, 2003; Wang *et al.*, 2003). Additionally, through a better understanding of rAAV transduction biology it was discovered that modulating the proteasome/ubiquitin pathway can improve rAAV transduction by enhancing intracellular

processing of the virus (Duan *et al.*, 2000b; Douar *et al.*, 2001; Yan *et al.*, 2002; Ding *et al.*, 2003; Yan *et al.*, 2004).

It is important to point out that transduction biology of wild-type and recombinant AAV may be different. Although recombinant AAV vectors are currently thought to be very similar to that of the wild-type virus in terms of virion structure, few studies have directly compared their biology in the context of intracellular trafficking and genome conversion. Additionally, recombinant vectors provide unique methodologic approaches to study aspects of the latent viral life cycle that are not applicable with the wild-type virus. This review will focus on discussing aspects of rAAV transduction biology.

rAAV transduction can be categorized into four discrete stages:

- binding and endocytosis;
- intracellular trafficking to the nucleus;
- nuclear entry and virion uncoating; and
- post-nuclear genome conversion events (Figure 38.1).

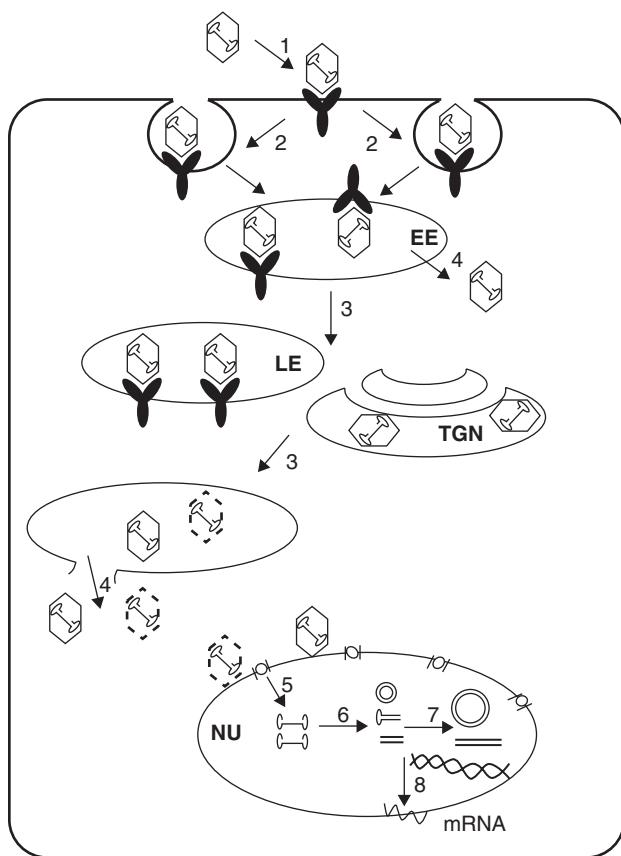
Our current understanding of each of these stages and our knowledge about virus–host interactions will be highlighted throughout this chapter. Since rAAV2 is the most extensively studied serotype in the AAV family, this chapter will focus mainly on rAAV2 transduction and to a lesser extent on other serotypes, such as rAAV5.

## INFECTIOUS ENTRY PATHWAYS FOR rAAV

### AAV receptors and tropism

Currently, eight isolated serotypes of AAV have been cloned and sequenced. Biological studies demonstrate that each serotype has unique binding and cell tropism characteristics (Muramatsu *et al.*, 1996; Chiorini *et al.*, 1997; Rutledge *et al.*, 1998; Bantel-Schaal *et al.*, 1999; Chiorini *et al.*, 1999; Xiao *et al.*, 1999; Gao *et al.*, 2002). Generally speaking, the host range for AAV is very broad and includes both dividing and post-mitotic cells. Omnipresent heparan sulfate proteoglycan (HSPG) has been identified as the primary attachment receptor for AAV2 (Summerford and Samulski, 1998) and in part explains why rAAV2 can infect a wide range of different tissues despite varying transduction efficiencies (Figure 38.2). Most likely, HSPG is also a primary attachment receptor for AAV3 (Rabinowitz *et al.*, 2002). Cell surface glycans, such as 2,3 O-linked and 2,3 N-linked sialic acid, are thought to be key components of the receptor complex responsible for cellular binding of AAV4 and AAV5 (Kaludov *et al.*, 2002; Walters *et al.*, 2001, 2002). It is still unclear, however, whether sialic acid in general serves as a receptor or whether the active component of sialic acid must reside on a specific cell-surface protein.

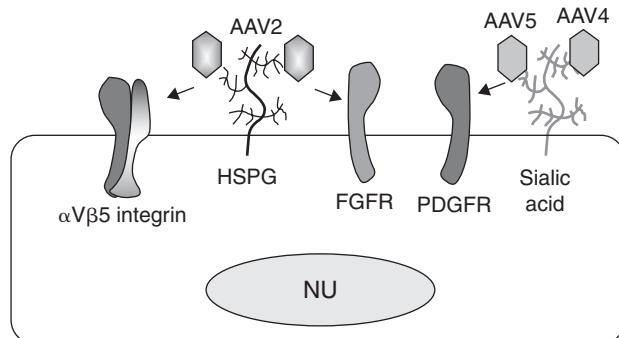
Cell-surface binding alone is insufficient to facilitate efficient entry of AAV. Additional protein interaction with



**Figure 38.1** A schematic model of rAAV transduction. The potential rate-limiting steps in rAAV transduction include: (1) receptor binding; (2) receptor-mediated endocytosis; (3) intracellular trafficking through various endosomal compartments; (4) endosomal escape into the cytoplasm; (5) translocation into the nucleus and virion uncoating; (6) conversion of single-stranded DNA genomes to double-stranded circular and linear intermediates; (7) long-term viral genome persistence by integration into chromosomal DNA or episomal concatamers; (8) transgene transcription. Each of these steps represents potential barriers to rAAV transduction as defined by expression of an encoded transgene. EE, early endosome; LE, late endosome; TGN, trans-Golgi network; NU, nucleus.

the viral particle or with the primary binding receptor is required for efficient endocytosis of the virion. For AAV entry, two potential co-receptors, including  $\alpha V\beta 5$  integrin (Summerford *et al.*, 1999) and human fibroblast growth factor receptor 1 (FGFR1) (Qing *et al.*, 1999), are thought to play critical roles. It has been observed that  $\alpha V\beta 5$  integrin is not required for AAV2 binding to HeLa cells, but it is required for effective internalization (Sanlioglu *et al.*, 2000b). The dependence of AAV2 infection on these two co-receptors in certain cell types is disputed (Qiu *et al.*, 1999), but it has been positively linked to a 150 kDa novel cell membrane protein (Mizukami *et al.*, 1996). In fully differentiated human airway cells, all known receptor and co-receptors for AAV2 are localized in a polarized fashion exclusively on the basolateral surface (Duan *et al.*, 1998b; 1999d). Despite the lack of HSPG, FGFR1, and  $\alpha V\beta 5$  integrin on the apical surface of polarized airway epithelia, rAAV2 particles are still efficiently endocytosed from the apical membrane. Internalization of rAAV2 from the basolateral membrane of polarized human airway epithelia is only 3–4 times greater than that from the apical membrane (Duan *et al.*, 1998b, 2000b). This slight difference in binding and internalization cannot presently explain the greater than 200-fold preference for rAAV2 transduction from the basolateral surface (Duan *et al.*, 1998b). These findings suggest several important biologic aspects of rAAV2 transduction in the airway:

- Unidentified receptor/co-receptors must exist on the apical surface of airway epithelia.
- Distinct entry pathways from the apical and basolateral surfaces of airway epithelia have different efficiencies



**Figure 38.2 Cell surface receptors and co-receptors known to mediate AAV transduction.** AAV2 uses heparan sulfate proteoglycan (HSPG) as one of its primary attachment receptors. Two potential co-receptors for AAV2 may be important in viral endocytosis and include  $\alpha V\beta 5$  integrin and human fibroblast growth factor receptor 1 (FGFR1). Other cell surface glycans such as 2,3 O-linked and 2,3 N-linked sialic acid are thought to be key components of the receptor complex responsible for cellular binding of AAV4 and AAV5. Platelet-derived growth factor receptor (PDGFR- $\alpha$  and PDGFR- $\beta$ ) also appears to act as a co-receptor for AAV5 infection.

for the latent life cycle of the virus. This implies that some intracellular barrier to rAAV2 transduction exists following apical entry that is not associated with basolateral entry pathways.

Although co-receptors have been suggested for AAV2, it is currently unclear if AAV5 shares a similar dependence on a co-receptor for efficient infection. However, a significant correlation was recently observed between the expression of the platelet-derived growth factor receptor (PDGFR- $\alpha$  and PDGFR- $\beta$ ) and the permissiveness of rAAV5 transduction (Pasquale *et al.*, 2003). Although the PDGFRs are sialoglycoproteins containing both N- and O-linked oligosaccharide chains with sialic acid, PDGFR-mediated endocytosis does not require sialic acid and is not sensitive to neuraminidase (Daniel *et al.*, 1987; Hosang, 1988). This evidence suggests that PDGFR plays a role much like 2,3 N-linked sialic acid in AAV5 infection (Walters *et al.*, 2001). Further identification and characterization of other AAV receptors and/or co-receptors will significantly influence the development of AAV-mediated gene transfer.

### AAV internalization and receptor-mediated endocytosis

The mechanism of rAAV endocytosis has recently been investigated using fluorescent-tagged AAV virions (Bartlett and Samulski, 1998; Bartlett *et al.*, 2000; Sanlioglu *et al.*, 2000a,b). The internalization of AAV2 appears to be a rapid process with a half-time of less than 10 minutes (Bartlett *et al.*, 2000). Co-localization of Cy3-labeled rAAV2 with known markers of fluid-phase and receptor-mediated endocytosis have been useful in determining pathways for rAAV2 movement through cells. To this end, fluorescein isothiocyanate (FITC)-labeled transferrin, as a marker for clathrin-dependent endocytosis, demonstrates significant overlap with Cy3-AAV particles following infection of HeLa cells (Daro *et al.*, 1996; Duan *et al.*, 1999a). In contrast, little overlap was seen between Cy3-AAV and FITC-Dextran (Shurety *et al.*, 1998). These experiments suggest that following binding, the majority of rAAV2 particles enter cells through clathrin-coated pits (Duan *et al.*, 1999a; Bartlett *et al.*, 2000). Dynamin is a 100 kDa cytosolic guanosine triphosphatase (GTPase) that selectively regulates clathrin-mediated endocytosis and has also been useful in studying entry pathways for rAAV2. Dynamin, which associates with clathrin-coated membrane invaginations, has been proposed to mediate the constriction of the coated pits and the budding of coated vesicles from the plasma membrane. Inhibition of rAAV endocytosis following expression of a dominant-negative dynamin mutant demonstrated that the rAAV2 internalization is dynamin-dependent (Duan *et al.*, 1999a; Sanlioglu *et al.*, 2000b). Together with the findings that rAAV2 is endocytosed into transferrin-containing vesicles, these data strongly suggest that the

process of rAAV2 entry in HeLa cells is clathrin-mediated endocytosis. It is important to point out, however, that HeLa cells predominantly utilize HSPG-dependent pathways of rAAV2 entry (Sanlioglu *et al.*, 2000b). Hence, this pathway of entry may not apply for other yet to be identified receptor entry pathways.

The AAV2 co-receptor,  $\alpha V\beta 5$  integrin, also participates in the process of viral internalization. Integrins are molecules involved in cell adhesion and motility (Brooks *et al.*, 1997; Klemke *et al.*, 1997). It is well known that integrins are also associated with multiple small intracellular signal molecules, including Rho, Ras, Cdc42, and GTPase. Through these integrins, actin fibers facilitate cell motility and endocytic movement of vesicles (Nobes and Hall, 1995a,b; Parsons, 1996). Molecular dissection of these processes and their relevance to rAAV2 internalization has been studied using methods of inhibition for  $\alpha V\beta 5$  integrin, Rac1, and phosphatidylinositol-3 kinase (PI-3K). Results from studies in HeLa cells suggest that  $\alpha V\beta 5$  integrin, Rac1, and PI-3K are all required for internalization and/or intracellular movement of rAAV2 (Sanlioglu *et al.*, 2000b). Blocking antibodies against  $\alpha V\beta 5$  integrin or expression of a dominant negative Rac1 mutant (N17Rac1) both prevent endocytosis of rAAV2 and transduction in HeLa cells. Subsequent to integrin-mediated activation, activation of PI-3K (determined by inhibition in the presence of Wortmannin) appears to be required for the initiation of rAAV2 intracellular movement to the nucleus. Hence, Rac1 and PI-3K pathways that have been demonstrated to be biochemically linked in their activation, affect distinct stages of AAV2 transduction.

## Intracellular barriers to rAAV transduction

It has been increasingly recognized that infectious entry pathways of rAAV are considerably more complex than previously thought. Efficient internalization of rAAV does not always lead to effective transduction, as judged by the end point of transgene expression. For example, in spite of the fact that the polarized human airway cells show no significant differences in their ability to internalize rAAV2 from the apical or basolateral surface, transgene expression following apical infection is as much as 200-fold less efficient than basolateral infection (Duan *et al.*, 1998b, 2000b; Ding *et al.*, 2003). This implies that impaired intracellular processing of rAAV2 is responsible for the observed reduced transduction from the apical membrane. Post-entry blocks in rAAV2 transduction have also been extensively demonstrated in fibroblasts (Hansen *et al.*, 2000, 2001a). The differing efficiencies of rAAV2 and rAAV5 transduction in HeLa cells also suggest the existence of serotype-specific intracellular barriers. Although AAV2 and AAV5 capsid-mediated infection in HeLa cells results in similar levels of virus endocytosis as judged by Hirt Southern blotting, significantly higher levels ( $\sim 5$ –7-fold) of transgene expression with rAAV2 are observed when compared with

rAAV2/5 pseudotyped virus containing an identical viral genome (Yan *et al.*, 2002). The lack of a direct correlation between viral uptake and transgene expression suggest the importance of post-entry barriers for successful rAAV transduction. These post-entry barriers may manifest in a cell type-specific, serotype-specific, or polarity-specific manner.

## INTRACELLULAR TRAFFICKING OF rAAV TO THE NUCLEUS

Although receptor-mediated endocytosis of AAV2 is fairly rapid, it has been clearly demonstrated that intracellular trafficking to the nucleus for rAAV2 is a slow, rate-limiting process for certain cell types (Hansen *et al.*, 2000, 2001a; Duan *et al.*, 2000b; Pajusola *et al.*, 2002). The recently recognized importance of nuclear trafficking for effective rAAV transduction has led to an increased interest in understanding mechanistic aspects of this process. By means of a real-time, single-molecule imaging technique, the movement of Cy5-AAV during infection of a living HeLa cell was visualized in real-time (Seisenberger *et al.*, 2001). Time-chase experiments of virions in living cells suggest the modes of AAV motion include:

- free and anomalous diffusion of the virus/endosome in the cytoplasm, and
- directed motion to nuclei by motor proteins in the cytoplasm and nucleus.

Remarkably, AAV was seen to freely diffuse in the cytoplasm, and then to suddenly start to move at a constant velocity, into the nuclear region along well-defined pathways (movie available at [www.single-virus-tracing.com](http://www.single-virus-tracing.com)). Insights into AAV movement within living cells, as visualized by this innovative technique, have been valuable. However, details regarding the process of virus sorting in the endosomal compartments and vesicular transport, remain ill defined.

## Endosomal sorting

rAAV2 has been demonstrated to be endocytosed through clathrin-dependent receptor endocytosis and then processed through endosomal compartments in a similar fashion to transferrin (Duan *et al.*, 1999a; Sanlioglu *et al.*, 2000b). Infection of HeLa or 293 cells, which are highly permissive for AAV2 transduction, showed rapid and efficient trafficking of virus to the nucleus or perinuclear area (Bartlett *et al.*, 2000; Sanlioglu *et al.*, 2000b). The use of fluorophore-labeled AAV2 or monoclonal antibodies against the capsid proteins suggests that viral escape from the early endosome may occur very early in the infectious process, followed by accumulation around the nucleus (Bartlett *et al.*, 2000; Xiao *et al.*, 2002). The perinuclear localization of fluorescently-labeled AAV2 can be very stable and persist up to

23 hours after infection (Pajusola *et al.*, 2002). The use of subcellular fractionation techniques followed by Southern blotting for viral DNA has also suggested that AAV2 perinuclear persistence can last for 16–24 hours (Xiao *et al.*, 2002). However, biochemical analyses using inhibitors of intracellular trafficking appear to contradict observations that AAV2 exits from the early endosome. Inhibitor studies with brefeldin A, a fungal antibiotic that causes the early endosome to form tubular networks and prevents early-to-late endosome transitions (Lippincott-Schwartz *et al.*, 1991; Pelham, 1991), have suggested that AAV2 virions are processed through the early endosome and routed toward the late endosomal compartment (Douar *et al.*, 2001). Other studies using an inhibitor of the vacuolar proton ATPase (bafilomycin A<sub>1</sub>), which efficiently inhibits endosomal acidification and therefore inhibits endosomal maturation, suggest that trafficking of AAV2 depends on acidification of the endosome (Douar *et al.*, 2001). These experiments suggest that AAV2 escape from the endosome is activated by low pH typically associated with the late-endosomal compartment.

Other studies in several cell types have suggested that AAV2 and AAV5 traffic to the Golgi compartment in permissive HeLa cells. Co-immunostaining of fluorescently-labeled AAV2 with the trans-Golgi marker galactosyl transferase has demonstrated significant co-localization in HeLa cells but not in a non-permissive endothelial cell line (Pajusola *et al.*, 2002). This observation is very reminiscent of what has been reported for AAV5. The endocytosis of AAV5 also predominantly occurs through clathrin-coated vesicles and is mainly routed to the Golgi area in the HeLa cell. In this context, AAV5 virions can be detected within cisternae of the trans-Golgi network, within vesicles associated with cisternae, and within dictyosomal stacks of the Golgi apparatus (Bantel-Schaal *et al.*, 2002). The functional significance of rAAV localization in the Golgi compartment has been difficult to dissect. Studies correlating rAAV localization in the Golgi with the ability of infected cells to express an rAAV2-encoded GFP gene have failed to demonstrate a correlation between retained localization in the Golgi and the ability of a cell to express GFP (Pajusola *et al.*, 2002). Hence, it was hypothesized that a minority of viral particles may be the functional component that expresses encoded transgenes. Whether this small fraction of virus that leads to transgene expression routes through the Golgi, or some other compartment, remains to be determined.

## Escape from endosomal vesicles

Early studies have suggested that AAV escapes from the early endosome, yet requires an acidic environment to activate movement into the cytosol. In support of this notion, lysosomotropic agents, such as ammonium chloride, inhibit AAV2 infection only during the first 30 minutes after infection (Bartlett and Samulski, 1998; Bartlett *et al.*, 2000).

Real-time single-molecule imaging analysis has also demonstrated that a substantial fraction of rAAV has a diffusion coefficient consistent with free viral movement in the cytoplasm shortly after infection (Seisenberger *et al.*, 2001). Subcellular fractionation and tracking of AAV2 viral DNA by slot-blot hybridization suggests that virus quickly escapes from the early endosome with half-time of ~10 minutes and subsequently associates within the perinuclear region of HeLa cells (Xiao *et al.*, 2002). No detectable virus or free DNA was associated with the dense or late endosomal compartments in this study. Despite the relatively slow rate of nuclear uptake of rAAV2 in HeLa cells, this study also demonstrated that co-infection with adenovirus significantly enhanced the rate of rAAV2 nuclear accumulation (Xiao *et al.*, 2002). All of these lines of evidence support the theory that AAV2 is rapidly released from the early endosome compartment into the cytosol. However, these findings partially contradict other observations in HeLa cells using brefeldin A and bafilomycin A<sub>1</sub> to inhibit the early-to-late endosome transition and rAAV transduction (Douar *et al.*, 2001). These observations implicating a critical role for the late-endosome in rAAV trafficking and transduction have also been confirmed by density-gradient fractionation of cytoplasmic vesicles from 293 cells (Hansen *et al.*, 2001a).

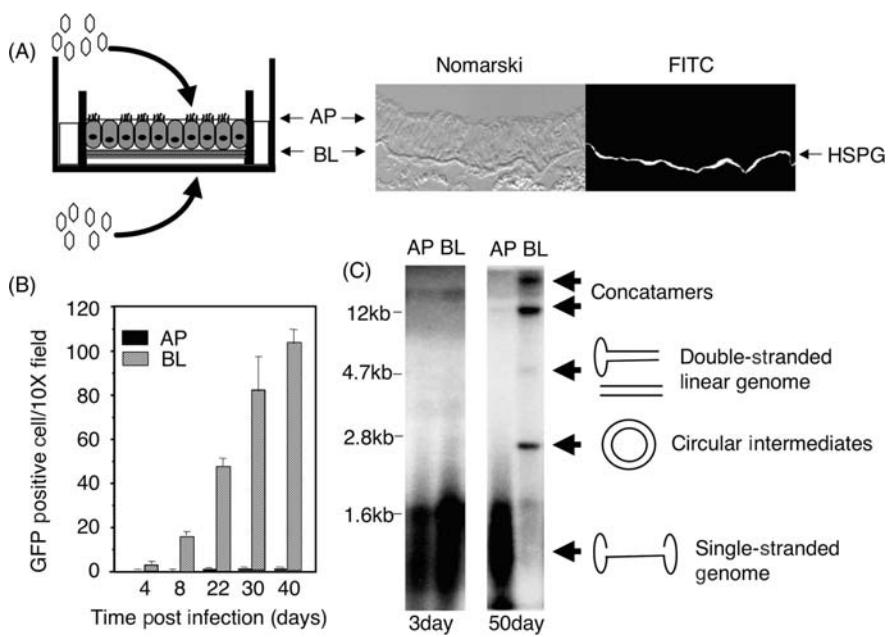
## Cell-type specificity of rAAV trafficking

The intracellular trafficking patterns of rAAV2 demonstrate significant cell-type specificity. Even among highly permissive cells, AAV2 appears to use distinct endosomal pathways for its cytoplasmic routing. For example, LN-229, a glioma cell line that is highly permissive for rAAV2, appears to use a different trafficking pathway than HeLa or 293 cells. In this context, bafilomycin A<sub>1</sub> has only a minor effect on AAV transduction in LN-229 cells (Pajusola *et al.*, 2002), a finding that differs from observations in HeLa and 293 cells (Douar *et al.*, 2001; Hansen *et al.*, 2001a). In contrast, rAAV transduction in several cell lines, such as murine fibroblast NIH3T3, human endothelial cells, Eahy-926, and IB3 human bronchial epithelial cells, is very low despite the efficient binding and internalization of rAAV2 (Hansen *et al.*, 2000; Pajusola *et al.*, 2002; Yan *et al.*, 2002; Smith *et al.*, 2003). Studies on AAV2 transduction in these less permissive cell lines reveal that impaired intracellular trafficking to the nucleus is predominantly responsible for their low transduction efficiency. For example, while AAV2 particles were found in both early endosomes and late-endosomes/lysosomes in 293 cells, they were localized predominantly to the early endosomal compartment in AAV2-infected NIH 3T3 cells (Hansen *et al.*, 2000). Moreover, treatment with bafilomycin A<sub>1</sub>, which decreases AAV2 transduction in 293 cells by inhibiting the acidification of the late endosome, had no effect on NIH 3T3 cells. All of these results suggest that in less permissive NIH 3T3 cells, AAV2 trafficking to the nucleus was retarded at the level of the early endosome.

Interestingly, this inefficient endocytic processing of AAV could be overcome by treatment of NIH 3T3 cells with hydroxyurea (HU). Although the mechanism of HU action remains unclear, treatment with this compound redirected virus to the late endosome/lysosome compartment sensitive to bafilomycin A<sub>1</sub> (Hansen *et al.*, 2001a). Altered patterns of intracellular movement for rAAV2 between permissive and non-permissive cell lines has also been observed in the context of Golgi localization. Eahy-926 cells, which are less permissive for rAAV2 transduction, demonstrate a lack of AAV2 targeting to the Golgi network in contrast to HeLa cells (Pajusola *et al.*, 2002).

In addition to cell type-specific differences in rAAV intracellular trafficking and transduction, cell polarity has also been demonstrated to significantly affect rAAV intracellular movement to the nucleus. Polarized airway epithelial cells grown at an air–liquid interface have provided a useful model for dissecting how rAAV transduction differs between apical and basolateral membrane infection. With reporter gene expression as an end point, rAAV2

transduction from the basolateral membrane is more than 200-fold higher than that from the apical surface (Figure 38.3) (Duan *et al.*, 1998b). Quantitative studies using radiolabeled rAAV2 demonstrated only slight differences (3–4-fold) in viral binding and uptake between these two membranes, a difference that cannot explain the 200-fold variance in expression from the basolateral and apical surfaces. Southern blotting analyses also confirmed near equivalent levels of viral DNA uptake and a similar pattern of single-stranded DNA genomes in cells at 3 days post infection, regardless of the membrane of entry (Figure 38.3C). However, at the later time point of 50 days post infection, only viral DNA following basolateral infection was efficiently converted to double-stranded circular and linear intermediates capable of expressing virally encoded genes. Viral DNA from apically infected cells was retained as single-stranded genomes (Figure 38.3C). These findings demonstrate that post-entry barriers specific to the apical membrane exist in polarized airway epithelial cells (Duan *et al.*, 2000b).



**Figure 38.3** Impaired intracellular processing, but not receptor-mediated endocytosis, represents a significant obstacle for AAV2 transduction from the apical membrane of human polarized airway epithelia. **(A)** Polarized human airway epithelia are cultured at an air–liquid interface and used as a model to study the polarity of rAAV2 transduction from the apical (AP) or basolateral (BL) membranes. Immunofluorescent localization of HSPG in human bronchus demonstrates predominant staining of this primary attachment receptor for AAV2 at the basolateral membrane (right panel of A). An identical pattern of staining is seen in primary polarized airway epithelial cultures (data not shown). **(B)** rAAV2 GFP encoding virus transduces polarized human airway epithelia from the apical surface (AP) much less efficiently than from the basolateral surface (BL). **(C)** Southern blot analysis of rAAV2 genomes from polarized airway epithelia following 3 and 50 days after infection. In spite of the lack of HSPG expression on the apical surface of polarized human epithelia, entry of rAAV2 from the apical surface at 3 days post infection is only 3- to 4-fold less efficient than entry from the basolateral surface. By 50-day post infection, the majority of viral DNA following apical infection remains as single-stranded viral genomes. In contrast, most of the viral genomes following basolateral infection are converted into double-stranded forms that migrate at 2.8 kb (circular monomer), 4.7 kb (linear monomer) and >12 kb (concatamers). Data presented in this figure were adapted from previous publications (Duan *et al.*, 1998b, 2000b). See also Color Plate 38.3.

## Cellular factors that influence intracellular trafficking of AAV

Cellular studies dissecting intracellular trafficking of AAV have suggested that the process is complex and dependent on multiple aspects of a cell's phenotype. Entry and movement of AAV through the cell is an active process facilitated by virus–host cell interactions at multiple levels. Beginning at the level of receptor binding, AAV attachment to its receptor and co-receptor may activate signaling pathways within the cell that initiate endocytosis. Once inside the endosomal compartment, AAV likely moves as a passive passenger directed by its cognate receptor or co-receptor exposed on the cytoplasmic face of the endosome. Once AAV breaks out of the endosome, it must again interact with cellular factors such as microtubules and/or microfilaments to make its way to the nucleus. Our understanding of these virus–host cell interactions presently remains very limited.

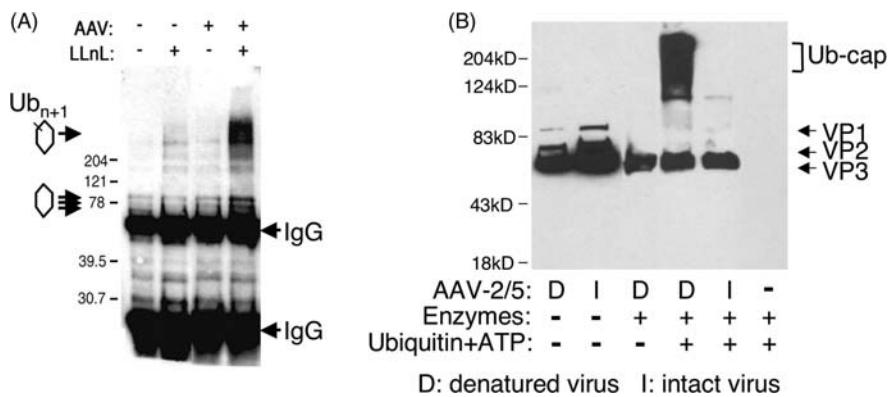
## Phosphatidylinositol-3 kinase (PI-3K) activation and the cytoskeletal network

Endocytosis and the sorting of integrin-linked receptors have been previously suggested to require phosphatidylinositol-3 kinase (PI-3K) activity (Ng *et al.*, 1999). The direct interactions between small GTP-binding proteins (i.e. Rac1, Rho, and Cdc42) and PI-3K have also been reported (Parsons, 1996; Brooks *et al.*, 1997). The activation of the PI-3K pathway is important for rAAV2 transduction since the intracellular movement of AAV2 particles to the nucleus via microtubule and/or microfilament is inhibited by

Wortmannin (Sanlioglu *et al.*, 2000b), which blocks PI-3K. Despite the fact that active Rac1 and PI-3K pathways are required for the internalization of the adenovirus (Li *et al.*, 1998a,b), inhibition of the PI-3K pathway does not appear to influence rAAV2 entry. In contrast Rac1 is required for rAAV2 endocytosis (Sanlioglu *et al.*, 2000b). Hence, the activation of Rac1 following rAAV2 binding to its receptor appears to be proximal to PI-3K activation. The activation of PI-3K and several small GTP-binding proteins can induce changes in the cytoskeletal network, such as the polymerization of monomeric actin (Tapon and Hall, 1997). The involvement of cytoskeletal elements in controlling the intracellular movement of viruses has long been recognized (Luftig and Lupo, 1994; Cudmore *et al.*, 1997; Dramsi and Cossart, 1998). The dependence of rAAV2 intracellular trafficking on an intact cytoskeleton is clear. For example, accumulation of AAV2 in the nucleus is dramatically reduced by treatment of cells with nocodazole or cytochalasin B, compounds that disrupt microtubules and microfilaments, respectively (Sanlioglu *et al.*, 2000b). Thus, in the context of rAAV2 infection, receptor activation of Rac1 appears to initiate endocytosis of virus and subsequent PI-3K-dependent endosomal movement of virus to its destined intracellular compartment.

## Ubiquitination of AAV and modulation of the proteasome pathway

An interesting discovery in the field of AAV intracellular processing was the recent finding that AAV particles are ubiquitinated in transduced cells (Duan *et al.*, 2000b) (Figure 38.4).



**Figure 38.4** Ubiquitination in rAAV transduction. **(A)** Inhibition of proteasome activity results in the accumulation of ubiquitinated AAV2 capsid in infected cells. rAAV2 infected human primary fibroblasts, in the presence (+) or absence (−) of proteasome inhibitor (LLnL), were harvested at 16 hours post infection. rAAV capsid proteins were first immunoprecipitated using an anti-AAV capsid antibody (Clone B1) followed by Western blotting with an anti-ubiquitin antibody. **(B)** Reconstitution experiments demonstrate that both rAAV2 and rAAV5 purified virions can be ubiquitinated in vitro.  $3 \times 10^8$  particles of rAAV5 (rAAV2 not shown) were incubated with ubiquitin conjugation enzymes at 37°C for 30 minutes and then resolved by SDS-PAGE. Western blotting with anti-AAV capsid antibody (Clone B1) showed an increase in the size of capsid proteins, reflecting modification by ubiquitination. Interestingly, the intact virus (I) was a poor substrate for ubiquitin conjugation, a process that was significantly increased following partial denaturation of viral particles (D) by heat treatment. These findings suggest that processing of the AAV capsid in the endosomal compartment, or following release into the cytoplasm, may prime AAV particles for ubiquitination. Data presented in this figure were adapted from previous publications (Duan *et al.*, 2000b; Yan *et al.*, 2002).

rAAV-mediated transduction can be enhanced by modulating proteasome activity in AAV-infected cells with proteasome inhibitors such as tripeptidyl aldehydes (LLnL, MG-132, Z-LLL) (Duan *et al.*, 2000b; Douar *et al.*, 2001; Yan *et al.*, 2002; Ding *et al.*, 2003) and anthracycline derivatives (aclacin or doxorubicin) (Yan *et al.*, 2004). Ubiquitination is a well-known targeting signal for endogenous or foreign protein degradation by the proteasome system. However, despite increased AAV-mediated transgene expression, the presence of proteasome inhibitors does not substantially prevent enzymatic degradation of the internalized virions. Nevertheless, rAAV nuclear trafficking increases, and ubiquitinated AAV capsid proteins accumulate, in the presence of these inhibitors (Duan *et al.*, 2000b; Yan *et al.*, 2004). *In vitro* reconstitution experiments have demonstrated that both AAV2 and AAV5 capsid proteins are substrates for conjugation with ubiquitin. Interestingly, it was found that the intact AAV particles were not preferred substrates for ubiquitination; ubiquitination of capsid proteins was substantially increased following a short period of heat denaturation of viral particles (Yan *et al.*, 2002). This result suggests that virion processing inside the endosomes might be important for priming AAV capsids for ubiquitination once they enter the cytoplasm. One hypothesis is consistent with current trafficking data and suggests that the acidic environment of the late endosome might lead to changes in viral particle conformation, allowing for the exposure or activation of lysine residues in the AAV capsid to become ubiquitin ligase substrates. Ubiquitin conjugation of AAV capsid proteins likely occurs after viruses escape from the endosomal compartment, as currently identified ubiquitination enzyme systems all exist outside the endosomal compartment. However, it is presently unclear if ubiquitination occurs in the cytoplasm and/or the nucleus. Ubiquitination of capsid proteins could act to direct viral movement to the nucleus through the association of ubiquitin-dependent nuclear receptors and/or may also play a role in the priming for virion uncoating.

Another interesting finding is that proteasome inhibition does not uniformly increase AAV transduction in all cell types. Inhibition of the proteasome in endothelial cells has little effect on rAAV transduction (Pajusola *et al.*, 2002). Furthermore, certain cell types appear to be induced to a greater or lesser extent with various classes of proteasome inhibitors (Yan *et al.*, 2004). For example, in two airway cell lines (A549 and IB-3), induction of rAAV2 and rAAV5 transduction demonstrates unique cell line-dependent specificities to either anthracycline or tripeptidyl aldehyde proteasome inhibitors. These findings suggest that proteasome-modulating agents may act by more than one mechanism to enhance rAAV transduction. In support of this hypothesis is the observed synergistic action of combined proteasome inhibitor classes to induce rAAV transduction in polarized airway epithelial cells (Yan *et al.*, 2004). Cell type-specific influences of the ubiquitin/proteasome pathway on rAAV transduction *in vivo* have also been

demonstrated. Administration of proteasome inhibitors significantly improved rAAV-mediated gene transfer in the lungs of mice, but not in muscle (Duan *et al.*, 2000b). It is presently unclear if these differences reflect a fundamental variation in the mechanisms of rAAV transduction in different cell types (i.e. no need for capsid ubiquitination in the life cycle of the virus) or if they simply reflect an altered capacity of various cell types to ubiquitinate and effectively deliver virus to the nucleus. Since proteasome inhibitors generally appear to be less effective at increasing rAAV transduction in highly transducible cell lines, the latter explanation is likely the case.

Although a detailed understanding of ubiquitin/proteasome function in rAAV transduction remains unclear, there are several plausible hypotheses that are consistent with current findings. First, inhibition of the ubiquitin/proteasome pathway may alter endosomal maturation and sorting of rAAV in a manner that more efficiently releases virus into the cytoplasm. Second, ubiquitination of the capsid may directly participate in trafficking virus to the nuclear pore. Third, ubiquitination may be a fundamental component of the viral life cycle required for uncoating. Further research is required to understand whether one or all of these mechanisms play a role in rAAV transduction and whether they are universal for all AAV serotypes.

## NUCLEAR TRANSLOCATION/VIRION UNCOATING

Indirect evidence using Cy3-labeled virus suggests that AAV particles accumulate in a perinuclear region before entering the nucleus. This work also reveals that the process of viral entry into the nucleus is particularly slow, as most of the viral signal is concentrated in the perinuclear region for up to 24 hours (Pajusola *et al.*, 2002; Xiao *et al.*, 2002). In contrast, other studies have suggested that viral DNA or intact virions can be detected in the nucleus within 2–4 hours (Bartlett *et al.*, 2000; Sanlioglu *et al.*, 2000b). It is not surprising that conflicting rates of AAV movement to the nucleus are reported for different cell lines. However, it is difficult to completely understand the reason for conflicting reports regarding the rate of AAV movement to the nucleus in the most highly investigated HeLa cell line. One plausible explanation for these differences observed in HeLa cells may include different methods for viral purification and labeling.

The location and mechanism(s) of AAV uncoating are unclear. Three-dimensional confocal reconstruction of nuclei, following Cy3-AAV2 infection, suggests that AAV2 can move through the nuclear pore complex prior to uncoating (Sanlioglu *et al.*, 2000a,b) possibly by free diffusion. Reconstitution experiments have suggested that AAV virions can accumulate within isolated nuclei that do not possess functional nuclear pore complexes (Hansen *et al.*,

2001b). The concept of AAV nuclear transport as a rate-limiting step in AAV infection has been increasingly appreciated. Treatment of cells with proteasome inhibitors or hydroxyurea appear to enhance nuclear uptake of virus and similarly enhance transduction (Duan *et al.*, 2000b; Pajusola *et al.*, 2002; Yan *et al.*, 2004). Furthermore, co-infection with empty adenovirus particles enhances nuclear uptake and transduction with rAAV (Xiao *et al.*, 2002). However, our current understanding of the molecular processes and mechanisms affected by these approaches remains very limited.

## rAAV GENOME CONVERSION

The last stage of the AAV infectious pathway is the molecular conversion of the single-stranded AAV genome into transcriptionally active intermediates. This step can only efficiently occur in the nucleus following virion uncoating. Latent infection can occur following infection with both wild-type AAV (wtAAV) and rAAV vectors. However, it should be stressed that molecular intermediates for latent wtAAV and rAAV may be very different. In support of this concept is rep-mediated integration at the AAV-S1 locus that occurs for wtAAV but not rAAV vectors (Giraud *et al.*, 1994; Kotin *et al.*, 1992) (see Chapter 16). Interesting, relatively little is known about the structure of latent wtAAV genome intermediates, since the wild-type viral genome cannot be manipulated to study genome structure as with rAAV vectors. This section will only discuss aspects of rAAV genome conversion, recognizing that these intermediates may be unique from those found with wtAAV.

### Second-strand synthesis

Full-length conventional rAAV vectors deliver a single-stranded DNA genome to the nucleus in infected cells. To express an encoded transgene, this single-stranded viral DNA must be converted into a duplex form through second-strand synthesis by host cell-mediated DNA synthesis. This step is recognized as a potentially important rate-limiting step in rAAV transduction (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). Others have suggested that second-strand synthesis is not the major pathway for double-stranded DNA formation in the liver. Instead, self-annealing of the incoming plus and minus single-stranded viral genomes was suggested to be responsible for AAV transduction at very high multiplicities of infection (Nakai *et al.*, 2000b). This possibility, however, does not appear to agree with most model systems. The development of a new class of rAAV vectors, which do not require second-strand synthesis for gene expression, has now led to the generally accepted notion that second-strand synthesis is an important rate-limiting factor for rAAV transduction in many systems. The rate-limiting aspect of second-strand synthesis can be overcome

by self-complimentary AAV (scAAV) or double-stranded AAV (dsAAV) vectors. These viruses which contain half-length genomes (<2.5 kb) have been shown to package either two annealed single-strand genomes with open ends (—, i.e. dsAAV) or replication form (Rf) monomer genomes with a single covalently closed end (—, i.e. scAAV) (McCarty *et al.*, 2001; Fu *et al.*, 2003; McCarty *et al.*, 2003; Wang *et al.*, 2003).

These scAAV/dsAAV vectors have been useful in addressing whether viral genome conversion is rate limiting in various systems. For example, in polarized human airway epithelial models, genome conversion does not appear to be rate limiting when the primary barrier to efficient transduction is intracellular trafficking of virus to the nucleus (Ding *et al.*, 2003). In these studies, transduction efficiencies with full-length rAAV and scAAV/dsAAV vectors are similar. However, when intracellular barriers are sufficiently circumvented by treatment with doxorubicin to inhibit the proteasome, second-strand synthesis becomes rate-limiting and scAAV/dsAAV vectors have a more rapid onset of gene expression as compared with full-length rAAV vectors (Yan *et al.*, 2004).

It is generally accepted that in most model systems only a small fraction of viral genomes effectively make it to the point of genome conversion capable of expressing an encoded transgene. The majority of viral DNA in most systems is retained as single-stranded genomes, most of which are metabolized and degraded over time. This is most evident by tracking viral genomes over time by Southern blotting and the observation that only a small fraction of input genomes persist as genome conversion products. The most traditional models of genome conversion suggest that second-strand synthesis is a self-priming process (similar to AAV replication during the lytic phase but with the use of the host DNA synthesis system) (Ferrari *et al.*, 1996; Fisher *et al.*, 1996). Molecular evidence to support this concept includes the discovery of a cellular single-strand D-sequence binding protein (ssD-BP) (Qing *et al.*, 1997). The efficiency of rAAV transduction in different cell types and tissues seems to correlate with tyrosine phosphorylation of a cellular single-stranded D-sequence binding protein (ssD-BP). The phosphorylation of ssD-BP by epidermal growth factor receptor protein tyrosine kinase (EGFR PTK) promotes binding of ssD-BP to the D-sequence in the inverted terminal repeat (ITR) and blocks DNA synthesis at the 3' hydroxyl end of the genome. Dephosphorylation facilitates the detachment of ssD-BP and activates second-strand synthesis by self-priming (Mah *et al.*, 1998; Qing *et al.*, 1997, 1998). It is now known that in HeLa cells, the ssD-BP is the cellular protein that binds the immunosuppressant drug FK506, termed the FK506-binding protein 52 (FKBP52) (Qing *et al.*, 2001, 2003).

rAAV2 transduction can be augmented by adenoviral gene products (Ferrari *et al.*, 1996; Fisher *et al.*, 1996; Duan *et al.*, 1999b), genotoxic stimuli such as UV irradiation and hydroxyurea (Yakobson *et al.*, 1989; Russell *et al.*, 1995;

Sanlioglu *et al.*, 1999), heat-shock treatment (Yakinoglu *et al.*, 1988; Zhong *et al.*, 2004), and cellular redox stress (Sanlioglu and Engelhardt, 1999). The augmentation in transgene expression is accompanied by an increase of *de novo* viral DNA synthesis. It is believed that most of these stimuli induce cellular factors important for the conversion of a single-stranded viral genome into an expressible duplex form. However, as various intracellular barriers to rAAV transduction have become clearer, this notion has been challenged for certain of these agents. Interestingly, induction of genome conversion intermediates appears to be stimulus-specific. For example, UV irradiation has been demonstrated to augment the generation of rAAV circular transduction intermediates (Sanlioglu *et al.*, 1999), while Rf linear intermediates are induced by adenoviral E4ORF6 (Ferrari *et al.*, 1996; Fisher *et al.*, 1996; Duan *et al.*, 1999b). Interestingly, the E2a gene product of adenovirus was seen to increase the abundance of circular intermediates (Duan *et al.*, 1999b). The dynamics of genome conversion products for rAAV seen under various cellular conditions has led to a renewed interest in understanding the mechanisms responsible for second-strand synthesis and genome recombination. Efforts in this area have led to the development of several novel rAAV vectoring strategies (see Chapter 39).

### rAAV genome circularization and concatamerization

In the absence of a helper virus or genotoxic stimuli, rAAV will enter a latent life cycle in which the viral genome has the potential to either integrate randomly into the genome or persist as an episomal provirus. It has been suggested that the formation of double-stranded circular transduction intermediates are very important for persistent transgene expression in certain tissue types such as muscle (Duan *et al.*, 1998a, 1999b, 1999c). The existence of episomal AAV genomes has been suggested for many years based on several indirect observations (Flotte *et al.*, 1994; Afione *et al.*, 1996). However, the first conclusive evidence for the existence of circular AAV genomes was obtained using a shuttle rAAV vector containing a bacterial replication origin and an ampicillin-resistant gene. Circularized AAV2 genomes were successfully retrieved from rAAV-infected cell lines and muscle tissue *in vivo* (Duan *et al.*, 1998a). Results of Southern blot analysis and sequencing on these transduction intermediates suggest that the generation of the monomer circular genome is the product of viral genome head-to-tail self-circularization through ITR homologous recombination at both ends of the genome (Duan *et al.*, 1999c). These studies also revealed the existence of larger circular intermediates containing two or more copies of the viral genome. Furthermore, the percentage of circular dimer and concatameric genomes of high molecular weight increased in a time-dependent manner, correlating with stable, long-term transgene expression in the muscle (Duan

*et al.*, 1998a). The formation of AAV circular intermediates has also been confirmed by independent studies from other laboratories (Nakai *et al.*, 1999; Vincent-Lacaze *et al.*, 1999; Musatov *et al.*, 2000). Whether circular AAV genomes represent pre-integration intermediates remains to be systematically determined. However, a recent study comparing integration of circular and linear AAV-based plasmids shows that circular molecules integrate three times more efficiently than the linear form in the presence of rep proteins (Tsunoda *et al.*, 2000).

Subsequent research evaluating the mechanisms of concatamerization has demonstrated these large AAV genome concatamers originated through intermolecular recombination. In these experiments, two independent rAAV vectors containing either enhanced green fluorescent protein (EGFP) or alkaline phosphatase reporter genes were co-administered into the tibialis anterior muscles of mice, and the molecular structure of circular intermediates was analyzed. Results from these experiments confirmed that the formation of circular concatamers was a result of intermolecular recombination rather than *de novo* replication from a single viral genome (Yang *et al.*, 1999). Molecular analysis of these concatamers suggested that ITR-mediated intermolecular recombination in muscle was the likely basis for the formation of single circular molecules containing two distinct AAV genomes. Based on these findings, new AAV vectoring strategies, which use two independent AAV vectors to deliver an oversized transgene expression cassette, have been developed to effectively double the 4.7 kb limited package capacity of a single rAAV vector (Sun *et al.*, 2000; Yan *et al.*, 2000; Duan *et al.*, 2000a; Nakai *et al.*, 2000a) (see Chapter 39).

### CONCLUDING REMARKS AND FUTURE CHALLENGES

Over the last decade, enthusiasm for rAAV as a gene delivery tool and therapeutic vehicle has propelled basic studies on the molecular virology of rAAV transduction to new heights. In turn, this expanding knowledge on mechanisms of rAAV transduction has greatly improved current rAAV vector systems. The identification of cell surface receptors/co-receptors, and the isolation of different AAV serotypes, has been critical for expanding the repertoire of rAAV vector best suited for a specific target tissue. Similarly, a better understanding of intracellular rate-limiting steps in rAAV transduction has led to methods capable of enhancing rAAV movement to the nucleus, uncoating, and/or genome conversion to transgene expressing forms. Despite these advances in AAV biology, our knowledge of rAAV transduction remains fairly superficial. For example, it remains unclear what cellular proteins and signaling processes influence rAAV binding, endocytosis, and movement of virus to the nucleus for most AAV serotypes. Similarly, little is known

about the molecular mechanisms that control virion uncoating and debate remains regarding whether this occurs in the cytoplasm or the nucleus. To this end, several important questions require further investigation including:

- What are the various receptor-mediated endocytic mechanisms for different serotypes of rAAV and how do these pathways differ between cell types?
- How do various entry pathways for rAAV influence virion intracellular fate?
- What are the cell signaling pathways involved in rAAV endosomal sorting and how do different endosomal pathways influence priming of rAAV for nuclear transport and/or uncoating?
- How does rAAV escape from the endosomal compartment and translocate into the nucleus?
- What are the mechanisms of virion uncoating, viral genome conversion, and circularization/concatamerization?

Each of these questions has potentially different answers for a given rAAV serotype and target cell type. However, it is also likely that common themes will also emerge for certain mechanisms of rAAV transduction as our understanding of these processes becomes clearer. As information on the structure of various AAV serotypes continues to emerge, and genetic approaches clarify the amino acids in the virion capsid necessary for efficient transduction, it will become increasingly easier to dissect how AAV capsid proteins direct various stages of rAAV transduction within the cell. With enhanced understanding of rAAV transduction biology, fundamental paradigms for improving rAAV vector system for human gene therapy will undoubtedly emerge.

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# Expanding the capacity of AAV vectors

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A long-standing challenge with the use of adeno-associated virus (AAV) vectors is the relatively small packaging capacity of the virus. AAV is one of the smallest viruses currently known with an average size of  $\sim 200\text{--}250 \text{ \AA}$  (Atchison *et al.*, 1965). In wild-type AAV (wtAAV), a  $\sim 4.7 \text{ kb}$  single-stranded DNA genome is surrounded by an icosahedral capsid protein shell consisting of 60 identical subunits (Xie *et al.*, 2002). In recombinant AAV (rAAV), the genome size can be increased up to 110 percent of the wtAAV (Dong *et al.*, 1996). However, the packaging efficiency suffers greatly when the genome size is increased to 5.15 kb and above (Dong *et al.*, 1996).

Despite the size limitation, rAAV has evolved into a leading gene therapy vehicle over the past decade. In this system, the therapeutic expression cassette, which includes a promoter, a gene, and a polyA sequence, is placed between two AAV-inverted terminal repeats (ITRs). The ITRs direct the packaging of the single-stranded viral genome into a pre-assembled capsid (King *et al.*, 2001). The ITRs also play a critical role in converting the single-stranded viral genome into a double-stranded, transcription-competent form *in vivo*. The AAV ITR is approximately 0.15 kb long. Assuming a 0.6 kb promoter and a 0.4 kb polyA signal are used in an rAAV construct, the maximal cDNA size that can be expressed by rAAV is  $\sim 3.9 \text{ kb}$ . Although this length is sufficient for many therapeutic genes, such as factor IX for hemophilia B, a significant number of other genes are excluded by this limitation. These include the cystic fibrosis transmembrane regulator (CFTR) gene for cystic fibrosis therapy and the minidystrophin gene for Duchenne and Beck muscular dystrophy (DMD and BMD) therapy.

Owing to the packaging limitations that accompany rAAV vectors, strategies for deleting less critical regions of therapeutic cDNAs have been developed. Shortcomings of this

approach include the potential for less effective transgenes that do not completely complement genetic diseases. For example, massively truncated microdystrophin genes are currently being explored for DMD/BMD gene therapy. Microdystrophins carry only 30 percent of the full-length dystrophin cDNA and do not contain the C-terminal domain. Preliminary studies in a mouse DMD model have revealed certain functional improvement in both limb muscle and cardiac muscle following expression of this transgene (Wang and Xiao, 2000; Harper *et al.*, 2002; Yue *et al.*, 2003). However, microgenes have failed to restore the diaphragm strength to a normal level (Harper *et al.*, 2002). In addition to a potential reduction in the functional efficacy of a gene product, gene truncation, as an approach to circumvent the packaging limitation of rAAV, must be individually tailored for each target gene. This process can often be a very daunting task requiring many years of basic research on the structure-function relationship of the protein product.

A more versatile alternative to creating minigene cassettes is to develop generic methods that expand rAAV packaging capacity. However, these methods must be compatible with the inherent physical properties of the AAV virion that set the maximal viral genome size limit. To overcome this hurdle, researchers have developed novel dual-vector methods capable of reconstituting intact transgene cassettes from two independent input vectors. In this context, the two independent recombinant viral particles are produced and each virus carries a portion of an intact transgene expression cassette. Therapeutic gene expression is achieved from a reconstituted viral genome following simultaneous infection of both viruses. Currently, three mechanistically distinct dual-vector methods have been described, including *cis*-activating vectors, *trans*-splicing vectors, and overlapping vectors (Duan *et al.*, 2000, 2001; Nakai *et al.*, 2000; Sun *et al.*, 2000;

Yan *et al.*, 2000; Chao *et al.*, 2002; Halbert *et al.*, 2002; Reich *et al.*, 2003). In this chapter, we will review the molecular basis and gene therapy applications of these higher capacity dual-AAV vector systems.

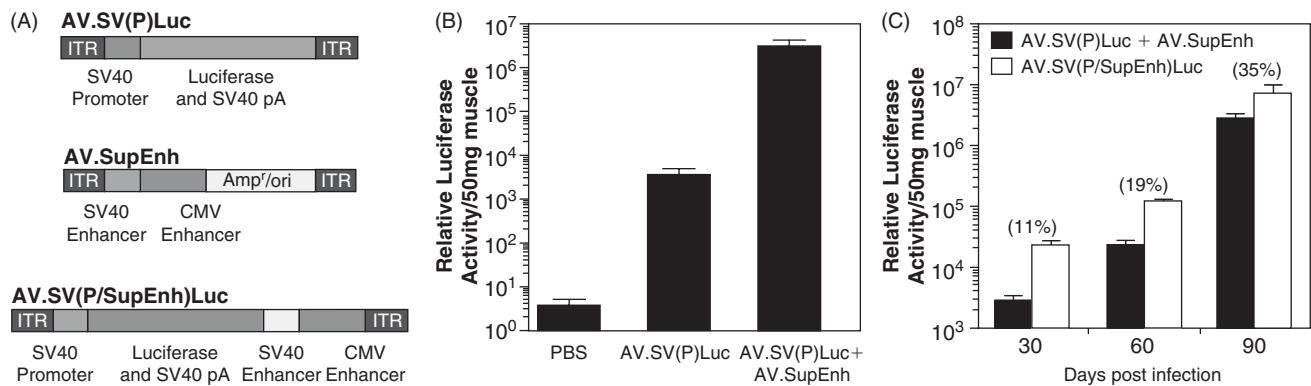
## MOLECULAR BASIS AND PROOF OF PRINCIPLE STUDIES

### Cis-activating vectors

Efficient transgene expression is tightly controlled by regulatory elements, such as promoters, enhancers, silencers, insulators, and polyA sequences. Many regulatory elements can act at a distance from the gene-coding sequence. A salient feature of AAV transduction is viral genome concatamerization. Integration of the concatameric proviral genome may occur within the liver (Miao *et al.*, 1998) and in skeletal muscle (Xiao *et al.*, 1996; Fisher *et al.*, 1997). More recently, however, it has been suggested that circular episomal genomes may be the predominant form in tissues such as skeletal muscle (Duan *et al.*, 1998; Schnepf *et al.*, 2003). Interestingly, with the use of an rAAV bacterial shuttle vector, circular episomal dimers, trimers, and multimers have been rescued from muscle tissue (Duan *et al.*, 1999a,b). To further detail the molecular mechanisms underlying rAAV concatamer formation, Yang *et al.* used a co-infection strategy in skeletal muscle to demonstrate that rAAV genome concatamers form, at least in part, through a process of intermolecular recombination (Yang *et al.*, 1999). In this study, a significant proportion of larger circular concatameric viral genomes were found to consist of physically linked genomes from two independent viruses revealing a

previously unknown aspect of AAV transduction biology and providing a theoretical foundation for splitting regulatory elements from the core expression cassette in a dual rAAV vector system. In principle, this approach could deliver regulatory elements, such as a tissue-specific enhancer and/or promoter, with one rAAV vector while delivering a therapeutic gene in a second rAAV vector. This method is clinically attractive because many disease genes, such as the CFTR gene, approach the packaging capacity of a single AAV vector leaving no space for strong promoter or enhancer sequences. The finding that the ITR can also function as a weak promoter (Flotte *et al.*, 1993) also suggested that transcriptional activation of the ITR using this approach might be useful for vectors that completely lack a promoter.

This dual rAAV vector approach predicts that intermolecular concatamerization will join the two viral genomes combining enhancer sequences with the transgene. This process should then allow for *cis*-activation of transcription within the same concatamer vector genome. Importantly, *cis*-activation of transcription is also predicted to be independent of the orientation of the recombined viral genome. To test these hypotheses, a 'super-enhancer' rAAV vector was constructed, carrying two potent viral enhancers (simian virus 40 enhancer and cytomegalovirus immediate early enhancer) (Figure 39.1). Two AAV luciferase viruses were also constructed, one driven by a minimal SV40 promoter, and the other driven by the intrinsically weak promoter activity of the AAV ITR. As expected, co-infection of the super-enhancer virus with either luciferase virus boosted luciferase expression by 200–600-fold in skeletal muscle (Figure 39.1) (Duan *et al.*, 2000). Molecular analysis of rescued concatamer genomes also confirmed that this *cis*-activation of transcription was independent of the direction of intermolecular concatamerization (Duan *et al.*, 2000).



**Figure 39.1** Cis-activation increases rAAV transduction in muscle. (A) Schematic outlines of the vectors used in this dual rAAV vector approach. (B) Levels of luciferase expression from PBS, single-vector, and super-enhancer AAV virus co-infected muscles at 90 days post infection. (C) The relative efficiency of intermolecular cis-activation following dual-vector infection, as compared to single-vector administration with all the enhancer sequences, was evaluated at 30, 60, and 90 days post infection. The data represent the mean ( $\pm$  SEM) of four to six independent muscle samples for each experimental condition. Percentages in brackets above each paired set represent relative percent of transgene expression achieved via the dual-vector approach as compared to maximal expression with a single vector (adapted from Duan *et al.*, 2000).

## Trans-splicing vectors

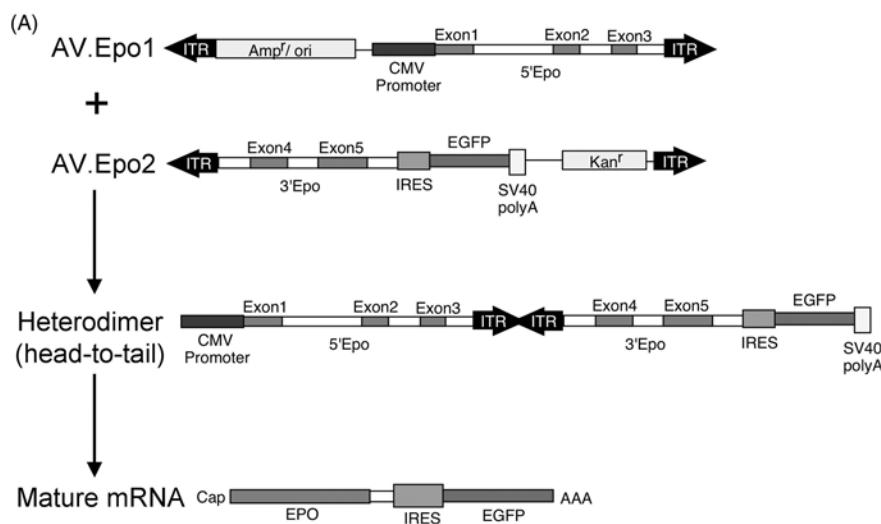
*Cis*-activation is an efficient way to enhance expression of therapeutic genes that fit into a single AAV particle. However, this approach will not work for many other genes whose coding sequences are longer than the ~5 kb limit inherent with rAAV. To address this issue, researchers have developed a *trans*-splicing rAAV vector approach (Nakai *et al.*, 2000; Sun *et al.*, 2000; Yan *et al.*, 2000; Duan *et al.*, 2001; Chao *et al.*, 2002; Reich *et al.*, 2003). This strategy also exploits intermolecular recombination of the rAAV genome.

During lytic infection, progeny AAV genomes are generated through self-priming and replication. The resultant multimer intermediates are in head-to-head and tail-to-tail tandem repeats. In latent infection, AAV concatamers are derived from ITR-mediated, intermolecular recombination. Interestingly, these latent concatamers are organized in a less random fashion with an apparent preference for head-to-tail and less frequently head-to-head and tail-to-tail arrays (Yang *et al.*, 1999). The existence of an apparently preferred head-to-tail arrangement of concatamers offered the opportunity for reconstituting two segments of a large therapeutic gene within concatamers. To this end, the approach for *trans*-splicing reconstitution of two mini-exons from two independent vectors was born as an approach to deliver large transgenes with rAAV vectors. However, a critical issue still remained. In the centre of a head-to-tail AAV concatamer is the double-D ITR junction (Xiao *et al.*, 1997; Duan *et al.*, 1999b). Strategies using this approach must then be developed to efficiently remove the ITR junction and restore an open reading frame (ORF) from the reconstituted transgene.

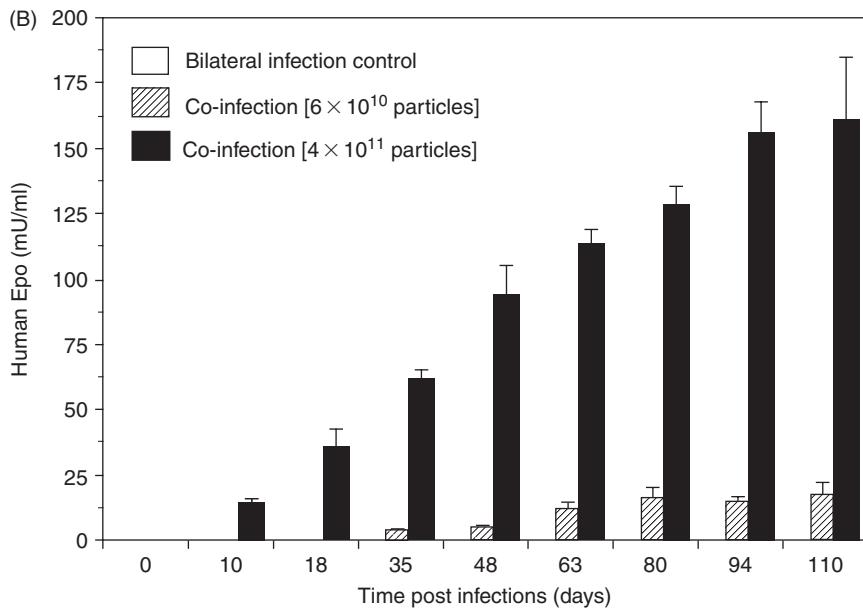
In contrast to prokaryotic genes, eukaryotic genes are composed of protein-coding sequences called exons and intervening non-protein coding sequences called introns. Both exons and introns are transcribed into pre-mRNA transcripts. Pre-mRNA transcripts are processed into mature mRNA by the removal of intronic sequences through a process called splicing. Splicing is mediated by

the spliceosome complex, which specifically binds to sequences in the junctions of introns and exons, to facilitate the catalytic removal of intronic sequences (Sharp, 1988; Nilsen, 2003). The use of this naturally occurring mechanism provided the ideal approach to process pre-mRNA transcripts derived from two independent rAAV genomes joined as heterodimers. In this context, a single heterodimer-derived pre-mRNA transcript can be processed by ‘*trans*-splicing’ across sequences coded by two vector genomes. This approach of vector *trans*-splicing should not be confused with other gene therapy techniques such as spliceosome-mediated mRNA *trans*-splicing (SMART) that mediates splicing across two independent transcripts (Liu *et al.*, 2002; Chao *et al.*, 2003). To test the feasibility of this *trans*-splicing approach, early studies divided the human erythropoietin (*Epo*) gene at its third intron and cloned 5' and 3' portions into two independent ‘donor’ and ‘acceptor’ rAAV vectors (Figure 39.2A) (Yan *et al.*, 2000). Based on the findings that co-infected rAAV genomes form head-to-tail concatamers, it was anticipated that head-to-tail concatamers between donor and acceptor viruses would functionally reconstitute an intact *Epo* pre-mRNA. Subsequent splicing of pre-mRNA would then lead to the production of *Epo* mRNA and protein. Consistent with this hypothesis, researchers found therapeutic levels of erythropoietin in the serum of mice following co-infection of the tibialis muscle with both donor and acceptor vectors (Figure 39.2B). Administration of either the donor or acceptor vector alone, however, did not produce erythropoietin *in vivo* (Yan *et al.*, 2000).

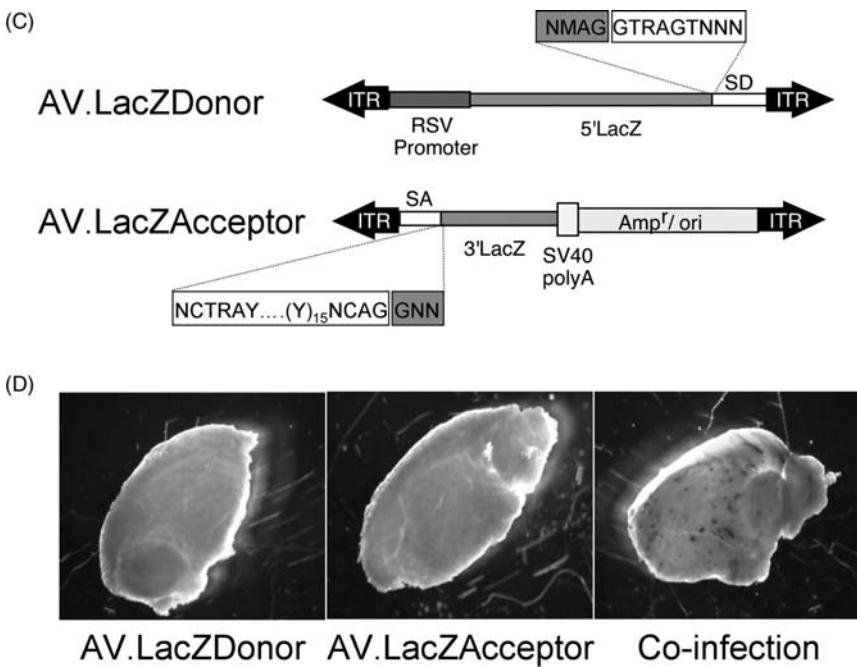
Although the use of the *Epo* gene locus was useful in demonstrating the feasibility of rAAV vector *trans*-splicing approaches, the usefulness of this approach to expand packaging capacity for large disease genes requires that it be applied in the context of cDNA sequences. To this end, a β-galactosidase cDNA was developed as a model system (Sun *et al.*, 2000; Duan *et al.*, 2001; Reich *et al.*, 2003). One critical issue is to define an appropriate site for dividing the cDNA into two segments. The conserved consensus



**Figure 39.2** Trans-splicing rAAV vector-mediated gene expression in muscle. (A) Schematic diagram of *Epo* trans-splicing vectors, AV.Epo1 and AV.Epo2. These vectors encode either the 5' or 3' genomic segment of the human *Epo* gene and various bacterial selectable markers.



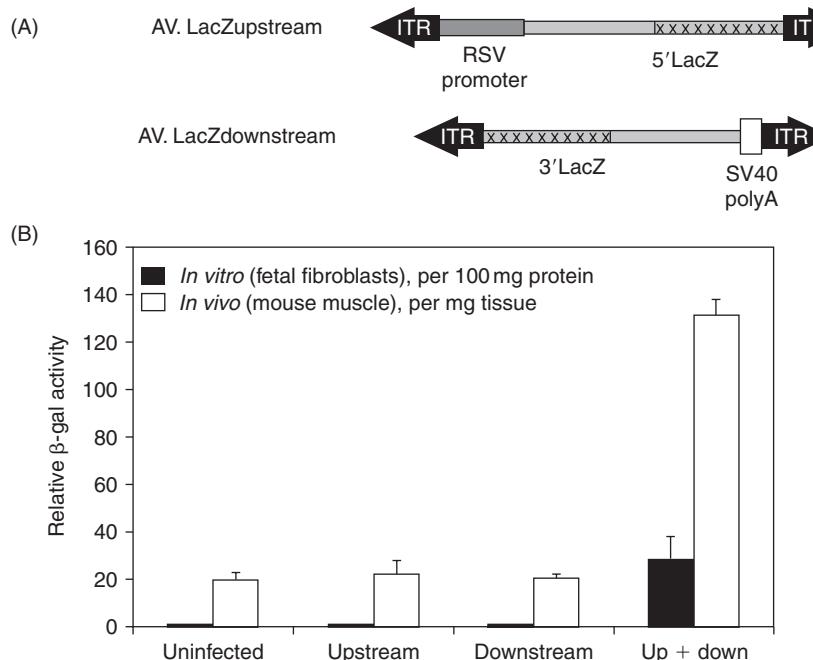
**Figure 39.2 (B)** Human Epo level in mice infected with Epo trans-splicing vectors. Results depict the mean ( $\pm$  SEM) for:  $N = 6$  (virally co-infected low dose group),  $N = 4$  (virally co-infected high dose group), and  $N = 4$  (control groups) independent animals in each group.



**Figure 39.2 (C)** Schematic diagram of the LacZ trans-splicing vectors. SD, splicing donor; SA, splicing acceptor. The consensus sequences at the exon/intron boundaries are highlighted for both donor and acceptor vectors. M, adenine or cytosine; R, adenine or guanine; Y, cytosine or thymidine. **(D)** The anterior tibialis muscles of 7-week-old BL6 mice were infected with either the donor or acceptor virus alone or co-infected with both viruses. In situ LacZ staining was performed at 7 weeks post-infection. LacZ positive myofibers were only seen in co-infected muscles. (Panel A and B are adapted from Yan et al., 2000; Panel D is adapted from Duan et al., 2000).

splicing sequences occur at the exon–intron junctions and certain regions within the interior of introns. These conserved sites act as binding site for the small nuclear ribonucleoproteins (snRNPs) that form the spliceosome complex. As such, potential sites for the introduction of splice consensus sequence within a cDNA includes the conserved motif ‘N-M-A-G//G-N-N’. The ‘N-M-A-G’ represents the end of the donor exon and the ‘G-N-N’ represents the beginning of the acceptor exon (Senapathy *et al.*, 1990). In a given therapeutic gene, if its genome structure has been

determined, the endogenous exon–exon junction can also be used for dividing a cDNA into two exons. A PCR-mediated approach can then be used to insert the intron splicing signals into a selected site within the cDNA. These intron splicing signals can be derived from a small endogenous intron, a heterologous intron, or a synthetic intron. As shown in Figure 39.2C, the *LacZ* gene has been used to test this approach. In this case, the *LacZ* cDNA was divided between nucleotide 2742 and 2743 and a 132 bp chimeric intron from the commercially available plasmid pCI



**Figure 39.3** Overlapping approach reconstitutes LacZ expression. (A) Schematic outline of rAAV overlapping vectors used to test dual vector delivery of LacZ. The homologous region is denoted by 'xxxxx'. (B)  $\beta$ -galactosidase expression from overlapping AAV viruses was assayed at 3 days and 7 weeks post infection in fetal fibroblasts and murine skeletal muscle, respectively. The data represent the mean ( $</>$  SEM) of three in vitro samples and four in vivo samples for each experimental condition. (Adapted from Duan et al., 2001.)

(Promega, #E1731) was inserted. Vectors generated from this approach were tested for their ability to reconstitute a functional *LacZ* minigene following infection in tibialis muscle of mice. Only muscles co-infected by both AV.LacZDonor and AV.LacZAcceptor vectors produced  $\beta$ -galactosidase enzyme activity (Figure 39.2D).

## Overlapping vector

Despite the exciting promise of *trans*-splicing approaches to expand the packaging capacity of the rAAV vector, this approach has its limitations:

- The therapeutic cDNA must be divided at a limited number of sites that conform to the conserved exon–intron motif.
- Vector construction is technically challenging and often requires complicated PCR amplification to divide the cDNA into two parts.
- Recombination events must occur in only one orientation to produce functional pre-mRNA transcripts.
- Since intermolecular recombination between vector genomes can also occur in head-to-head and tail-to-tail orientations (although apparently at somewhat lower frequency), truncated protein products can also conceivably be generated. This last issue, although not observed in the *Epo* and *LacZ* examples discussed above, could potentially be more of a concern for proteins whose domains might function in a dominant manner.

To explore alternative approaches to circumvent these limitations, researchers have developed a homologous

recombination strategy to reconstitute large transgenes using overlapping vectors (Duan et al., 2001; Halbert et al., 2002).

Homologous recombination was first suggested to occur with AAV genomes following the observation that a deleted region of a terminal palindrome could be self-repaired by an intramolecular correction mechanism (Senapathy and Carter, 1984). Homologous recombination involves the pairing of two identical DNA fragments and subsequent strand invasion and resolution. This recombination process requires a break in the DNA backbone to initiate strand invasion (Radding, 1982; West, 2003). Since rAAV is a single-stranded DNA virus and retains a free end for invasion, it seems plausible that rAAV might efficiently mediate recombination with sites of DNA homology in the vector genome. The finding that rAAV is capable of efficient recombination with homologous sites in genomic DNA supports this notion (Russell and Hirata, 1998; Liu et al., 2004). If indeed similar recombination events could occur between two independent vector genomes with overlapping homology, this approach could be used to expand rAAV packaging capacity. An rAAV overlapping vector delivery approach was tested by two laboratories using either the alkaline phosphatase (Halbert et al., 2002) or  $\beta$ -galactosidase (Duan et al., 2001) cDNAs. The approaches used by these two groups were similar and involved dividing the cDNA into two overlapping segments for packaging into two independent rAAV vectors. An example of this technology is illustrated in Figure 39.3 where the Rous sarcoma virus (RSV) promoter and the first two-thirds of the *LacZ* gene were cloned into an 'upstream' rAAV vector and the 3' two-thirds of the *LacZ* gene and the SV40polyA were cloned into a 'downstream' rAAV vector. Overlapping regions between these two vectors included 1 kb of the *LacZ*

cDNA (Figure 39.3A). As shown in Figure 39.3B, co-infection with both the upstream vector and the downstream vector resulted in *LacZ* expression in fetal fibroblasts *in vitro* and in mouse limb muscle *in vivo*. Single infection with either vector alone, however, did not yield any detectable *LacZ* protein. Similar results in the lung were seen with overlapping alkaline phosphatase vectors (Halbert *et al.*, 2002). These pilot studies proved the feasibility of using rAAV to deliver large genes with overlapping vectors.

## APPLICATIONS OF DUAL-rAAV VECTOR METHODS IN GENE THERAPY

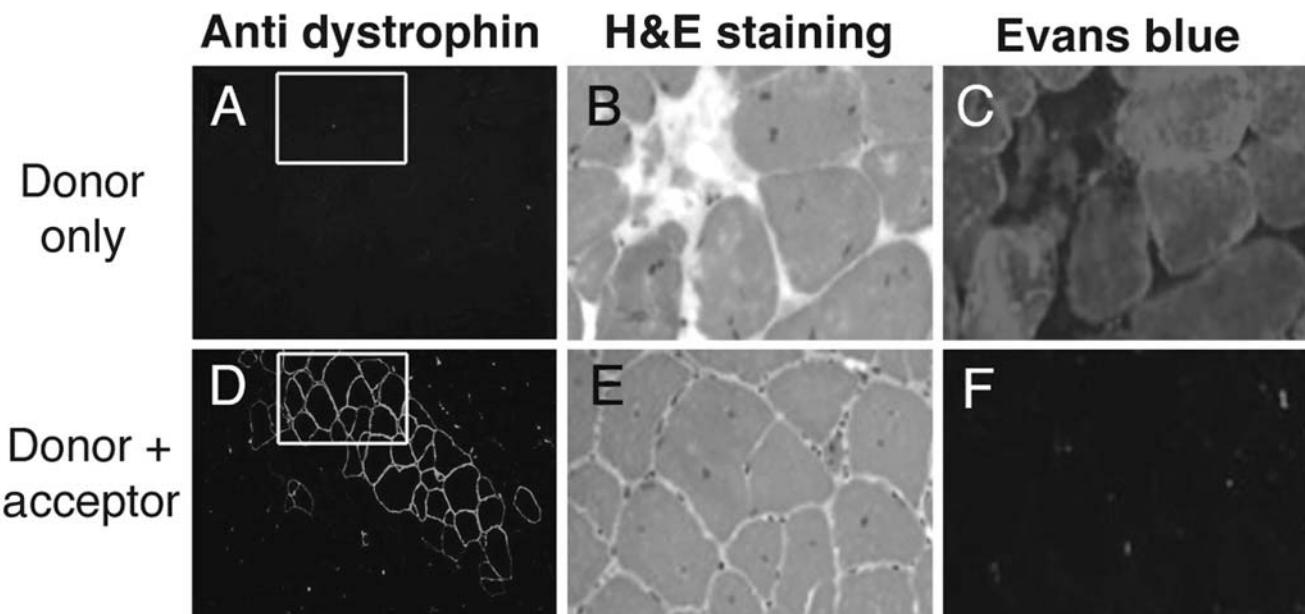
The three newly developed dual-vector technologies described above have greatly enhanced the therapeutic potential of rAAV. Each method bears unique advantages and limitations. *Cis*-activation has the most notable application for genes approaching the packaging limits of rAAV. However, this application may be limited by potential safety concerns associated with integrated regulatory elements (such as a super-enhancer), which could alter expression of endogenous genes. Advantages of the overlapping vector strategy include its simplicity and the ease in the cloning of vectors. However, this overlapping vector approach accommodates less DNA than the *trans*-splicing method (Duan *et al.*, 2003). Furthermore, the overlapping method may have transgene-specific and/or tissue-specific differences in efficiency. In lung, for example, the efficiency of alkaline phosphatase gene delivery from overlapping vectors appears

quite high (Halbert *et al.*, 2002). However, in murine skeletal muscle overlapping vectors were much less efficient than *trans*-splicing vectors in delivering the *LacZ* gene (Duan *et al.*, 2001). Clearly, further investigation is needed to improve the efficiency of these methods and to evaluate which of these approaches are most clinically useful for various organ systems and diseases. Below we describe two examples of applying the *trans*-splicing dual-vector approach in the context of a therapeutic gene.

### *Trans*-splicing rAAV vector delivery of a secreted protein

The first therapeutic secretory protein expressed from the *trans*-splicing AAV vector was erythropoietin (Yan *et al.*, 2000). To determine whether *trans*-splicing vector-mediated expression was sufficient to treat anemia, both donor and acceptor vectors (Figure 39.2A, p. 527 and 39.2B) were co-injected into the limb muscle of adenine-treated mice. Adenine is toxic to the proximal tubules and glomeruli in the kidney and induces chronic renal failure and anemia. In uninfected mice, adenine administration led to a significant drop in hematocrit. Encouragingly, *trans*-splicing AAV infection protected the adenine-treated mice from anemia (Yan *et al.*, 2000).

While this study provided proof-of-concept for delivery of a therapeutic protein using the *trans*-splicing approach, it did not address the issue of protein production from larger genes. In this regard, Chao *et al.* conducted studies in which they divided the 7 kb factor VIII gene into *trans*-splicing



**Figure 39.4** *Trans*-spliced minidystrophin expression prevents sarcolemma damage in *mdx* skeletal muscle. Serial sections from donor-infected (Panel A) or donor- and acceptor-co-infected (Panel D) muscle samples were evaluated by antidystrophin immunostaining. Panels B, C, E, and F are enlarged photomicrographs of the H&E staining and Evans blue dye uptake in the boxed region in Panels A and D, respectively. See also Color Plate 39.4.

vectors (Chao *et al.*, 2002). Consistent with the finding of Yan *et al.*, factor VIII expression was observed following a portal vein injection of the two *trans-splicing* viruses. Furthermore, the level of expression was sufficient to convert severe hemophilia A into a milder form of the disease. In summary, these results suggest that *trans-splicing* is a viable method to deliver therapeutic levels of secretory proteins encoded by various sized genes.

### **Trans-splicing rAAV vector delivery of a structural protein**

The requirements for achieving therapeutic delivery of a structural protein can be quite different from those for a secreted protein. In the latter case, therapeutic protein levels can be achieved from relatively few cells that produce high levels of the protein. In contrast, to ameliorate a structural defect, a significantly larger population of cells must be transduced in the relevant tissue. For example, dystrophin is missing from virtually all myofibers in DMD skeletal muscle. Although functional recovery does not require absolute correction, at least half of the myofibers must express dystrophin to reverse the severe skeletal muscle damage (Hoffman *et al.*, 1988; Arahata *et al.*, 1989; Kunkel and Hoffman, 1989; Chamberlain, 2002). To explore the therapeutic potential of *trans-splicing* rAAV vector systems for DMD gene therapy, our laboratory has cloned the 6 kb minidystrophin gene in *trans-splicing* vectors (Yue *et al.*, unpublished results). Type-2 AAV donor and acceptor viruses were directly injected into the anterior tibialis muscle of 2-week-old mdx mice, a mouse model for DMD. At 1 year post infection, minidystrophin expression was examined. Large bundles of 20–40 dystrophin-positive fibers were detected in donor and acceptor co-infected muscles (Figure 39.4). Histological examination revealed a significant improvement in minidystrophin-positive myofibers. To determine whether *trans-splicing*-mediated minidystrophin expression could improve muscle structure, and presumably function, we performed an Evans blue dye (EBD) uptake assay. EBD is a small vital dye that diffuses into leaky myofibers. Extensive EBD uptake was observed in muscle infected only by the donor virus. Minidystrophin expression from co-infected donor and acceptor viruses significantly improved sarcolemma integrity, preventing EBD uptake (Figure 39.4).

breakthrough, among these discoveries, is the development of dual vector technology to double the packaging capacity of rAAV vectors. Many therapeutic genes that have been traditionally excluded from rAAV gene therapy applications are now being revisited. Among these are the minidystrophin gene, for DMD and BMD gene therapy, and the photoreceptor-specific ATP-binding cassette transport (ABCR) gene, for the treatment of Stargardt's disease. The clinical success of such gene therapy approaches will eventually depend on the transduction efficiency of the dual vector systems. Promising transduction efficiencies have been demonstrated in certain cases, such as with rAAV6 alkaline phosphatase overlapping vectors in the lung (Halbert *et al.*, 2002). However, the current inefficiencies seen in other tissues remain a focus for improvement. The challenge will be to not only dissect potential rate-limiting steps involved in the recombination process, but more importantly to develop novel strategies to overcome these barriers and to achieve clinical success in patients.

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### **SUMMARY AND FUTURE DIRECTION**

Over the past few years, the use of rAAV vectors for gene therapy has entered a new phase of research and clinical application. Accumulated efforts from many research laboratories have begun to unmask many previously unrecognized features of rAAV transduction biology. A major

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# Tailoring of the AAV capsid: designing the second generation vector

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As noted in earlier sections, it is now clear that recombinant AAV (rAAV) can efficiently transduce non-dividing cells *in vivo* to generate long term persistent expression of a transgene. This occurs with little if any inflammatory response or pathology. Thus, rAAV has provided basic biologists with an alternative to transgenic and knockout mice for the study of gene function in the whole animal, and raised the hope that at least some diseases might be treated successfully in humans.

Most of the work to date has been done with the AAV2 serotype but, as noted in the previous section, a significant amount of information is now available about some of the other serotypes as well. In general, although each serotype seems to have a distinct tissue tropism profile *in vivo*, all of the serotypes studied to date can transduce a broad range of tissues and cell types. This is not a serious problem for the early animal and clinical applications that are currently underway. However, it would be extremely useful to design vectors that are targeted specifically to a narrow range of cell types or tissues. Increasing the specificity of transduction inherently increases the safety of the vector, reduces the amount of vector that needs to be administered, and in many cases would allow for simpler delivery strategies to be used (for example, simple intravenous injection). Furthermore, there are some cell types that seem to be poorly transduced by all of the rAAV serotypes tested to date; endothelial cells, astrocytes, and pancreatic islet cells are just some of the examples. Targeting these cell types could provide therapeutic strategies that

would be useful for cardiovascular disease, diabetes, and neurodegenerative diseases.

A successful AAV infection requires attachment to a cell surface receptor, followed by endocytosis, release into the cytoplasm and entry and uncoating in the nucleus. In principle, tissue tropism could be determined or modified at any one of these steps. To date, however, the details of intracellular trafficking and the capsid regions responsible for various trafficking steps have not been defined well enough to allow retargeting of an AAV capsid at an intracellular stage. Virtually all of the attempts to retarget AAV have focused on changing the surface of the AAV2 capsid so that it will bind different cellular receptors. In principle, if a ligand can be engineered into the AAV capsid that targets an alternative endocytosed receptor, then the virus tropism can be expanded to include cell types that are not normally infected. In addition, if the viral surface features that determine binding to the native AAV2 receptor, heparan sulfate proteoglycan (HSPG), can be eliminated, then a vector with an entirely different tropism will be generated. Of course, to be useful, any modification will have to have minimal effects on viral capsid assembly and stability, and not interfere with other aspects of viral entry, such as trafficking to the nucleus and uncoating. There are essentially four strategies currently being explored to engineer targeted vectors that will infect specific cell types, or that will have an expanded tropism (i.e. will infect cells that are not normally infected). These are discussed below in no particular order along with what is known about the viral features that determine normal tropism.

## INTRODUCTION OF NEW RECEPTOR LIGANDS INTO THE CAPSID SEQUENCE

As noted in earlier sections, the AAV capsid open reading frame (ORF) codes for three capsid proteins by using alternative splicing and alternative initiation codons. All three proteins contain the same 532 C-terminal amino acids that are present in the most abundant capsid protein, VP3. The two minor capsid proteins, VP2 and VP1, contain additional 66 and 223 N-terminal amino acids, respectively. The amino acid numbering system most commonly used starts at amino acid 1 of VP1; VP2 then begins at 138 and VP3 at 203. The stoichiometry of the three capsid proteins has not been precisely established but is believed to be 80–90 percent VP3 and 5–10 percent each of VP1 and VP2 (McPherson and Rose, 1983; Wistuba *et al.*, 1995). Since there are a total of 60 capsid proteins in the  $T = 1$  icosahedral AAV particle, this means that there are approximately 48–54 VP3 molecules per capsid and 3–6 molecules each of VP1 and VP2. Insertion of a foreign receptor ligand into VP3 would modify all 60 of the AAV capsid polypeptides, while insertion of a ligand into VP2 or VP1 would modify approximately 6–12 (VP2 region) or 3–6 (VP1 region) capsid proteins. Thus, by choosing the appropriate insertion site in the capsid ORF, it is possible to have some control over the number of foreign ligands that decorate the surface of the modified capsid. Three approaches have been used to identify regions of the capsid sequence that would accommodate the insertion of foreign ligands.

### Scanning mutagenesis

The first was to scan the capsid ORF with either in-frame insertion or alanine scanning mutagenesis to identify regions of the capsid sequence that had no effect on capsid assembly or infectivity. These so called non-essential sites were then targets for insertion of foreign ligands and, if the ligand was displayed on the surface of the capsid, it might give the capsid a new tropism. Three groups tried this approach and, in general, the results were disappointing (Rabinowitz *et al.*, 1999; Wu *et al.*, 2000; Shi *et al.*, 2001). The most aggressive attempt along these lines by Wu *et al.* identified some 18 positions that would accommodate an alanine scanning mutation but, when these positions were used for substitution of short (6–10 amino acid) antibody epitopes, only one produced viable capsid particles in good yield (Wu *et al.*, 2000). The lone positive site was amino acid 34 of VP1, which displayed the epitope on the capsid surface. Substitutions at all of the other sites produced either no intact capsids or capsids that were non-infectious.

### Focusing on surface loops

An alternative approach took advantage of the fact that parvovirus capsid sequences are conserved within the core

antiparallel beta sheets that form the primary contacts at the 2-fold, 5-fold, and 3-fold axes of symmetry (Xie *et al.*, 2002; Chapman and Rossmann, 1993). Regions between these beta sheets form loops that are not conserved and are potentially on the surface of the particle. These surface loops have been shown in other parvoviruses to be targets for neutralizing antibodies as well as sites for receptor binding (Chapman and Rossmann, 1993; Huttner *et al.*, 2003). Girod *et al.* were the first to target these loops and found several positions that would tolerate an insertion of a 14 amino acid ligand carrying the arginine, glycine, aspartic acid (RGD) motif (Girod *et al.*, 1999), a binding site for several integrin receptors (see Table 40.1). These modified viruses produced reasonable levels of virus particles (0–3 logs lower than wild type) and displayed the ligand on the surface of the particle as determined by immunoprecipitation. One position in the GH loop at amino acid 587 (Figure 40.1) produced nearly wild-type levels of virus. The GH loop is so named because it lies between beta strands G and H and is in fact the largest of the surface loops as subsequently shown by the viral X-ray crystal structure (Xie *et al.*, 2002). The GH loop is also called loop IV by some groups. The mutant virus carrying the insertion at amino acid 587 was capable of infecting a cell line, B16F10, that was normally not susceptible to AAV infection. A peptide inhibitor that blocked RGD binding eliminated the infectivity of the virus with the 14 amino acid insertion, while heparin sulfate, which blocks normal AAV2 infection, had no effect. Thus this was the first demonstration of a virus that could be made in good yield, apparently bound to an alternative receptor and used successfully to transduce cells carrying that receptor. Impressively, the increase in infectivity of the modified virus on the previously non-permissive B16F10 cell line was 4–6 logs, and nearly matched the infectivity of wild-type virus on cells permissive for wild type. Interestingly, on cells normally permissive to wild-type virus, the RGD-modified virus was also sensitive to heparin sulfate inhibition, suggesting that the mutant virus retained the ability to use both types of entry pathways.

Several other groups followed a similar strategy (Wu *et al.*, 2000; Shi *et al.*, 2001; Grifman *et al.*, 2001). In addition to the amino acid 34 position in VP1 mentioned earlier, Wu *et al.* identified a number of surface loop positions in VP3 that would accommodate ligand insertions with relatively modest decreases in viral yields (Wu *et al.*, 2000) (Table 40.1 and Figure 40.1). Shi *et al.* found additional sites in the GH loop (amino acids 459 and 584) but also identified sites in VP2 (amino acid 161) and VP1 (amino acid 46) that would accept an insertion (Shi *et al.*, 2001). Insertions of an RGD motif into the VP1 and VP2 sites were subsequently found to be non-infectious, possibly because these insertions interfered with post-entry pathways in the infection process (Shi and Bartlett, 2003). Insertions at amino acids 459 and 584, however, were infectious in the presence and the absence of heparin sulfate, suggesting that the modified viruses could use an integrin

**Table 40.1** Capsid positions successfully used for altering the capsid surface<sup>a</sup>

Amino acid position <sup>b</sup>	Capsid	Loop <sup>c</sup>	Epitope or ligand inserted <sup>d</sup>	Altered tropism <sup>e</sup>	Heparin binding <sup>f</sup>	Reference
34	VP1	NA	HA tag	ND	+	Wu <i>et al.</i> , 2000
	VP1	NA	serpin	+	+	Wu <i>et al.</i> , 2000
	VP1	NA	apoE	+	+	Loiler <i>et al.</i> , 2003
46 <sup>g</sup>	VP1	NA	RGD	ND	+	Shi and Bartlett, 2003
115 <sup>g</sup>	VP1	NA	RGD	ND	+	Shi <i>et al.</i> , 2001; Shi and Bartlett, 2003
138	VP2	VP2 N	HA tag	ND	+	Wu <i>et al.</i> , 2000
	VP2	VP2 N	serpin	+	+	Wu <i>et al.</i> , 2000
	VP2	VP2 N	fractalkine	ND	+	Warrington <i>et al.</i> , 2004
	VP2	VP2 N	leptin	ND	+	Warrington <i>et al.</i> , 2004
	VP2	VP2 N	GFP	ND	+	Warrington <i>et al.</i> , 2004
	VP2	VP2 N	CD34 sFv	+	ND	Yang <i>et al.</i> , 1998
	VP2	VP2 N	BPV	ND	+	Shi <i>et al.</i> , 2001
	VP2	VP2 N	LH	+	+	Shi <i>et al.</i> , 2001
	VP2	VP2 N	apoE	+	+	Loiler <i>et al.</i> , 2003
	VP2	VP2 N	RGD	+	+	Shi and Bartlett, 2003
161 <sup>g</sup>	VP2	NA	BPV	ND	+	Shi <i>et al.</i> , 2001
	VP2	NA	LH	ND	+	Shi <i>et al.</i> , 2001
	VP2	NA	RGD	ND	+	Shi and Bartlett 2003
261	VP3	BC	RGD	ND	ND	Girod <i>et al.</i> , 1999
266	VP3	BC	HA tag	ND	+	Wu <i>et al.</i> , 2000
381	VP3	EF	RGD	ND	ND	Girod <i>et al.</i> , 1999
447	VP3	GH	HA tag	ND	+	Wu <i>et al.</i> , 2000
	VP3	GH	RGD	ND	ND	Girod <i>et al.</i> , 1999
449	VP3	GH	NGR	ND	+	Grifman <i>et al.</i> , 2001
459 <sup>g</sup>	VP3	GH	BPV	ND	ND	Shi <i>et al.</i> , 2001
	VP3	HG	LH	ND	+	Shi <i>et al.</i> , 2001
573	VP3	GH	RGD	ND	ND	Girod <i>et al.</i> , 1999
584	VP3	GH	BPV	ND	+/-	Shi <i>et al.</i> , 2001
	VP3	GH	RGD	+	+	Shi and Bartlett, 2003
587	VP3	GH	RGD	+	+	Girod <i>et al.</i> , 1999
	VP3	GH	NGR	+	+	Grifman <i>et al.</i> , 2001
	VP3	GH	EYH	+	+	Work <i>et al.</i> , 2004
	VP3	GH	SIGYPLP	+	-	Nicklin <i>et al.</i> , 2001, 2003
	VP3	GH	Z34C <sup>h</sup>	+	-	Ried <i>et al.</i> , 2002
	VP3	GH	MSL	-	+/-	White <i>et al.</i> , 2004
	VP3	GH	MTP	+	+/-	White <i>et al.</i> , 2004
	VP3	GH	NSSRDLG <sup>i</sup>	+	-	Muller <i>et al.</i> , 2003
	VP3	GH	RGDAVGVi	+	-	Perabo <i>et al.</i> , 2003
588	VP3	GH	RGD	+	+/-	Shi and Bartlett, 2003
591	VP3	GH	HA tag	ND	-	Wu <i>et al.</i> , 2000
664	VP3	HI	HA tag	ND	+	Wu <i>et al.</i> , 2000
735	VP3	VP3 C	His tag	ND	+	Zhang <i>et al.</i> , 2002

<sup>a</sup> The table lists the capsid positions into which ligands have been successfully inserted to produce nearly wild-type titers of DNA containing AAV particles that display the ligand on the capsid surface.

<sup>b</sup> Ligands were generally inserted immediately after the amino acid listed in the table. The numbering of capsid amino acids begins with the N terminal met of VP1, VP2 begins at amino acid 138 and VP3 begins at amino acid 203.

<sup>c</sup> NA means not applicable because the crystal structure of AAV2 did not map the coordinates of amino acids 1–216. VP2 N indicates that the ligand was inserted immediately after the N terminal amino acid of VP2. VP3 C indicates the C terminal end of VP3.

<sup>d</sup> Ligands are named by their parent molecule (e.g. serpin), the key amino acids in the ligand (e.g. RGD) or an abbreviation used in the text (e.g. HA). See text for more details.

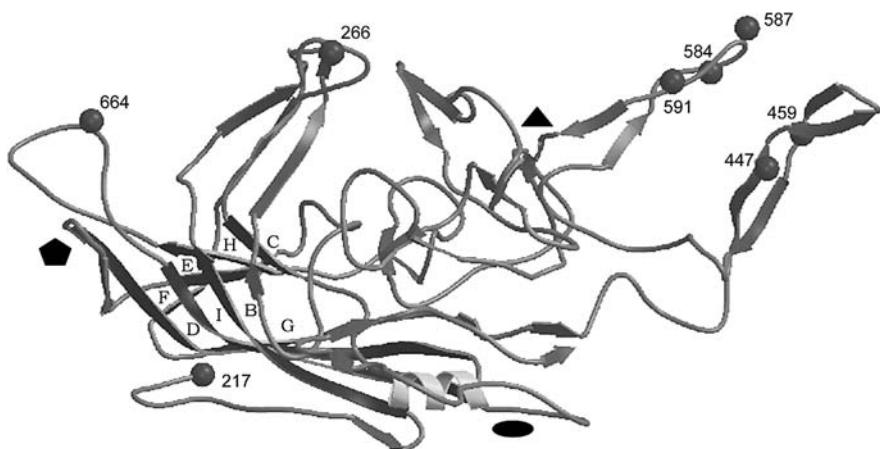
<sup>e</sup> ND = not done.

<sup>f</sup> Depending on the investigator, heparin binding was determined by binding to heparin sulfate columns or by inhibition of infection with soluble heparin sulfate. ND, not done.

<sup>g</sup> Insertions at these positions displayed the ligand on the surface but produced virus that was relatively non-infectious.

<sup>h</sup> This ligand codes for the immunoglobulin binding domain of protein A.

<sup>i</sup> One of several examples of ligands that emerged from a random library inserted into AAV2.



**Figure 40.1** AAV2 VP3 monomer. The approximate 2-, 3-, and 5-fold icosahedral axes are shown by the filled oval, triangle, and pentagon, respectively. Some of the positions for peptide insertions (Girod et al., 1999; Shi et al., 2001; Wu et al., 2000) that successfully displayed the new epitope on the capsid surface, are shown by the black balls and labeled with the amino acid number (based on VP1 numbering). The 217 position indicates the most N-terminal amino acid whose atomic coordinates could be deduced by X-ray crystallography. The strands in the eight-stranded antiparallel  $\beta$ -barrel domain are shown as arrowed ribbons in blue and are labeled BIDG and CHEF for the two sheets. The small helix (residues 293–303) that is observed in all parvoviruses is shown in cyan. The rest of the VP3 polypeptide is shown in red, with strands shown as arrowed ribbons and the remaining residues as coils. (The figure was kindly prepared by Mavis Agbandje-McKenna.) See also Color Plate 40.1.

receptor for entry (Shi and Bartlett, 2003). This was confirmed by showing that the RGD-modified virus could bind to integrin and transduce cells such as K562 that lack significant amounts of heparan sulfate proteoglycan but have  $\alpha V\beta 5$  integrin. Infection of HeLa cells, which express both integrin and HSPG, could not be inhibited by either RGD peptides or heparin sulfate alone but required both inhibitors to abolish infection, suggesting that both receptor pathways could be used.

Grifman et al. focused on the GH loop and found two sites, amino acids 449 and 588, that were near those found earlier by other groups and would accommodate insertions (Grifman et al., 2001). Both Wu et al. and Grifman et al. (Wu et al., 2000; Grifman et al., 2001) asked the question whether it was better to delete a surface loop and substitute it with a foreign ligand, or to simply insert an additional sequence into the wild-type AAV capsid sequence. The answer from both groups seemed to be that insertions produced better viral titers than substitutions. Two groups also asked the question whether it was useful to flank the foreign ligand with additional residues within the surface loop. Grifman et al. reported somewhat better transduction of cells targeted by an RGD ligand when the ligand was flanked by two to four cysteine residues to form a miniloop containing the ligand sequence (Grifman et al., 2001). This had been seen earlier with phage display (Arap et al., 1998). Shi et al. used cyclic cysteine constructs as well, but also incorporated five amino acid flanking peptide linkers in which the ligand was preceded by TG and followed by one of three amino acid sequences, GLS, ALS, or LLA (Shi et al., 2001; Shi and Bartlett, 2003). They found that the nature of

the peptide linkers often had a major effect on the viral particle titer, the accessibility of the ligand to antibody precipitation, and on the infectivity of the virus. Shi et al. also made the observation that whether a ligand is tolerated at a specific location often depends on the amino acid sequence of the ligand (Shi et al., 2001). A six amino acid sequence from bovine papillomavirus (BPV) inserted at site 459 was well tolerated and generated fully infectious particles, but a 10 amino acid luteinizing hormone (LH) peptide, inserted at the same position with the same flanking linker sequences was not infectious. Furthermore, the size of the ligand also makes a difference. With few exceptions ligands  $>20$  amino acids have not been tolerated at loop sites within VP3 and generally produce low virus titers, no particles, or particles that are not infectious (Buning et al., 2003).

Of all of the loop sites tested to date (Table 40.1), the sites near amino acid 587 in the GH loop have been tested most extensively. In addition to the studies already mentioned (Girod et al., 1999; Grifman et al., 2001; Shi and Bartlett, 2003), Nicklin et al. inserted a 7 amino acid peptide (SIGYPLP) at position 587 that appeared to be specific for endothelial cells by phage display (Nicklin et al., 2001). The modified virus infected human umbilical vein and saphenous vein endothelial cells but not vascular smooth muscle cells or human hepatocytes, which are targets for the wild-type capsid. Unlike the wild-type virus, the modified virus was not inhibited by baflomycin A1 (an inhibitor of endosomal acidification), suggesting that intracellular trafficking of the SIGYPLP virus had been altered. Similarly, Work et al. used another phage display-derived peptide with the EYH motif at position 587 to demonstrate differential

ability to infect vascular smooth muscle cells but not endothelial cells (Work *et al.*, 2004). Finally, White *et al.* identified a peptide by phage display containing an MTP motif that was inserted at the 587 site (White *et al.*, 2004). When injected intravenously, this virus was approximately 2- to 3-fold reduced in its ability to infect liver, spleen, and lung, and significantly better (approximately 10-fold) at transducing the vasculature of the vena cava compared with wild type.

## N and C terminal positions

In addition to the putative surface loops, Wu *et al.* tested the possibility of insertions at the N-terminus of the three capsid proteins and the common C-terminus (Wu *et al.*, 2000). With the exception of the N-terminus of VP2, none of these positions produced viable particles. The N-terminus of VP2 (amino acid 138), however, readily accommodated small insertions and consistently produced wild-type or nearly wild-type virus yields with the ligand present in the VP1 and VP2 proteins. In one set of experiments, they demonstrated that a serpin ligand insertion, either at the N-terminus of VP2 (just after amino acid 138) or at amino acid position 34 in VP1, increased infectivity on a normally non-permissive cell line, IB3, by 15- and 60-fold, respectively (Wu *et al.*, 2000). In this case, normal heparin binding was still necessary for infectivity as shown by inhibitor studies with soluble heparin sulfate. This suggested that the serpin-modified virus bound heparan sulfate proteoglycan-like wild-type virus but used an alternative serpin receptor for endosomal uptake.

Similarly, Bartlett and his colleagues (Shi *et al.*, 2001) inserted a 10 amino acid peptide just after amino acid 139 for human luteinizing hormone, which was known to bind the luteinizing hormone receptor (LHR). Like the serpin-modified vector described above, the LH-containing vector could be grown to wild-type titers and its infectivity was inhibited by more than two logs in the presence of heparin sulfate on HeLa cells, which do not express LHR. However, when it was tested on OVCAR-3 cells, a human ovarian cancer cell line that expresses the LH receptor, significant transduction was seen in the presence of heparin sulfate and this infectivity was increased even further by inducing LH receptor synthesis in the cells with progesterone treatment. In contrast, transduction of OVCAR-3 cells was severely depressed in the presence of soluble LH peptide. Like the RGD virus described earlier by Girod *et al.* (Girod *et al.*, 1999), the LH-containing virus appeared to be capable of infecting cells by two distinct pathways, one of which involved HSPG and the other LHR. Loiler *et al.* also showed that insertions at the N-terminus of VP2 were viable (Loiler *et al.*, 2003). This group inserted a 28 amino acid sequence containing an ApoE-derived ligand that normally binds to the LDL-R receptor. Their goal was to engineer a virus that could infect pancreatic islet cells that normally are not

susceptible to infection by AAV2. The ApoE-modified virus increased vector transduction of murine islet cells by as much as 3 logs *ex vivo* and murine hepatocytes by as much as 4-fold *in vivo*. As in the case of the RGD- and LH-modified viruses described above, the ApoE virus appeared to be capable of using both the HSPG and low-density lipoprotein receptor (LDL-R) receptor pathways.

## IDENTIFICATION AND ELIMINATION OF THE HSPG BINDING SITE ON THE CAPSID SURFACE

As noted earlier, the AAV2 serotype has been shown to bind primarily to HSPG on the cell surface (Summerford and Samulski, 1998). Three co-receptors have been identified,  $\alpha V\beta 5$  integrin, fibroblast growth factor 1 receptor (FGFR1) and hepatocyte growth factor receptors (Qing *et al.*, 1999; Summerford *et al.*, 1999; Kashiwakura *et al.*, 2005). If AAV2 is to be successfully targeted to a particular cell type, then two things must be accomplished; a new receptor ligand has to be attached or inserted into the capsid that is unique to that cell type, and the existing binding site to HSPG needs to be eliminated. Earlier mutagenic studies (Rabinowitz *et al.*, 1999; Wu *et al.*, 2000) had identified two regions within the GH loop that reduced binding of mutant virus to heparin sulfate, amino acids 509–522 and 561–591. Because heparin sulfate binding is essentially a charge interaction that requires basic amino acids to be positioned in a certain orientation and distance from each other, insertions or substitutions at distant sites might affect heparin binding by causing conformational changes in the loop. Determination of the atomic coordinates of the AAV2 capsid proteins (Xie *et al.*, 2002) showed that the 585–588 region mapped to a basic charge cluster at the 3-fold axis of symmetry and was, therefore, the most likely to be directly involved in heparin binding.

To determine precisely which amino acids were involved in heparin sulfate binding, two groups independently made substitution mutants in all of the likely basic residues within VP3 (Kern *et al.*, 2003; Opie *et al.*, 2003). Capsid particles that contained only VP3 had previously been shown to bind heparin with the same affinity as wild-type particles (Rabinowitz *et al.*, 1999). Both groups essentially agreed that there were five basic residues involved in heparin sulfate binding: R484, R487, K532, R585, and R588 (Kern *et al.*, 2003; Opie *et al.*, 2003). When viewed perpendicular to the 3-fold axis of symmetry, the five amino acids (which are contributed by two different VP polypeptides) collectively form a basic patch on one side of each 3-fold related spike. Thus, there are three such patches at the 3-fold symmetry axis, which presumably could cooperate in binding HSPG. Precisely how they bind heparin sulfate has not yet been established.

Of the five amino acids involved in heparin binding, mutation of R585 and R588 eliminated virtually all of the heparin sulfate binding activity without affecting particle

assembly and stability or the particle titer (Opie *et al.*, 2003). Virus particles containing the R585,588A double mutation were approximately 3 logs reduced for infectivity in cell culture assays compared with wild type (Opie *et al.*, 2003). The remaining three amino acids, R484, R487, and K532, had comparatively less effect on heparin binding, although some of these mutants had significant effects on infectivity, presumably due to interference of other steps in the infection process (Kern *et al.*, 2003; Opie *et al.*, 2003). The fact that wild-type particle titers can be achieved with simple point mutants that do not bind heparin sulfate means that it should be possible to eliminate a significant amount of the normal tropism of wild-type AAV2. Indeed, Kern *et al.* went on to show that intravenous injection of the double mutant R484E, R585E, eliminated virtually all of the normal transduction of liver and kidney tissue in mice and significantly enhanced the level of transduction in heart (Kern *et al.*, 2003).

The key roles played by R585 and R588 in heparin binding explain some of the results of earlier studies in which ligands were inserted at position 587. Any of these insertions would have changed the spatial relationship between these two amino acids and therefore should reduce heparin binding. Indeed, this was shown by several groups (Grifman *et al.*, 2001; Nicklin *et al.*, 2001; White *et al.*, 2004). The caveat to this is that, if the insertion contained basic amino acids (R, K, or H), then heparin binding might be preserved, and this was also seen in several instances (Grifman *et al.*, 2001; Work *et al.*, 2004; White *et al.*, 2004).

HSPG is not normally internalized when bound by virus; therefore its role is believed to be primarily to bind virus and bring it into close proximity with the co-receptor FGFR1 or  $\alpha V\beta 5$  integrin. A similar situation has been shown for adenovirus 5, in which the primary receptor for binding the cell surface is the coxsackievirus and adenovirus receptor (CAR) but the receptor for endosomal uptake is  $\alpha V\beta 5$  integrin. In the case of adenovirus (Ad), modification of the CAR binding site on the Ad fiber protein reduces normal Ad tropism by approximately 3 logs while modification of both the CAR binding site and the integrin binding site in the penton base reduces normal tropism by up to 6 logs (Akiyama *et al.*, 2004). The challenge for AAV targeting studies, therefore, is to identify the co-receptor binding sites and eliminate them in the same fashion as the HSPG binding site.

## **IN VITRO SELECTION OF VECTORS WITH MODIFIED TROPISM FROM CAPSID LIBRARIES**

Most groups have tested ligands that were either identified by phage display or had previously been determined by biochemical analysis of larger peptides. Insertion of a ligand into the context of the AAV capsid, however, does not necessarily ensure that it will allow capsid assembly, that it will be correctly presented to the appropriate receptor, or that it will not interfere with a subsequent step in

trafficking. To address these issues, two groups have explored the possibility of using virus libraries carrying randomly generated ligands that can be selected for new tropisms by using *in vitro* cell culture techniques. Perabo *et al.* created a library carrying a randomized seven amino acid sequence (flanked by two or three alanines) at position 587 (Perabo *et al.*, 2003). The resulting 12 amino acid insertion size was chosen on empirical grounds, as longer insertions tend to interfere with particle assembly. The library was then sequentially passaged on M07e or Mec1 cells, both of which are non-permissive for AAV2. After five passages, viruses with unique sequences were isolated that were significantly improved for infection on the non-permissive cell lines. One of the viruses isolated on Mec1 cells, a B-cell chronic lymphocytic leukemia (B-CLL) line, showed improved transduction of primary B-CLL cells, suggesting that it might be useful for therapy of this disease.

Muller *et al.* followed a similar strategy (Muller *et al.*, 2003). In this case a seven amino acid random peptide library was inserted at position 588 (flanked by G and A) into a vector that would have a frameshift in the capsid gene unless an insert was present. The library was initially amplified in the presence of wild-type capsid genes by transfection and then amplified further in 293 cells at low multiplicity of infection (MOI) to produce the final library. The random library was then selected for sequences with improved transduction on primary human coronary artery endothelial cells; again, a cell type that is not infected well by AAV2. Two rounds of selection were sufficient to isolate several different sequences that had as much as 600-fold improved replication on endothelial cells. One of the selected ligands also was tested *in vivo* for biodistribution and displayed approximately 5-fold higher transduction of heart and lower transduction of liver compared with wild type after intravenous injection into mice.

The use of libraries to select ligands in the context of the AAV capsid is a promising approach to isolating new modified viruses with altered properties. One difficulty with the approach is the relatively short peptide that is used for selection. Another difficulty is that the method is limited by the availability of the target cells used for selection. Finally, a major difficulty that is common to all the approaches discussed here is that no simple selection has been devised for targeting *in vivo*. Acquiring good specificity in cell culture is far different from engineering specificity in intact animals. In the hope that larger ligands might produce more stringent specificity in targeting, several alternative targeting strategies have also been tried and are discussed below.

## **CONJUGATE-BASED TARGETING OF RECOMBINANT AAV**

Several groups have devised methods for conjugating ligands to the AAV capsid that could potentially have any size. Bartlett *et al.* used an antibody specific for the  $\alpha V\beta 3$

integrin receptor, which is present on megakaryocyte related lines like M07e and DAMI but not on HeLa or K562 cells (Bartlett *et al.*, 1999). To attach this antibody to the AAV capsid, they chemically cross-linked the Fab' fragment of the integrin antibody to the Fab' fragment of the A20 antibody (Wobus *et al.*, 2000), an antibody that recognizes a capsid epitope that is present only on intact AAV capsids. The resulting bispecific heterodimeric antibody was then allowed to bind to AAV2 via the A20 portion and tested on the megakaryocyte lines, which are poorly infected by AAV. It was clear that the addition of the bispecific antibody to the capsid surface significantly improved transduction on the megakaryocyte lines over wild-type background levels by as much as 70-fold. Inhibition studies in the presence of the soluble integrin antibody demonstrated that the increase in transduction was due to the addition of the bispecific antibody. However, the level of transduction on the megakaryocyte lines was lower than that seen on HeLa or K562 cells by wild-type virus. This may have been due to the fact that A20 is a neutralizing antibody that appears to inhibit infection of AAV at a post-entry step during the infection process (Wobus *et al.*, 2000).

Ponnazhagan *et al.* genetically engineered fusion proteins between core-streptavidin and either epidermal growth factor (EGF) or fibroblast growth factor 1 $\alpha$  (FGF1 $\alpha$ ) (Ponnazhagan *et al.*, 2002). The fusion proteins were then purified from bacterial expression systems and bound to wild-type capsids that had been chemically conjugated to biotin. The EGF and FGF1 $\alpha$  conjugated viruses increased transduction of cell lines carrying the appropriate receptor by 2–3 logs over the wild-type virus.

Ried *et al.* took a different and less complicated approach to creating a conjugate-based targeting vector that had the advantage of requiring the least amount of *in vitro* manipulation (Ried *et al.*, 2002). This group inserted a 34 amino acid peptide (Z34C) that contained the IgG binding domain of protein A into the 587 position of AAV2. Insertion of the binding domain reduced the viral titer, but insertion into a mutant containing a deletion of capsid amino acids 581–590 produced wild-type titers. The mutant capsids were then bound to antibodies to various cell surface receptors, including CD29 ( $\beta_1$  integrin), CD117 (c-kit) or CXCR4 (coreceptor for HIV). As expected the Z34C-modified virus was 3–4 logs reduced in infectivity on HeLa cells, presumably because it lacked the ability to bind HSPG. However, when the Z34C-modified virus was bound to specific antibodies, the infectivity on cell lines expressing the antibody specific receptor was increased from a level that was undetectable (when antibody was not bound to the capsid), to a titer of approximately 10<sup>3</sup>/ml when antibody was present on the capsid surface. In all cases, soluble antibody inhibited the antibody-specific transduction, but the titer of 10<sup>3</sup> was approximately 2 logs lower than background titers of wild-type AAV on the same cell lines. Thus, specificity had been achieved at the expense of efficiency of transduction.

## INSERTION OF LARGE LIGANDS DIRECTLY INTO THE CAPSID SEQUENCE

Yang *et al.* tried what promises to be the most direct approach to targeting AAV virus with large ligands (Yang *et al.*, 1998). This group fused a single-chain fragment variable region (sFv) of a monoclonal antibody to human CD34 (a cell surface receptor on hematopoietic stem cells) to the N-terminus of VP1, VP2, or VP3. In each case they attempted to complement the fusion protein with wild-type capsid proteins (for example, VP1 fusion protein with wild-type VP2 and VP3), but they were unable to recover any virus from any combination of wild-type and fusion capsid genes. They then tried each fusion protein plasmid in combination with a plasmid that synthesized all three wild-type capsid proteins, thus generating mosaic viruses that contained both wild-type and fusion protein in different ratios. They found that N-terminal fusions with VP2 in the presence of wild-type VP1, 2, and 3 were successful in generating virus stocks that contained the sFv in the capsid. The modified virus also was able to infect CD34 $^+$  human myoleukemia cell line KG-1, which is normally refractory to rAAV transduction. Although the mosaic virus stocks were low in titer (200 infectious units per ml) and contained a mixture of wild-type and modified capsids, this was the first time that a modified AAV virus particle was used to extend the tropism of an AAV vector, and it is one of the few times that a large ligand (35 kDa) was successfully inserted into an AAV capsid gene. As noted earlier, several groups subsequently showed that the N-terminus of VP2 could readily accommodate small ligands (Wu *et al.*, 2000; Shi *et al.*, 2001). However, these insertions were also present in the VP1 protein at the same position.

Warrington *et al.* have recently re-examined the requirements for successfully introducing a large ligand at the N-terminus of VP2 (Warrington *et al.*, 2004). This group created plasmids that expressed only one or two of the capsid proteins under the control of the native AAV promoters. The idea was to use a single capsid protein for ligand insertion, which then could be complemented with the remaining two wild-type capsid proteins. Unlike earlier groups (Ruffing *et al.*, 1992; Muralidhar *et al.*, 1994; Steinbach *et al.*, 1997; Hoque *et al.*, 1999), Warrington *et al.* found that VP2 is non-essential. Particles composed only of VP1 and VP3 could be made with essentially the same titer and infectivity as wild-type particles. In addition, they confirmed an earlier report (Rabinowitz *et al.*, 1999) that it was possible to make capsids that contained only VP3. The VP3 only particles had approximately the same particle titer as wild type but were approximately 3–4 logs lower in infectivity due to the lack of the VP1 phospholipase activity (Zadori *et al.*, 2001; Girod *et al.*, 2002). The observation, however, that VP3 alone is sufficient to make intact virus particles, contradicts earlier reports (Ruffing *et al.*, 1992; Muralidhar *et al.*, 1994; Hoque *et al.*, 1999), and the discrepancies

between different laboratories have not been resolved. Warrington *et al.* went on to insert three different peptides exclusively at the VP2 N-terminus, fractalkine (76 amino acids), leptin (146 amino acids), and green fluorescent protein (238 residues, GFP). In the case of the two smaller ligands, they succeeded in generating viral stocks that had essentially the same DNA-containing particle titer and infectivity as wild-type virus. This was accomplished in part by overexpressing the VP2-ligand fusion protein and in some cases, overexpression of the modified VP2 increased the relative amount of VP2 related protein in the resulting virus particles. As in the case of smaller ligands, insertion of the 30 kDa GFP protein into VP2 generated DNA-containing particle titers that were similar to wild type. However, the GFP-modified virus was approximately 3 logs lower in infectivity (Warrington *et al.*, 2004). This was partly due to the fact that VP1 was underrepresented in the particles containing the GFP insert. When VP1 expression was increased during vector production, the infectivity of the GFP-containing particles approached wild type (Muzyczka and Warrington, 2005).

Thus, Warrington *et al.* essentially demonstrated that at least some large ligands can be directly inserted into an AAV capsid protein without sacrificing viral titer or infectivity (Warrington *et al.*, 2004). The key to accomplishing this was to insert the ligand into only one of the three capsid proteins. When large ligands were inserted into both VP1 and VP2, infectivity dropped dramatically (Warrington *et al.*, 2004). A similar observation was made by Zhang *et al.* who successfully inserted a his tag at the C-terminus of VP3, whereas earlier attempts by Wu *et al.* to insert a his tag into the C-terminus of all three viral capsid proteins had failed to produce viable particles (Wu *et al.*, 2000; Zhang *et al.*, 2002). In addition, since GFP is similar in size to single chain monoclonal antibodies, this approach may allow the incorporation of highly specific ligands into AAV vectors, which are targeted to only a limited number of cell types. Finally, the incorporation of large ligands directly into the capsid gene also simplifies the production of large virus lots in a reproducible way for therapeutic purposes, and bypasses the problems of non-covalent linkage of ligands to the capsid. It remains to be seen, however, whether such vectors will function for viral entry and intracellular trafficking. The ligands tested by Warrington *et al.* retained HSPG binding and presumably used the normal AAV2 entry pathway (Warrington *et al.*, 2004).

## FUTURE PROSPECTS

In summary, several methods for modifying recombinant AAV capsids have been described that have proven successful in changing the tropism of the virus. To date, the work described provides proof of principle for the concept that virus particles can be engineered with high specificity for some cell types. At the very least, it seems clear that the

tropism of AAV2 can be expanded by insertion of foreign peptide sequences into the capsid. In addition, all of the methods described so far can be adapted to other AAV serotypes. However, relatively little work has been done so far *in vivo*. Given the multiple routes of entry that many viruses use, the usefulness of targeted AAV vectors will not be clear until conventional biodistribution experiments are performed in animal models.

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# rAAV vectors for gene targeting

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TONI CATHOMEN

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Molecular medicine based on gene therapy has the potential to achieve long-term correction of a genetic defect. Although the problems are still manifold, some gene therapy protocols have proven successful in clinical trials in recent years (Gene therapy clinical trials worldwide, 2004). Long-term gene therapy often demands the integration of the therapeutic nucleic acid into the host genome to maintain it over many cell divisions. Strategies based on retroviral gene transfer take advantage of the innate ability of these viruses to integrate their genome into the host chromosome, and have been the vectors of choice in many gene therapy protocols. It is important to keep in mind, however, that retroviral systems preferentially integrate into active genes (Bushman, 2003). The inherent risk of insertional mutagenesis associated with randomly integrating vectors has been highlighted when two of nine patients in a gene therapy trial involving children with X-linked severe combined immunodeficiency (X-SCID) developed leukemia (Hacein-Bey-Abina *et al.*, 2003). At least in part, the leukemia was triggered by the retroviral insertion and activation of an oncogene in the hematopoietic progenitor cells (Baum *et al.*, 2004). Although the leukemic proliferation observed in this trial may be unique to the particular experimental setting, every genetic manipulation poses a risk, especially in stem and progenitor cells with their high proliferative potential. This incident emphasizes the obligation to improve current strategies for gene therapy and highlights the need to search for alternatives to systems based on random integration.

## GENE TARGETING VERSUS GENE ADDITION

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Animal models created by knockout and knock-in technology facilitated the investigation of innumerable biological pathways and human diseases. The procedure to generate these

animals is called gene targeting and involves homologous recombination (HR) in embryonic stem (ES) cells, in which part of the endogenous gene is replaced by vector sequences contained on plasmid DNA. The low frequency of gene targeting in mammalian cells ( $10^{-5}$  to  $10^{-7}$ ) and the high levels of random integration of the targeting vector ( $10^{-2}$  to  $10^{-4}$ ) classically requires a double-selection strategy and thus prevented this methodology from being broadly applied for therapeutic gene corrections (Vasquez *et al.*, 2001). In the last decade, however, several technologies to introduce specific genetic changes into homologous chromosomal sequences have been developed, many of which reach gene targeting frequencies that are four orders of magnitude higher than the conventional approach based on plasmid DNA transfection.

Analogous to conventional gene therapy, gene targeting produces a permanent alteration of the genome, which is inherited by the daughter cells. In contrast, however, gene targeting modifies the genomic DNA directly at the site of the mutation in the chromosome. By its ability to revert a mutation to the wild-type sequence, this method can be employed to address both recessive and dominant disorders. In the case of dominant mutations there is an alternative to correcting the sequence. If the second allele is sufficient to restore function, the mutant allele can simply be knocked out. Therapeutic gene targeting hence pursues two main strategies: gene correction and gene knockout.

## THERAPEUTIC GENE TARGETING

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### Non-viral approaches

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All strategies aiming at therapeutic gene correction have in common the fact that the therapeutic molecule contains

sequences, which are homologous to the target sequence with the exception of the mutation to be corrected. Importantly, the modification of a gene at the site of the mutation has the advantage to ensure sustained and tissue-specific expression of the gene product because it remains under control of its endogenous promoter.

The nature of the therapeutic molecule varies. Chimera-plasts or chimeric RNA-DNA oligonucleotides (RDOs) have been successfully used *in vitro* and *in vivo* (summarized in Kren and Steer, 2002; Liu *et al.*, 2003). Although repair frequencies of up to 40 percent have been reported, the method is controversial and success of gene correction using RDOs is highly variable and difficult to reproduce (Kren and Steer, 2002; Yoon *et al.*, 2002). Single-stranded oligonucleotides (SSOs) are less difficult to synthesize and have been shown to produce more robust results. Single nucleotide changes approach a frequency of 1 percent in transfected cell lines and  $10^{-4}$  in mouse ES cells (Pierce *et al.*, 2003). Triplex-forming oligonucleotides (TFOs) have the characteristic of forming a stable DNA triple helix by binding to the major groove of the target DNA. The combination of TFOs and SSOs has led to the development of bifunctional oligonucleotides that promote gene correction in transfected cell lines with frequencies of up to 1 percent (Chan *et al.*, 1999; Culver *et al.*, 1999).

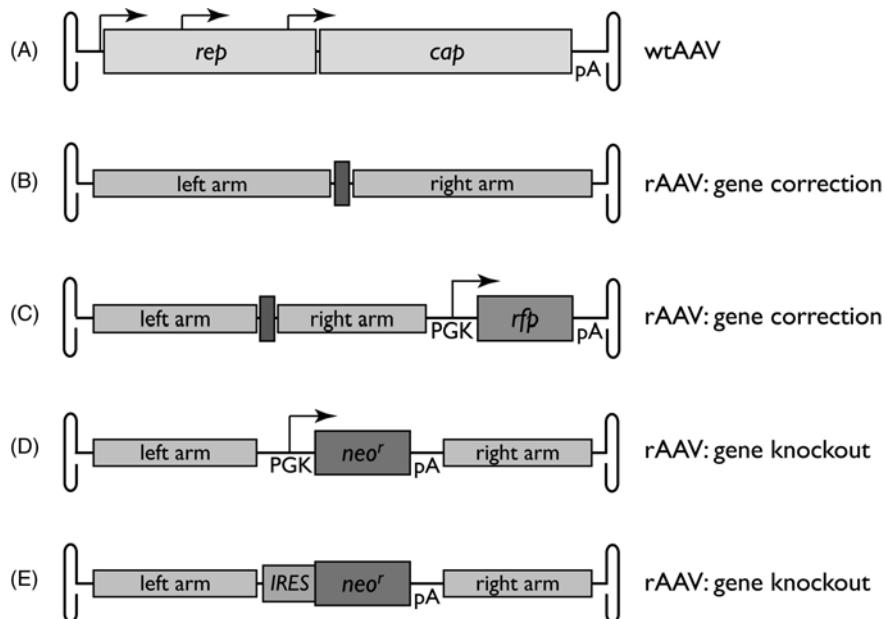
In contrast to oligonucleotide-based strategies, short DNA fragments have been shown to promote the replacement of several base pairs within the mutated area of a gene. These DNA fragments are typically a few hundred

nucleotides in length and single stranded. Experiments in human cells revealed that genomic DNA could be modified with a frequency of 1–10 percent after transfection into cultured cells or microinjection into hematopoietic stem cells (Gruenert *et al.*, 2003).

## Viral approaches

The previously described strategies rely on artificial means to deliver the therapeutic nucleic acid to the target locus. Classically, liposome-mediated transfection, electroporation, and microinjection were employed. An alternative to transfection is the use of natural vehicles, such as viruses, which have co-evolved with their respective hosts to efficiently deliver their nucleic acid to the site of replication. Russell and co-workers were the first to show that recombinant vectors based on adeno-associated virus (rAAV) could be exploited to correct small insertions, deletions or single-base mutations (Russell and Hirata, 1998; Inoue *et al.*, 1999, 2001; Hirata and Russell, 2000). Three recent reports, including ours, have confirmed these findings (Kohli *et al.*, 2004; Liu *et al.*, 2004; Porteus *et al.*, 2003).

All rAAV vectors used for gene targeting thus far have been based on adeno-associated virus type 2 (AAV2). As described in Part 1 of this book, AAV2 is a human parvovirus with a single-stranded linear DNA genome flanked by inverted terminal repeats (ITRs; Figure 41.1A). Only upon infection is the AAV2 genome slowly converted into a



**Figure 41.1** rAAV gene-targeting vectors. Schematic diagrams of wild-type AAV (A) and rAAV gene targeting vectors (B–E). The illustration displays the location of the ITRs, the respective genes (large boxes), promoters (arrows) and polyadenylation signals (pA). Left and right arm refer to sequences homologous to the target sequence whereas the dark box in (B) and (C) depicts the non-homologous sequence set to correct the genetic mutation. PGK denotes the phosphoglycerate kinase promoter but stands for any heterologous promoter of choice. rfp and neor<sup>r</sup> represent any arbitrary fluorescent marker gene or selection marker, respectively, while IRES indicates an internal ribosomal entry site.

double-stranded template allowing the expression of the viral genes (Muzychka and Berns, 2001). The slow conversion from a single-stranded into a double-stranded genome is also relevant for rAAV vectors. There is growing interest in rAAV as a gene transfer vector, mainly as a result of the low immunogenicity and the ability to transduce multiple tissue types including non-dividing cells. Transgene expression is realized from both episomal and integrated forms of the vector. However, as opposed to the wild-type virus, rAAV vectors integrate at random (see Chapter 2).

## rAAV GENE TARGETING

### Design of vectors

Conventional rAAV-based gene therapy vectors contain an ITR-flanked transgene cassette including promoter and polyadenylation signal. Gene targeting vectors, on the other hand, can exclusively contain sequences homologous to the target locus (Figure 41.1b). The size capacity of 4.5 kb of exogenous DNA in between the ITRs is thereby ample to promote the introduction of specific genetic changes into homologous chromosomal sequences. It was found that the frequency of rAAV-mediated gene correction was improved when the non-homologous sequences, i.e. the mutation to be corrected, were placed in the centre of the rAAV vector genome (Hirata and Russell, 2000). Correction frequencies also improved by increasing the length of homology between the rAAV targeting vector and the chromosomal locus (Hirata and Russell, 2000; Liu *et al.*, 2004). This effect levelled out at a length of 600–1000 nucleotides for the short arm of homology (Hirata and Russell, 2000). As a matter of fact, two homology arms of 900 nucleotides were shown to be sufficient to promote rAAV-mediated gene targeting (Kohli *et al.*, 2004). Although not based on systematic examination, the consideration of a few additional observations may be useful when rAAV gene targeting vectors are being designed. A number of experiments implied that rAAV-mediated insertion of DNA is more efficient than the deletion of chromosomal sequences (Hirata *et al.*, 2002; Inoue *et al.*, 1999; Miller *et al.*, 2003; Kohli *et al.*, 2004). Two studies suggested that single-stranded genomes are the preferred substrate for rAAV-mediated gene targeting (Hirata and Russell, 2000; Hendrie *et al.*, 2003). A simple way to avoid the generation of rAAV particles with double-stranded genomes is to keep the total length of the vector genome larger than half of the wild-type size AAV genome, i.e. >2.4 kb (Hirata and Russell, 2000). With a parvoviral vector based on minute virus of mice (MVM), which has preserved the characteristic of packaging only one specific DNA strand, it was found that there is no difference in the frequency of gene correction for the coding and non-coding strand. However, the overall frequency of gene correction was lower with MVM vectors when compared with rAAV (Hendrie *et al.*, 2003).

### rAAV-mediated gene correction

The initial experiments by Russell and colleagues were performed with up to 500 000 vector genomes per cell to correct a single copy selection marker. The correction frequency in these experiments approached 1 percent, as determined by counting drug-resistant colonies (Russell and Hirata, 1998; Inoue *et al.*, 1999, 2001; Hirata and Russell, 2000). As vector design improved the vector dose could be considerably lowered (20 000 particles/cell) without a decline in the targeting frequency (Hirata *et al.*, 2002). Because drug selection can distort the experimental outcome, gene correction was assessed in unselected cell populations as well. An elegant and quantitative method is the enhanced fluorescent green protein (EGFP) rescue assay, in which a mutation abolishing fluorescence of the green fluorescent protein is corrected (Porteus *et al.*, 2003; Liu *et al.*, 2004). In a human cell line carrying a chromosomal copy of the mutant EGFP reporter gene, about 0.1 percent of cells turned green after infection with 10 000 particles/cell. Considerably less correction (0.006 percent) was attained in primary fibroblasts of a transgenic mouse carrying the mutant EGFP gene, and no correction could be detected *in vivo* after injecting the rAAV repair vector into the tibialis muscle (Liu *et al.*, 2004). This result is disappointing and further experimentation is needed to find out whether the failure to correct the mutant EGFP gene *in vivo* was species- and/or tissue-specific.

The ratio between gene correction and random integration is called the targeting ratio. It is a marker of the quality of gene correction and is as important a number as the absolute gene correction frequency. For the initial experiments it was estimated that the frequency of random integration was about 10-fold higher than gene correction (Russell and Hirata, 1998), thus giving rise to a targeting ratio of 1:10. As random integration is a matter of concern, this number must not be disregarded. In order to keep an eye on such random integration events, we opted to incorporate a small expression cassette for a fluorescent protein on the gene targeting vector (Figure 41.1c). Expression of the marker gene is only possible in the case of a non-favored event, such as episomal latency or integration into the host genome, but not if the rAAV vector genome is being used for gene correction (Cathomen and Weitzman, unpublished results). This safety measure not only permits to monitor directly non-favored events but will also be a valuable experimental tool for finding the optimal conditions for gene targeting.

### rAAV-mediated gene knockout

Besides correcting defective genes, the rAAV gene targeting system has also been used to insert transgene cassettes at a prechosen site in the chromosome. As already mentioned, gene disruption through HR is a major technique

to investigate the function of a gene in a specific cell type or organism. rAAV-mediated gene targeting therefore provides a simple means to accomplish gene knockouts both for therapeutic benefit or to assess biological pathways (Hirata *et al.*, 2002; Chamberlain *et al.*, 2004; Kohli *et al.*, 2004). Two different strategies to accomplish gene knockouts have been described. The first strategy aims at inserting a whole expression cassette for a selection marker into a gene of interest. The homology arms on either side of the expression cassette ensure site-specificity of the insertion (Figure 41.1d). The second approach uses intron–exon junctions to drive expression of the selection marker through an internal ribosomal entry site (IRES; Figure 41.1e). Both strategies have proven successful to precisely insert the transgene into an exon without the loss of any intronic sequences (Hirata *et al.*, 2002; Kohli *et al.*, 2004). After selection with the appropriate antibiotic, targeting frequencies of 0.4–13 percent for the promoter strategy and 31–90 percent for the approach involving the IRES were reported. Such approaches could also be combined with the Cre/lox system to excise the selection marker after successful knockout. By this means, null phenotypes or even the role of a specific exon could be investigated.

A preclinical application of this technology has recently been published for the brittle bone disorder osteogenesis imperfecta (Chamberlain *et al.*, 2004). rAAV vectors were used to disrupt the dominant-negative mutant COL1A1 allele in human mesenchymal stem cells (MSCs). The possibility to insert specific modifications in the genome of adult stem cells has great implications for molecular medicine as therapies involving transplantations with autologous, genetically modified stem cells could be used to treat numerous diseases.

## rAAV-mediated gene targeting and DNA double-strand breaks

A DNA double-strand break (DSB) leads to the activation of a multitude of different pathways critical to the survival of the affected cell, including cell-cycle check point responses and appropriate DNA repair pathways (Petrini and Stracker, 2003). The cell copes with DSBs in two different ways. It either re-ligates the broken ends by a mechanism called non-homologous end-joining (NHEJ) or by homology-directed repair, which involves HR between the broken chromosome and its sister chromatid (reviewed in Friedberg, 2003). As opposed to NHEJ, the second mechanism repairs DNA without the loss of sequence information.

Intriguingly, the cellular DNA repair system also accepts exogenous DNA as a template for homology directed repair. Studies in yeast and vertebrate cells have indicated that the intentional creation of a DSB in the target sequence augments HR-mediated gene targeting up to 1000-fold (reviewed in Johnson and Jasin, 2001). The rare cutting meganuclease I-SceI, an intron-encoded endonuclease present in the mitochondria of *Saccharomyces cerevisiae*, was initially used

to produce the DSBs (Jasin, 1996). I-SceI recognizes an 18 basepair target sequence, which is not present in the human genome. In order to test whether DSBs stimulate rAAV-mediated gene targeting, artificial target loci with an I-SceI binding site close to the mutation were generated. In two independent reports, gene correction was stimulated more than 100-fold in the presence of the I-SceI endonuclease (Miller *et al.*, 2003; Porteus *et al.*, 2003). Importantly, the creation of a DSB in the target locus also improved the targeting ratio 50-fold, hence reducing the risk of insertional mutagenesis considerably (Cathomen and Weitzman, unpublished results).

Although the I-SceI system makes a great model to study the mechanism of rAAV gene targeting, the therapeutic usefulness of the meganuclease is limited by the need to introduce the corresponding recognition site into a target locus. Enzymes that are designed to specifically cleave at prechosen chromosomal sequences are required. Such enzymes can be generated by molecular evolution or rational design based on the structure of existing meganucleases (Epinat *et al.*, 2003). Alternatively, chimeric nucleases can be designed by attaching a non-specific endonuclease domain to a specific DNA-binding domain (Jamieson *et al.*, 2003). Artificial DNA-binding domains can be synthesized by assembling predefined Cys<sub>2</sub>-His<sub>2</sub> zinc finger modules, each one recognizing a 3 basepair motif of the respective target sequence (Segal, 2002). The modules can be assembled in any order necessary to recognize any given sequence in the target locus. Fusion with the non-specific DNA-cleavage domain of the FokI endonuclease generates the so-called zinc finger nucleases (ZFNs). ZFNs were shown to stimulate gene targeting in cultured cells and in *Drosophila*, although some cytotoxicity issues, most likely due to cleavage at additional sites in the genome, remain unresolved (Bibikova *et al.*, 2001, 2003; Porteus and Baltimore, 2003; Wilson, 2003). Only a few ZFNs have been successfully created and used in cells since the concept has been established eight years ago (Kim *et al.*, 1996), suggesting that the technology is not yet robust enough to be broadly applied. Progress has been made with regard to the FokI cleavage domain (Miller *et al.*, 2004), but more research addressing affinity and specificity of the DNA-binding domain has to be done. A powerful method to optimize multidomain proteins in a cellular selection system has been recently described (Hurt *et al.*, 2003). Zinc finger-based transcription factors with improved DNA-binding affinity and specificity were isolated by employing a strategy in which the single zinc finger modules were optimized concurrently and context-dependent. The same strategy may work to improve affinity and specificity of ZFNs.

## The mechanism of rAAV-mediated gene targeting

The mechanism underlying rAAV-mediated gene targeting is poorly understood. Mismatch repair or HR have been

initially proposed to be directing rAAV-mediated gene targeting (Inoue *et al.*, 1999; Hirata *et al.*, 2002; Kren and Steer, 2002). Recent data suggest HR to be the more likely mechanism. First, the reports demonstrating that expression cassettes encompassing up to 2.7 kb can be inserted at prechosen loci in the human genome (Hirata *et al.*, 2002; Chamberlain *et al.*, 2004; Kohli *et al.*, 2004) excludes other DNA repair pathways. Second, the stimulation of rAAV-mediated gene correction upon intentional creation of DSBs in the target gene implies repair of the DSBs by HR (Miller *et al.*, 2003; Porteus *et al.*, 2003). Still, there must be some mechanistic differences to standard HR since the rAAV-mediated reaction involves only three strands, rather than the conventional four-stranded reaction (Figure 41.2). Moreover, the T-shaped hairpin structures of the ITRs are recognized by miscellaneous cellular proteins, including DNA repair factors, that might contribute to gene targeting

(Cathomen *et al.*, 2001; Qing *et al.*, 2001; Zentilin *et al.*, 2001).

Similar to transduction with rAAV vectors (Chapter 2), rAAV-mediated gene targeting increases with time and vector dose (Hirata and Russell, 2000; Inoue *et al.*, 2001; Porteus *et al.*, 2003). Furthermore, a report using cell-cycle blockers suggested that, as for rAAV transduction, cells in the G1/S-phase are more susceptible to gene targeting than cells in other phases of the cell cycle (Liu *et al.*, 2004). However, two of these studies clearly pointed out that there is no correlation between the level of rAAV transduction and gene targeting. Although genotoxic stress and protease inhibitors were shown to augment rAAV transduction in cultured cells, both strategies failed to enhance gene correction (Hirata and Russell, 2000; Liu *et al.*, 2004). This implies that different cellular factors are engaged in rAAV transduction and rAAV-mediated gene targeting.

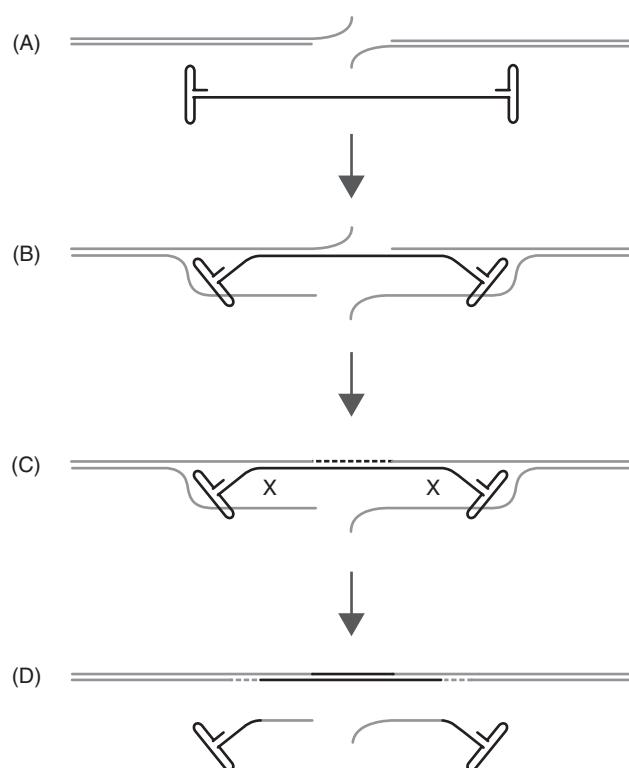
In summary, the single-stranded nature of the rAAV genome, the length of homology, and the number of targeting molecules that can be delivered to a single cell are crucial factors for the efficacy of this approach. Nonetheless, more work has to be done to better understand the molecular events underlying rAAV gene targeting, including the vector parameters and the cellular factors involved in this process.

## Safety aspects

The safety of rAAV vectors has been an issue of controversy ever since a study has demonstrated that rAAV vector genomes integrate into active genes in the mouse liver (Check, 2003; Kay and Nakai, 2003; Kohn and Gansbacher, 2003; Nakai *et al.*, 2003; Russell, 2003). Understandably, this has raised concerns given that rAAV vectors are currently being used in clinical trials of liver-directed gene therapy for hemophilia B and in view of the adverse events in the X-SCID gene therapy trial. Where and how rAAV vectors integrate has therefore implications for their use in gene therapy. It is important to emphasize the substantial differences between retroviral and AAV-mediated integration.

- Retroviral regulatory elements are more likely to activate a cellular gene next to the insertion site than the AAV ITRs.
- Retroviral vectors contain a protein machinery that actively integrates the viral genome – rAAV vectors do not.
- While a prerequisite for transgene expression by retroviral vectors, rAAV vector integration is relatively uncommon.

It has been suggested that rAAV integration is an inefficient process that takes place at low frequency into pre-existing chromosomal double-strand breaks by a mechanism involving NHEJ (Russell, 2003). In the mouse muscle, more than 99 percent of transgene expression may derive



**Figure 41.2** Possible mechanism of rAAV-mediated gene targeting. Schematic of rAAV-mediated gene targeting. (A) The cellular DNA repair machinery recruits homologous sequences, including the rAAV vector genome (black line), to the site of the DSB within the cellular target locus (gray lines), where it is used as a template for homology-directed repair. (B) The single-stranded nature of the rAAV vector genome facilitates strand invasion and allows the vector genome to basepair with the complementary sequences of the broken chromosome. (C) The rAAV genome serves as a template for de novo DNA synthesis of the top strand (dotted line) and/or as a substrate for double cross-over between the host and the vector genome. (D) After recombination is realized, the cellular repair machinery fills the gaps (dotted lines) and degrades the extra DNA.

from episomal copies of the rAAV genome (Thomas *et al.*, 2003), suggesting a very low risk of insertional mutagenesis. This observation also explains why rAAV-mediated long-term expression *in vivo* has been reported only from quiescent tissues. In addition, data obtained from hundreds of mice treated with rAAV vectors do not suggest a risk of cancer (Kay and Nakai, 2003). With regard to rAAV gene targeting, the selective creation of a DSB in the target locus significantly shifts the balance from random integration to gene targeting and thus lowers the risks associated with random integration even more.

## CONCLUSION

Ultimately, gene targeting has to be combined with cell targeting. For rAAV vectors enormous progress has been made in the recent past. The discovery of additional AAV serotypes (Gao *et al.*, 2002), the creation of mosaic capsids (Rabinowitz *et al.*, 2004) and the ability to redirect rAAV infection to specific tissues (Büning *et al.*, 2003; Müller *et al.*, 2003; Perabo *et al.*, 2003) provides us with an extensive and flexible vector system for a variety of applications.

Even though rAAV gene targeting has been realized in various cultured cells and in MSCs, the optimal vector parameters and the cellular factors governing this process have not yet been determined. Improvement of the gene targeting frequency and the targeting ratio will strongly depend on enhanced vector design and innovation regarding the ability to create a DNA damage with sufficient selectivity. Random integration is a serious side effect of all strategies aimed at introducing genetic modifications. Thus, quantitative model systems including transgenic mice will be invaluable for predicting and reducing the risk of insertional mutagenesis and for working towards the goal of permanent correction of inherited diseases.

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# rAAV gene transfer to the liver

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The liver has often been considered a prime target for gene transfer because of its central roles in the production of secreted proteins, in intermediary and drug metabolism, and in the excretion of bile. The preclinical experience with recombinant adeno-associated virus (rAAV) gene transfer to the liver has provided the rationale for a phase I clinical trial of rAAV-factor IX gene transfer to the liver, which has recently been performed in patients with hemophilia B (Snyder *et al.*, 1997, 1999; Nakai *et al.*, 1998; Mount *et al.*, 2002; High, 2004). These studies, along with numerous other *in vitro* and *in vivo* studies, have helped to define several advantages and limitations of this system and this information has been used to develop a number of new vector designs in an attempt to overcome these limitations.

The potential barriers to hepatocyte transduction and the potential means of overcoming these barriers are listed in Table 42.1. These include a paucity of certain cell surface receptors, proteasome-mediated degradation of vector, barriers to nuclear entry, slow conversion to double-stranded DNA, the establishment of stable integration of vector DNA in a form that will persist within the target cell population, and the development of humoral and cell-mediated immune responses to AAV capsid proteins and transgene products. In addition, one must consider other particular risks of rAAV-mediated gene transfer, including the potential for vertical germline transmission and the theoretical possibility of insertional mutagenesis leading to carcinogenesis. New approaches to surmount these barriers have included the introduction of new rAAV serotypes, capsid mutants, proteasome inhibitors, self-complementary AAV vectors, tyrosine phosphatase inhibitors, and AAV-rep-mediated site-specific integration. One other intrinsic

**Table 42.1** Barriers to rAAV vector transduction of the liver and the corresponding vector strategies to avoid these barriers

Barrier	Vector strategy
Small packaging capacity	Dual vector approaches
Paucity of receptors	Alternate serotypes
Proteasome-mediated degradation	Targeted capsid mutants
Inhibition of second-strand synthesis	Proteasome inhibitors
	Self-complementary AAV vectors
	Tyrosine kinase inhibitors
Immune responses to vector capsid	Alternate serotypes
Immune responses to transgene products	<i>In utero</i> gene transfer

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limitation of rAAV, its small packaging capacity, has recently been approached using dual vector approaches. Finally, one must consider the potential for immune responses, both to vector capsid components and to transgene products. Each of these barriers and the new vector strategies to overcome them will be considered in the context of rAAV-mediated gene transfer to the liver.

## FIRST GENERATION rAAV2 VECTOR GENE TRANSFER TO THE LIVER

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Recombinant AAV2 vectors have been shown to mediate gene transfer to the rodent liver in a number of different contexts with a number of transgenes of interest (Ponnazhagan *et al.*, 1997b; Miao *et al.*, 1998; Snyder *et al.*, 1997, 1999;

Koeberl *et al.*, 1997; Song *et al.*, 2001a; Xu *et al.*, 2001). The sum total of these studies tends to indicate that rAAV2-mediated gene transfer to the liver of healthy animals generally results in a high level of transgene expression that is stable over time. Further examination has shown, however, that only about 5 percent of hepatocytes account for all of the expression seen long term after rAAV injection into the portal or systemic venous circulation (Miao *et al.*, 2000). Furthermore, vector genomes within this subset of stably transduced cells are predominantly episomal (Nakai *et al.*, 2000, 2001; Song *et al.*, 2001a). Recent data suggest that a host nuclear protein, DNA-PKcs, is responsible for inhibition of integration and favoring of recombination between input rAAV vector genomes (Song *et al.*, 2001b, 2004).

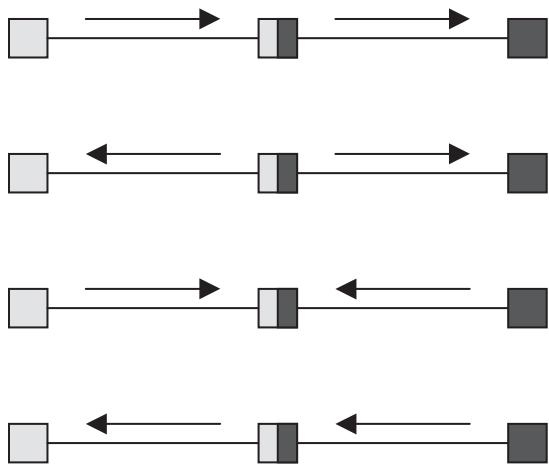
Recombinant AAV2-mediated gene transfer has recently been tested within the livers of patients with hemophilia B (reported by K. High at the 2004 American Society of Gene Therapy meeting). These studies have provided several valuable lessons:

- Gene transfer and expression was feasible in these patients and was generally well tolerated.
- Some patients did experience a number of potentially adverse findings, including the presence of vector DNA within the semen, transient elevations of the transaminases in some patients, and the development of cell-mediated immune responses to AAV capsid proteins.
- Also of note, transgene expression appeared to be transient in this context.

In considering the future use of rAAV in the liver, a number of potential limitations of this system will be considered below.

## LIMITED PACKAGING CAPACITY OF rAAV VECTORS

One inherent limitation of rAAV vectors in the liver is their small packaging capacity of approximately 5 kilobase. One approach to circumventing this limitation is to exploit the fact that rAAV genomes form concatemeric episomes within the nucleus of transduced cells. One rAAV vector combination was designed using a split intron (Sun *et al.*, 2000; Yan *et al.*, 2000; Duan *et al.*, 2001). The combination consists of two constructs. In the first construct was inserted a promoter, an initial portion of the gene of interest, a splice donor site and the upstream portion of an intron. In the second construct was inserted the downstream portion of the intron, the splice acceptor, 3' portion of the gene, and the polyA signal. Of the four possible orientations in dimerization between these two species that could occur, only one produces the intact protein of interest (Figure 42.1). The alternative design for a dual vector tandem was devised by inserting a transcriptional enhancer in the first vector and



**Figure 42.1** Four possible orientations of products from a split intron-dual vector approach. When one vector carries the entire transgene and the other an enhancer, all four will be active. When the two vectors carry the two halves of a single gene coding region with an intervening intron, only the first of these will be active (with permission from Flotte, 2000).

an inverted terminal repeat (ITR)-only promoter/gene vector in the other (Duan *et al.*, 2000). In this case, the low-level endogenous transcriptional activity of the AAV-ITR is greatly augmented after vector–vector dimerization occurs.

## OVERCOMING BARRIERS TO CELL ATTACHMENT AND ENTRY

Prior to the late 1990s, most studies of AAV biology and rAAV vectors were performed with AAV serotype 2. The emergence of pseudotyping of AAV2-ITR-flanked genomes into capsids of different serotypes, such as type 3, type 5, and type 6, was initially conceived as a potential means to bypass neutralizing antibodies to AAV2 (Beck *et al.*, 1999; Zabner *et al.*, 2000; Halbert *et al.*, 2000, 2001). Subsequently, it became clear that the AAV serotypes present in primates were very numerous and differed greatly in their transduction efficiency in a wide range of cell targets including hepatocytes (Gao *et al.*, 2002, 2003).

Some studies with AAV5- and AAV1-pseudotyped vectors have indicated that its primary receptor, N-linked sialic acid (N-SA), was present in higher abundance on the surface of cells in organs like liver and lung than was that of AAV2, the heparan sulfate proteoglycan (Teramoto *et al.*, 1998; Summerford and Samulski, 1998; Xiao *et al.*, 1999; Zabner *et al.*, 2000; Kaludov *et al.*, 2001). Subsequent studies have also shown greater potency for hepatic gene transfer from other rAAV pseudotypes, such as type 8. Recent work by Gao *et al.* has indicated that over 100 serotypes and novel genomic variants are present in the non-human primate and human population (Gao *et al.*, 2003). Some of these appear to have greater gene transfer efficiency in the liver as

compared with rAAV2. The impact of novel rAAV serotypes may affect the success of future protocols which target the liver. For example, gene therapy for AAT deficiency has been conceived of as targeting either skeletal muscle (as a platform for ectopic secretion of AAT protein) or the lung (which is its primary site of action). Recent data would suggest that rAAV8 might offer significant advantages for liver delivery (Gao *et al.*, 2002).

In a similar vein, novel receptor-targeted capsid mutagenesis approaches have emerged (Girod *et al.*, 1999; Rabinowitz *et al.*, 1999; Wu *et al.*, 2000b; Wendtner *et al.*, 2002; Buning *et al.*, 2003; Huttner *et al.*, 2003; Perabo *et al.*, 2003). Early studies demonstrated the feasibility of using AAV capsid mutagenesis to insert peptides of at least 28 amino acids into the capsid gene sequence, so that they are expressed on the surface of the recombinant virion. These include the N-terminal region of VP-2 and the heparan sulfate binding loop, including key arginine residues 585 and 588. A number of different short peptides have been inserted successfully, including those targeting receptors in high abundance in the liver, including the low density lipoprotein receptor and the serpin enzyme complex receptor. *In vivo* gene transfer to the liver with a rAAV2-LDL-receptor-targeted mutant showed a 4-fold advantage in efficiency over a standard rAAV2 vector (Wu *et al.*, 2000a). While some preliminary data have indicated some significant advantages of these capsids over the standard AAV2 vectors, it remains to be seen whether there will be any advantage over the new pseudotypes with greater efficiency for liver gene transfer. The recent publication of the crystal structure of AAV2 and the anticipated availability of structural data on newer AAV serotypes might afford an opportunity for further enhancements of rationally designed vectors of this type (Xie *et al.*, 2002, 2003).

## POST-ENTRY BLOCKADES TO GENE TRANSFER

Post-entry limitations may be the primary barrier to gene transfer in certain cell types that are relatively difficult to transduce with rAAV (Yan *et al.*, 2002). Specifically, vector particles were shown to have increased transduction efficiency after inhibition of proteosome-mediated degradation. This effect was shown with both tripeptide proteosome inhibitors and with anthracycline antibiotics and has been demonstrated *in vivo* in both liver and lung (Ding *et al.*, 2003; Yan *et al.*, 2004). Meanwhile, inhibition of ubiquitination actually blocks AAV2-apical mediated expression (Yan *et al.*, 2002). This suggests that ubiquitination and export from the endosomal lumen enhance vector expression, but that proteosomal degradation subsequent intercepts vector particles that would otherwise be bound for the nucleus and destined for expression of the transgene. The possibility exists that proteosome inhibitors might play a significant role as an adjunct to gene therapy, even with the existing vector technology. Potential side-effects of these agents clearly must also be considered.

## TYROSINE PHOSPHATASE INHIBITORS

Another significant advance in recent years has been the discovery of another barrier to rAAV-mediated expression presented by a nuclear protein, FKBP52, which binds to the AAV2-d-sequence and inhibits leading strand synthesis (Qing *et al.*, 1997, 1998, 2001). Binding of this protein to the ITR keeps the majority of rAAV genomes in the single-stranded DNA form, which is transcriptionally inactive. However, FKBP52 only binds the ss-d-sequence when the protein is in its dephosphorylated state. Tyrosine phosphatase inhibitors, such as genestein and tyrphostin, can increase the proportion of FKBP52 remaining phosphorylated state and thus hasten the conversion of rAAV genomes to double-stranded (ds)-DNA. This in turn will shorten the delay in transcription and translation of the transgene product.

Importantly, the role of FKBP52 as a limiting factor in rAAV-mediated gene transfer has recently been confirmed in transgenic mouse studies. In these studies, either knockout of FKBP52 or overexpression of a T-cell-derived phosphatase (TC-PCP), which will dephosphorylate FKBP52, resulted in a more rapid onset of transgene expression within the liver. These transgenic studies are important, for they confirm these effects in a more precisely defined manner than can be accomplished with pharmacologic inhibitors. Nonetheless, the inhibitors of the class mentioned above have been used clinically in other contexts. Although their safety and efficacy have not yet been demonstrated *in vivo*, it would be conceivable to consider a strategy where one might augment a rAAV gene therapy to the liver with a tyrosine phosphatase inhibitor.

Another approach to this problem that operates at a more molecular level is to use a rapidly-expressing self-complementary rAAV (sc-AAV) vector to bypass the need for leading strand synthesis (McCarty *et al.*, 2001; Ding *et al.*, 2003; Wang *et al.*, 2003). This approach involves including both sense and reverse-complement copies of the expression cassette and transgene with a hairpin inbetween, allowing vector DNA to immediately fold back upon itself into a ds-DNA form as soon as it is uncoated. The feasibility of this technique has been demonstrated by a number of groups. The primary limitation of this approach is that it further decreases the effective packaging capacity by 2-fold. A particularly inventive way around this has been to use a rapidly-expressing sc-AAV to express TC-PCP in cells prior to introduction of a conventional single-stranded rAAV vector (Zhong *et al.*, 2004).

## LONG-TERM PERSISTENCE OF TRANSGENE SEQUENCES

Conventional rAAV2 gene therapy vectors appear to lack a mechanism for efficient and stable vector DNA integration. Episomal persistence is the primary mode of rAAV2 vector

persistence in liver, lung, and muscle (Flotte *et al.*, 1994; Afione *et al.*, 1996; Kearns *et al.*, 1996; Ponnazhagan *et al.*, 1997a; Nakai *et al.*, 2001; Song *et al.*, 2001a,b, 2002, 2004). In healthy animals, this does not limit the duration of transgene expression, since these cell types are normally in the G<sub>0</sub> state. Thus, an rAAV-AAT gene therapy approach targeting one of these two organs might have the opportunity for long-term gene transfer even if no further modifications to the vector were made.

The primary caveat to that assumption, however, is that patients with AAT-deficient liver disease often experience ongoing hepatocellular injury, which very well might cause the liver in these individuals to continue proliferating at some ongoing rate. In this context, facilitating rAAV integration might be advantagous. The AAV non-structural protein, rep 78/68, has been shown to target integration of AAV-inverted terminal repeat (ITR)-containing genomes to sequences within the AAV-S1 site (Kotin *et al.*, 1990, 1991; Surosky *et al.*, 1991, 1997; Bertran *et al.*, 1998; Pieroni *et al.*, 1998). This approach has been used in rodent models, but requires further study *in vivo*. rep 68/78 has been shown to repress transcription from heterologous promoters, so that ongoing expression would likely be undesirable. The co-administration of rep protein or transient expression of rep 68/78 along with ITR-containing vectors might be preferable. Nuclear factors might also affect the rate of rep-independent integration. Recent work has shown that DNA-dependent protein kinase catalytic subunit (DNA-PKcs) blocks integration of rAAV integration and favors the formation of rAAV episomes (Song *et al.*, 2004).

## IMMUNE RESPONSES TO AAV AND OTHER VECTOR COMPONENTS

rAAV vectors do not elicit the innate immune cytokine responses that have characterized the administration of recombinant adenovirus vectors. Furthermore, all current rAAV vectors are devoid of viral coding sequences. Based on these facts, it has often been stated that rAAV is relatively immune privileged.

However, every rAAV vector consists of a protein capsid surrounding the vector DNA, and numerous preclinical and clinical studies have demonstrated humoral immune responses to these capsid proteins after administration of vector to a variety of sites (Xiao *et al.*, 1996; Halbert *et al.*, 1998; Aitken *et al.*, 2001; Moss *et al.*, 2004). These antibodies do appear to decrease the efficacy of subsequent administrations of rAAV vectors packaged into that same serotype. The use of alternative serotypes has been shown to overcome this humoral immune response to capsid (Manning *et al.*, 1998; Beck *et al.*, 1999; Halbert *et al.*, 2000, 2001). Interestingly, one study in rhesus macaques has demonstrated that while humoral immune responses to capsid are ubiquitous after administration of wild-type AAV to any of

a number of sites, cell-mediated immune responses were seen only after co-administration of a helper-adenovirus (Hernandez *et al.*, 1999). Preliminary evidence has emerged suggesting that hemophiliacs involved in the phase I clinical trial of liver delivery of rAAV2-factor IX may have also developed cell-mediated immune responses to vector capsid components (reported by K. High at the 2004 American Society of Gene Therapy meeting). It remains to be seen whether this patient population was particularly predisposed to such a phenomenon because of coincident infection with hepatitis C virus, or whether the use of a serotype (serotype 2), to which they had been previously exposed, was a critical factor.

In general, reports of humoral or cell-mediated immune responses to transgene products expressed from AAV vectors have been seen primarily when foreign (cross-species) transgenes have been used. Two recent reports of humoral immune responses after intramuscular administration of rAAV-erythropoietin vectors in macaques are somewhat troubling in this regard (Chenuaud *et al.*, 2004; Gao *et al.*, 2004). In the latter cases, the endogenous *Epo* gene was used and self-tolerance appears to have been broken after vector-mediated expression of *Epo* within the muscle. Such a phenomenon has not been seen in numerous other intramuscular injection experiments, however, and such a finding has not yet been reported after liver administration of rAAV. Nonetheless, the concern about humoral immune responses to transgene is a very real one, particularly since rAAV-mediated gene transfer may be used to deliver gene products in patients genetically deficient in those proteins. One approach to this problem has recently been demonstrated by the Byrne laboratory in a mouse model of glycogen storage diseases type 2 – deficiency in acid maltase (GAA). In that study, post-natal administration of the rAAV-GAA vector resulted in immune responses to GAA, but *in utero* gene transfer of the same vector did not (Rucker *et al.*, 2004). While a number of other safety and biodistribution issues must be addressed prior to adopting the *in utero* strategy for clinical gene therapy, this approach also has the theoretical advantages of requiring a lower absolute dose of vector and having the potential to prevent sequelae of the disease that might begin to develop very early in life.

## FUTURE DIRECTIONS

It seems likely that the coming years will witness more studies that seek to avoid potential side-effects of rAAV-mediated gene transfer to the liver while exploiting the best vector designs to optimal advantage for efficient, stable gene transfer. Of greatest interest, perhaps, will be those strategies aimed at avoiding immune responses to the transgene. If these can be avoided the intrinsic ability of rAAV to mediate long-term persistence may well prove to be the key to successful clinical gene therapy.

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# AAV vectors for retinal gene therapy

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The mammalian retina is an ideally suited target organ for developing gene-based therapies for several key reasons:

- The cell types primarily affected in known retinal diseases, retinal ganglion cells on the inner face of the retina, photoreceptors on its outer surface, and retinal pigmented epithelial cells adjacent to and interdigitated with photoreceptors, are all readily accessible to the skilled surgeon. This has provided the retinal specialist with a deep arsenal of techniques and tools with which to safely manipulate the retina. Thus efficient surgical delivery of a viral vector to the retina does not need to be invented, it already exists and is well within the expertise of virtually all retinal surgeons.
- There is perhaps a deeper appreciation of the range of genetic diseases of the retina than for any other organ, i.e. there are currently about 150 known genetic retinal disease loci in the human genome, of which approximately 90 are known at the complete sequence level ([www.sph.uth.tmc.edu/RetNet/](http://www.sph.uth.tmc.edu/RetNet/)). In this latter category, their biochemical roles in vision are known for nearly half.
- Non-invasive assessment of retinal function and structure has been the focus of clinical ophthalmology for more than a century. This has led to wide array of sensitive and reproducible techniques for measuring retinal morphology and vision status in humans. Thus clinical endpoints for determining the success or failure of a given gene therapy are already in hand for many retinal diseases.
- The profound impact of vision loss on an affected individual's life has prompted prospective clinical studies of many progressive retinal diseases, often beginning at relatively early ages, sometimes well

before the patient is aware of a vision deficit. Such data on the natural history of disease will greatly aid clinical trial planning with regard to optimal treatment ages and endpoint analysis.

- Finally, and not least, the development of sensitive ways to monitor visual status has led to identification of many natural animal models of retinal disease. This has greatly supplemented our ability to generate animal models of genetic retinal disease transgenically. There are currently dozens of natural and transgenic higher animal models that closely mimic the corresponding human retinal disease in at least five experimentally assessable species: mouse, rat, dog, cat, and chicken.

Thus, unlike the situation for many other diseases primarily affecting one organ, those of the retina have a broad diversity of genetically understood causes that can be placed in the biochemical context of vision, the tools for non-invasively monitoring disease progression are well established, and the animal models with which to test and perfect gene-based therapies are identified and available.

Against this backdrop, AAV vectors have emerged as the most versatile and effective gene delivery modality for the retina. This chapter is organized into two main topics: the tools used to efficiently and specifically direct AAV-mediated gene delivery to the desired retinal cell type, and a review of the current status of retinal gene therapy using AAV. This latter topic has been arranged according to genetic etiology: dominant retinal diseases, recessive (including most X-linked diseases that can be treated by gene replacement therapy) retinal diseases and retinal diseases of complex or unknown cause, primarily retinal neovascular diseases. Each class demands a somewhat different gene therapy approach.

## BEHAVIOUR OF AAV VECTORS IN THE RETINA

### Vector injection site options

Because of the laminated and compartmentalized nature of the mammalian retina, the choice of injection site is a key variable in AAV-vectored gene delivery. Clearly this choice is based on where a therapeutic gene needs to be expressed. Subretinal injections target the rod and cone photoreceptors and cells of the retinal pigment epithelium (RPE), the cells that contact the subretinal space. If the therapeutic gene is a secreted protein, subretinal injection can lead to effects on more distant cells such as the choroidal blood vessels presumably by diffusion across Bruch's membrane that separates the RPE from the choroidal blood vessels. Subretinal injections are typically administered via a thin long injection syringe via either a corneal or scleral puncture. The resulting vector bleb, really a subretinal detachment resulting from the injection volume of 0.5–2 µl in rodents, covers roughly 30–90 percent of the retina with a single injection. The trapped fluid in the subretinal space is both passively and actively transported towards the choroid by the RPE. This RPE fluid 'pump' function plays a major role in reabsorption of subretinal fluid and in promoting and maintaining retinal attachment. Reduction of the induced subretinal bleb is normally completed within a day after injection (Timmers *et al.*, 2001). This route of vector delivery has generated a certain amount of concern since subretinal fluid accumulation can be associated with a variety of retinal problems. Recently, chemicals such as INS37217, a synthetic P2Y<sub>2</sub> agonist against intracellular calcium signaling, have been shown to stimulate this RPE pumping process leading to enhanced retinal reattachment in a rodent model (Maminishkis *et al.*, 2002; Meyer *et al.*, 2002). Such strategies could be potentially beneficial in minimizing injection-related retinal injury, particularly following the larger volume subretinal injections used in larger species.

Intravitreal injection transduces primarily retinal ganglion cells (RGCs) and possibly amacrine cells (Mori *et al.*, 2002b). Passenger gene expression in rodent RGCs, because of their axonal projections to the brain, may also deliver some encoded protein to the brain presumably through optic nerve anterograde transport (Dudus *et al.*, 1999; Harvey *et al.*, 2002). In addition, several experiments have shown that intravitreal injection can transduce photoreceptors and RPE cells if neonates are treated (Harvey *et al.*, 2002; Tolentino *et al.*, 2002; Surace *et al.*, 2003). For example, after intraocular injection at P<sub>0</sub>, about 50 percent of transduced cells are photoreceptors with the remaining 30–40 percent being amacrine or bipolar cells. This observation, together with immunohistochemical analyses of the developing rodent retina (Rich *et al.*, 1997), suggest that the tight junctions of retina such as the inner limiting

membrane may be not fully developed at these early ages, thus allowing the penetration of vector beyond the RCG layer. Given that subretinal injection generally leads to a limited area of transfection, largely due to the small injection volumes, intravitreal injection currently provides the best means for dispensing vector to the whole retina in a single administration. An additional advantage is that intravitreal injections are technically easier and generally less invasive than the subretinal injections. Therefore, for many gene therapy applications, particularly those involving secreted proteins, intravitreal administration is the preferred route, as long any potential protein transport to the brain is monitored carefully.

While subretinal and intravitreal injections are currently the most commonly used delivery routes, both have potentially undesirable effects including cataract, retinal detachment, hemorrhage, and inflammation. This is particularly true for the subretinal injection. Even for a skilled person, up to 20 percent of mouse eyes suffer permanent injury as measured by electroretinogram (ERG), although such procedure-related adverse events are much less evident in a larger animal species. In addition, although proper serotype and promoter selection largely alleviates the problem (see below), there remains concern regarding possible toxicity from ectopic expression of vectored proteins in unaffected retinal cells, for example when a therapeutic gene is needed in the RPE, co-expression in photoreceptors could be toxic. These considerations have led to the search for safer alternative injection routes. Recent studies have shown that periocular (or subconjunctival) administration of vectors expressing secretable agents may be a good alternative. Several groups have exploited this option with promising results (Kim *et al.*, 2002; Gehlbach *et al.*, 2003a,b). Gehlbach *et al.* reported that periocular transfer of an adenoviral vector containing either sFlt-1, an antagonist of vascular endothelial growth factor (VEGF), or pigment epithelium-derived factor (PEDF) cDNAs suppressed choroidal neovascularization, suggesting that the encoded proteins can pass through the sclera and enter the choroids (Gehlbach *et al.*, 2003a,b). Whether they can reach the retina properly remains to be determined. These data, together with earlier studies with periocular injection of biotraceable proteins (Kim *et al.*, 2002; Gehlbach *et al.*, 2003a,b), suggest that this route may offer a useful alternative for delivering a therapeutic gene, at least one that is intended to become extracellular.

### Behaviour of AAV-serotypes in the retina

The transduction efficiency of AAV vectors in the retina is acutely dependent upon the vector serotype used. As noted in previous chapters, there are distinct differences among the cell targeting specificities of eight currently analyzed serotypes, and this holds for the various retinal tissues as well. Analysis of the known AAV serotypes/pseudotypes in the rodent eye has shown clear serotype dependence on

retinal cell-type specificity (Auricchio *et al.*, 2001; Yang *et al.*, 2002). Upon subretinal injection of either AAV2 or AAV5 vectors expressing green fluorescent protein (GFP), transduction of both RPE cells and photoreceptor cells (PRs) is seen. In contrast, AAV1 vectors transduce predominantly the RPE, being approximately 15 times more efficient than AAV2 (Li *et al.*, 2002). Like AAV1 to which it is closely related, AAV6 transduces primarily the RPE while AAV3 poorly transduces all retinal cells. In mammalian retina the transduction efficiency of AAV5 appears to be significantly greater than for AAV2: at 15 weeks post injection the ratio of transgene expressing photoreceptor cells transduced with AAV5 versus AAV2 is 400:1, and the viral genome copies per eye was 30 times higher for type 5. Additionally, AAV5 vectors showed a quicker onset and greater level of transgene expression (Yang *et al.*, 2002).

The unique environment of the eye plays a significant role in inhibiting the development of an antigen-specific response. The subretinal space is separated from the blood supply on one side by the RPE and Bruch's membrane, and on the other by a tight intracellular junction. These barriers contribute significantly to the protection of the retina from the immune-mediated damage by sequestering antigens from the systemic circulation. Recipient animals do not elicit responses to most AAV2-encoded products, even if these proteins contain foreign epitopes. This effect appears to reflect an inability of AAV2 to infect antigen-presenting cells (Jooss *et al.*, 1998). The subretinal administration of AAV2 as well as AAV5 and AAV1, although not eliciting neutralizing antibodies, does evoke an immune response similar to anterior chamber-associated immune deviation (Auricchio *et al.*, 2001; Anand *et al.*, 2002).

### Promoters to target specific retinal cell types

A key element in vector design is a promoter that allows proper retinal cell-type regulation and expression of the passenger gene. In the initial reports devoted to the retinal transduction by AAV, the ubiquitous immediate-early cytomegalovirus (CMV) enhancer-promoter, and later, the CBA promoter (a fusion of the chicken beta-actin promoter and CMV immediate-early enhancer (Sawicki *et al.*, 1998), drove efficient, stable, and non-toxic GFP or beta-galactosidase reporter expression in multiple retinal cell types. Following a single injection of 1–3 µl in rodents, more than half of the total retinal area was often seen to be transgene positive (Flannery *et al.*, 1997; Grant *et al.*, 1997). Cell-targeting specificity was strongly dependent on the site of administration. Subretinal injections resulted in RPE and photoreceptor transduction, as noted above, while intravitreal injections transduced various cells of the inner nuclear layer, primarily RGCs (Martin *et al.*, 2003) as well as a smaller number of Muller cells (Ali *et al.*,

1998; Auricchio *et al.*, 2001; Martin *et al.*, 2002), perhaps related to retinal injury (W. W. Hauswirth, unpublished). Passenger gene product spread to the optic nerve via RGC axons and perhaps beyond (Dudus *et al.*, 1999; Guy *et al.*, 1999).

Clearly one principal advantage of promoter choice is the possibility of limiting passenger gene expression to a single retinal cell type, and, in the context of gene therapy, to the affected retinal cell type. The mouse rod opsin promoter was the first to be tested in the context of an AAV vector in the retina (Flannery *et al.*, 1997). Employing the sequence (-386/+85) of the murine rhodopsin promoter/gene to drive expression in AAV2, the GFP reporter product is found exclusively in photoreceptors, not in any other retinal cell type or in the adjacent retinal pigment epithelium after subretinal administration into adult mice or rats. GFP-expressing photoreceptors were quite evident over 25–30 percent of the total retinal area surrounding a single 2 µl injection. Photoreceptors were transduced with nearly 100 percent efficiency in this region. Our current, more careful analysis of photoreceptor cell types transduced by rod opsin promoter-containing vector show that in both mice and rats, although there is a clear preference for rod photoreceptors, there is incomplete subtype specificity *in vivo* with transduction of some cones as well (Glushakova and Hauswirth, unpublished).

Cone-directed expression has also proven to be feasible using the appropriate cone opsin promoters (Li and Hauswirth, unpublished). The human 2.1 kb red/green opsin gene upstream sequence targeted GFP expression only to a subset of photoreceptors using AAV2 vectors. This promoter construct contained a proximal domain (495 bp) and an upstream domain from -3.1 to -4.6. This upstream region contains a locus control region (LCR) that is essential for expression of both red and green pigment genes and is highly conserved among mammals (Wang *et al.*, 1992). As shown by co-localization of GFP fluorescence and cone-specific antibody staining, expression was cone specific. Additionally, in rats, only M-cones supported reporter gene expression because no S-cone opsin-positive cones exhibited GFP fluorescence. In parallel experiments, a human blue cone proximal promoter (540 bp) was found to target GFP reporter expression to predominantly rodent S-cones. The relative transduction efficiency of AAV5 vector with this promoter driving GFP is 1500:34:1 for S-cones versus M-cones versus rods, respectively (Glushakova *et al.*, 2003).

The RPE plays a pivotal role in the development and function of the retinal photoreceptors, and, not surprisingly, mutations in RPE genes are the cause of a variety of retinal degenerations. Hence the RPE-specific expression of the therapeutic genes to restore normal RPE function is an important gene therapy aim. Two well-studied RPE specific genes whose mutations cause severe vision defects are VMD2 bestrophin gene, responsible for Best disease (Sun *et al.*, 2002) and RPE65 gene responsible for one form

of Leber congenital amaurosis (LCA) (Gu *et al.*, 1997; Marlhenes *et al.*, 1997). Transgenic mouse studies with several VMD2 promoter regions revealed that a -253/+38 bp fragment is minimally sufficient to direct RPE-specific expression *in vivo* (Esumi *et al.*, 2004). The rodent and human RPE65 genes share several *cis*-acting elements; an octamer sequence, a nuclear factor one (NF1) site, and two E-box sites, suggesting a conserved mode of regulation. In transgenic mice the 655/+52 bp fragment of the RPE65 promoter was sufficient to direct high RPE-specific expression, whereas shorter fragments (-297/+52 bp and -188/+52 bp) generated only background activity (Liu *et al.*, 1997). Candidate 5'-upstream regions of the human VMD2 and RPE65 genes were tested in AAV1 vectors for their ability to target RPE-specific expression (Glushakova and Hauswirth, unpublished). Both the 585/+38 bp VMD2 promoter and the 814 bp proximal RPE65 promoter in AAV1 limited expression of GFP almost exclusively to the RPE, with only an occasional photoreceptor cell also GFP-positive. Thus there appear to be two current promoter options for targeting RPE expression, the VMD2 and RPE65 promoters. In each case, in combination with AAV serotype 1 vectors, a high level of cell specificity is possible.

In the context of a human gene therapy, strong constitutive expression of a therapeutic gene in retinal tissues could be deleterious for function either because the product is overexpressed or because its duration of expression is too long, or both. This limitation can be overcome in theory by the use of exogenously regulatable promoter systems in the vector. Two such systems have been tested in the retina using AAV vectors (McGee Sanftner *et al.*, 2001; Auricchio *et al.*, 2002b). The tetracycline-inducible expression system employed in one a silencer/*trans*-activator AAV2 vector, and an inducible doxycycline-responsive GFP in another AAV2 vector that were co-injected subretinally (McGee Sanftner *et al.*, 2001; Auricchio *et al.*, 2002b). The system demonstrated tight regulation of the gene expression in photoreceptors and RPE cells in response to doxycycline levels in the drinking water. The second employed the rapamycin-induced dimerization system, and also used co-injection of two AAV vectors (McGee Sanftner *et al.*, 2001; Auricchio *et al.*, 2002b). Induction of high levels of the passenger gene product, a secreted erythropoietin, was achieved rapidly after the drug consumption. Upon the withdrawal of the inducer, erythropoietin levels decayed rapidly. Whether such regulated expression systems would be useful in a therapeutic setting in the retina is yet to be shown. For full usefulness, however, incorporation of the cell type-specific retinal promoters, as outlined above, into the AAV constructs would be desirable and probably necessary.

In a matrix format, the behaviour of AAV vector serotypes 1, 2, and 5 as a function of promoter is summarized for subretinal vector delivery in Table 43.1 and intravitreal delivery in Table 43.2.

**Table 43.1** Transduction behavior of AAV vectors when delivered subretinally. The font size is a general indication of the relative efficiency of transduction in that retinal cell type.

Promoter	Serotype		
	1	2	5
CBA	RPE, rods, cones	RPE, rods, cones	RPE, rods, cones
mouse opsin	rods, cones	rods, cones	rods, cones
human RPE65	RPE, rods,	RPE, rods,	RPE, rods,
human VMD2	cones	cones	cones
human red opsin	ND	M-cones	M-cones
human blue opsin	ND	S-cones	S-cones

**Table 43.2** Transduction behavior of AAV vectors when delivered intravitreally. The font size is a general indication of the relative efficiency of transduction in that retinal cell type.

Promoter	Serotype		
	1	2	5
CBA	RGC	RGC	RGC
mouse opsin	-0-	-0-	-0-
human RPE65	ND	ND	ND
human VMD2			
human red opsin	ND	ND	ND
human blue opsin	ND	ND	ND

## AAV-VECTORED RETINAL GENE THERAPY

### Gene specific treatment for autosomal dominant retinal disease

Retinitis pigmentosa (RP), the most common form of retinal degeneration, represents a family of inherited diseases leading to progressive night blindness, gradual narrowing of the visual field, and loss of visual acuity (van Soest *et al.*, 1999). Rhodopsin, the G-protein-coupled receptor responsible for phototransduction, comprises more than 85 percent of the total amount of rod outer segment proteins, and for that reason it is essential for maintaining the structure of the photoreceptor outer segments, in addition to having a central role in the phototransduction cascade. More than 100 mutations in the human rhodopsin gene have been associated with autosomal dominant RP ([www.sph.uth.tmc.edu/RetNet/](http://www.sph.uth.tmc.edu/RetNet/)) and appear to affect protein folding, stability, and/or intracellular trafficking. These mutants account for the largest proportion of autosomal dominant RP cases of known genetic origin (30–40 percent). The most frequent rhodopsin mutation identified, P23H, is responsible for 12 percent of all autosomal dominant retinitis pigmentosa (ADRP) cases in North America.

Since autosomal dominant diseases are usually caused by an encoded mutant protein that interferes with cellular function and stability, and/or the remaining wild-type protein is insufficient for normal cellular function (haploinsufficiency), the principal therapeutic aim is to downregulate the level of the mutant protein, and AAV-delivered ribozymes that can be designed to target and destroy a mutant mRNA have been often suggested for this purpose (O'Neill *et al.*, 2000; Lewin and Hauswirth, 2001; Fritz *et al.*, 2002; Guy *et al.*, 2002, 2003; Qi *et al.*, 2002, 2003a, 2003b, 2003c, 2004; Lewin *et al.*, 2003; Sun *et al.*, 2003). The efficiency of the ribozyme approach has been proven in one transgenic rat model of ADRP containing the P23H opsin gene. AAV-delivered hairpin and hammerhead ribozymes were optimized (Drenser *et al.*, 1998) and used to specifically cleave the mutant mRNA transcript of P23H *in vivo* (Lewin *et al.*, 1998). When injected at postnatal day 15 (P15), ribozyme expression markedly slowed the rate of photoreceptor degeneration. After 8 months, ribozyme-treated eyes retained four to five rows of photoreceptor nuclei, compared with one row of nuclei in the contralateral control eyes (LaVail *et al.*, 2000). In addition, the treatment resulted in significantly greater ERG amplitudes up to 6 months after treatment compared with control eyes. AAV-ribozyme treatment was also found to be effective when injected at P30 or P45, after more than 40 percent of the transgenic P23H rat photoreceptor cells had been lost (LaVail *et al.*, 2000), suggesting that late stage autosomal dominant RP may still be amenable to ribozyme therapy.

## Gene replacement for autosomal recessive retinal disease

Autosomal recessive retinal degenerations are characterized by the absence of an essential gene product necessary for cell function or viability. In this case the main therapeutic approach is to provide the wild-type gene for such homozygous loss-of-function mutations. The gene replacement technique was successfully used in a canine model of LCA, a severe form of autosomal recessive, childhood-onset retinal dystrophy (Acland *et al.*, 2001). Mutations in RPE65, a protein abundantly expressed in the RPE cells, are responsible for approximately 10–15 percent of LCA cases, as well as some cases of recessive RP. Patients with LCA are born with significant vision deficits and usually lose all vision within 10 years. RPE65 is involved in the regeneration of 11-cis-retinol from all-trans-retinol in the RPE and binds all-trans-retinyl esters in the RPE to present them to the isomeroxydrolase, the enzyme responsible for the isomerization of all-trans- to 11-cis-retinol (Mata *et al.*, 2004). RPE65 knockout mice (RPE65<sup>-/-</sup>) accumulate all-trans-retinyl esters in the RPE and have no detectable 11-cis-retinoids, hence photoreceptors contain almost no 11-cis-retinaldehyde chromophore and cannot absorb input light to trigger the phototransduction cascade. A 4 bp deletion in the RPE65

gene leads to a stop codon in a strain of homozygous Swedish Briard dogs (RPE65<sup>-/-</sup>; Aguirre *et al.*, 1998; Veske *et al.*, 1999) and results in the absence of a functional RPE65 protein. This effectively disrupts the visual cycle and causes an autosomal recessive retinal degeneration very similar to LCA in humans. Before treatment, all RPE65<sup>-/-</sup> dogs had severe visual deficits, with very low amplitude ERG responses to light stimuli and large lipid-like inclusions in their RPE. Subretinally injected AAV2 vector carrying wild-type canine RPE65 (AAV2-CBA-cRPE65) stably restored visual function in this large animal model of childhood blindness, as assessed by ERG analysis, immunohistochemistry, and behavioural testing (Acland *et al.*, 2001, 2002; Narfstrom *et al.*, 2003). Function was preserved for at least 3 years after a single treatment (Bennett, 2004). In a recent study, delivery of human RPE65 using an AAV1-CMV-hRPE65 vector to the subretinal space of RPE65 knockout mice at embryonic day 14 also resulted in efficient transduction of retinal pigment epithelium and rescue of visual function, suggesting that *in utero* retinal gene transfer might be useful for retinal diseases with early phenotypes (Dejneka *et al.*, 2004).

In naturally occurring rodent models for other forms of recessive RP, AAV-vectored gene replacement strategies have thus far shown only a temporary delay in the degenerative process. The Royal College of Surgeons (RCS) rat is an animal model for another RPE-based inherited disorder, in this case caused by a 409 bp deletion in the RPE membrane receptor tyrosine kinase gene *Mertk*. This leads to impaired phagocytosis of shed outer segments, accumulation of outer segment material in the subretinal space, followed by photoreceptor dystrophy and death. Retinal degeneration in RCS animals occurs rapidly, initiating around P20, when outer segment debris in the subretinal space is already generated, and by P60 only a few photoreceptor nuclei remain. Mutations in the human *Mertk* gene are also responsible for some cases of human autosomal recessive RP. Both adenoviral and AAV-based delivery methods have been used to deliver a functional wild-type *Mertk* gene to the retina of RCS rats (Vollrath *et al.*, 2001; Smith *et al.*, 2003). Subretinal injections of recombinant AAV carrying a mouse *Mertk* cDNA under the control of either the CMV or RPE65 promoter were performed in 10-day-old RCS rats and were found to significantly prolong photoreceptor cell survival. The effects on retinal morphology 6 weeks after treatment with the AAV vector were similar to those observed in an earlier study, in which an adenoviral vector carrying a *Mertk* cDNA driven by a CMV promoter was used (Vollrath *et al.*, 2001). The number of photoreceptors was 2.5-fold higher in AAV-*Mertk* treated compared with control eyes 9 weeks after injection, and the amount of debris in the subretinal space decreased, suggesting functional RPE. Four weeks after treatment, ERG recordings from treated eyes were significantly higher than in contralateral untreated control eyes. However, after this point ERG signals slowly declined. The accumulation of a substantial amount of

debris in the subretinal space by the time transgene expression occurs, combined with the inability to completely clear pre-existing debris have been suggested as factors leading to the transient beneficial photoreceptor rescue in this case.

The retinal degeneration slow (rds) mouse is homozygous for a null mutation in the rds/peripherin gene, which encodes a structural protein in rod outer segment disks. This membrane glycoprotein, peripherin2, is required for proper formation of photoreceptor outer segment discs. Mice heterozygous for the null mutation have short, highly disorganized outer segments compared with those found in wild-type animals and thus, the intracellular level of peripherin2 appears critical for normal outer segment structure. Rds mice, which completely lack a functional gene encoding peripherin2, fail to develop outer segments, exhibit loss of photoreceptors and suffer a progressive retinal degeneration initiating soon after birth. The outer nuclear layer of the retina is reduced from 11 rows of cells at P10 to a single row at 12 months. A recombinant AAV2 carrying the peripherin2 gene driven by a rhodopsin promoter was delivered by subretinal injection into P10 rds mice and led improved photoreceptor structure and function, as shown by transmission electron microscopy and ERG recordings (Ali *et al.*, 2000). Peripherin2 immunoreactivity was detectable by 2 weeks after injection and the protein was localized to the correct outer segment layer of the retina. Production of a few short but well-defined outer segments containing both peripherin2 and rhodopsin were evident even 9 months after injection. However, the potential for morphological improvement was dependent on the age of the animals at the time of injection, with more outer segments evident in younger treated animals (Schlichtenbrede *et al.*, 2003a). Independent of the age of vector administration, however, the structural integrity of these restored outer segment structures declined over time even in young treated animals. Moreover, there was no significant improvement in the number of photoreceptors in treated animals versus age-matched controls. In spite of the relative lack of permanent structural rescue, photoreceptor functionality in treated eyes, as determined by ERG analysis, persisted for over 14 weeks, whereas ERG amplitudes in untreated eyes declined to virtually undetectable levels by 8 weeks. A recent study has also determined that, despite the modest structural impact, gene replacement therapy in the rds mouse results in improved central visual function based on recordings from visually responsive neurons in the superior colliculus (Schlichtenbrede *et al.*, 2004).

### **Neurotrophic/anti-apoptotic factors for retinal disease**

Neurotrophic or anti-apoptotic factors that either prolong neuronal survival or block the apoptosis cascade represent an alternative treatment for retinal degenerations that does

not require disease genes to be known. ‘Survival factor’ gene therapy may therefore be applicable to a broader range of maladies than direct gene replacement. Apoptosis seems to be the proximal cause of photoreceptor death in most forms of retinal dystrophies (Reme *et al.*, 1998), as determined by the analysis of DNA fragmentation and by the absence of an inflammatory response (Chang *et al.*, 1993; Hopp *et al.*, 1998; Lolley *et al.*, 1994; Reme *et al.*, 1998; Green *et al.*, 2001). For rod diseases like RP, the use of growth factors and anti-apoptotic reagents to delay retinal degeneration may prove beneficial to the patient even though it is unlikely to rescue rod function. By preventing or retarding rod apoptosis, the therapy could, in theory, spare the cone photoreceptors that appear to require a survival factor provided by rods (Leveillard *et al.*, 2004).

An AAV2 vector carrying the cDNA for glial cell line-derived neurotrophic factor (GDNF) was evaluated in the S334ter-4 opsin line of transgenic rats (Sanftner *et al.*, 2001). In this model, the opsin transgene contains a termination codon at residue 334, resulting in the expression of a truncated rhodopsin lacking the last 15 amino acids of the C-terminus and ultimately leading to retinal degeneration. An AAV2-CBA-GDNF vector, delivered to the subretinal space, led to the expression of the human *GDNF* gene in the inner and outer segments of photoreceptor cells, increased rod photoreceptor survival and correspondingly improved ERG function relative to partner control eyes.

Ciliary neurotrophic factor (CNTF) has been perhaps the best-studied survival factor in the retina and has been found to transiently delay photoreceptor loss in several animal models of recessive and dominant retinal degenerations. These include rds and transgenic rhodopsin knockout mice and transgenic P23H and S334ter rhodopsin rats (Liang *et al.*, 2001a,b; Bok *et al.*, 2002; Schlichtenbrede *et al.*, 2003b). The extent of protection as assessed by histological analysis was found to vary among the various animal models and to depend on whether the animals received a subretinal or intravitreal injection of AAV-CNTF. Interestingly, CNTF gene therapy has also been reported to result in unwanted side effects, primarily decreases in ERG amplitudes even in the presence of rod cell rescue and photoreceptor cell body abnormalities (Liang *et al.*, 2001a; Bok *et al.*, 2002; Schlichtenbrede *et al.*, 2003b). Although no mechanistic studies have been reported, it has been proposed that vector-expressed CNTF may induce the activation of genes whose products are mildly toxic in the retina. Finally, in a rat model for cancer-associated retinopathy, there was significant protection from apoptotic death after subretinal delivery of AAV2-CBA-CNTF (Adamus *et al.*, 2003). Therapy in this model of autoimmune-mediated damage to the retina may suggest a role for CNTF in other, potentially immune system-related retinal diseases such as age-related macular degeneration (see below).

Several other neurotrophic factors have also been shown to retard photoreceptor cell death. AAV2 delivery of fibroblast growth factors FGF-2, -5 or -18 preserve

photoreceptors from apoptosis in rhodopsin S334ter-4 transgenic rats (Lau *et al.*, 2000; Green *et al.*, 2001). More photoreceptors survived in the AAV2-FGF2 treated retinas of S334ter-4 transgenic rats than in partner untreated controls as indicated by the morphometric analysis of the outer nuclear layer thickness. ERG amplitudes in AAV2-FGF2-injected eyes were also significantly greater than those in uninjected retinas, but were not significantly different to in AAV-reporter gene treated eyes, suggesting the possibility of injection-related rescue. For FGF-5 and -18, both rod cell counts and ERG amplitudes were significantly improved relative to controls. BDNF was beneficial in a rat glaucoma model involving laser ablation of trabecular meshwork outflow leading to a rise in intraocular pressure and RGC death over the ensuing 4 weeks (Martin *et al.*, 2003). Two weeks prior to laser treatment AAV2-CBA-BDNF was administered intravitreally to transduce RGCs; 4 weeks after the laser, RGC axon loss was reduced in vector-treated eyes by approximately 40 percent. Thus AAV vectored expression of neurotrophin cDNAs not only protects photoreceptors when delivered subretinally (data for CNTF, GDNF, FGF-2, -5 and -18) but also RGCs (data for BDNF) when delivered intravitreally.

In addition to enhancing expression of growth/survival factors directly, the potential to increase the responsiveness of retinal cells to such factors by vectored expression of the cognate cell receptor has been tested for TrkB, the cell-associated receptor for BDNF. Cheng *et al.* tested the idea that, upon RGC injury by axotomy, expression of neurotrophin receptors may be repressed, thus reducing the cell's ability to respond to its cognate neurotrophin (Cheng *et al.*, 2002). Upon transection of the rat optic nerve, RGCs are lost and TrkB message was found to rapidly decrease. AAV2 vector-mediated TrkB cDNA transfer into RGCs significantly increased cell survival when BDNF protein was co-administered. At a post-axotomy time when most RGC neurons are lost without treatment, vector-mediated TrkB expressing RGCs remained viable. Thus AAV delivery of neuronal survival factor receptor cDNAs may be a complementary mode of protecting retinal cells from physical or genetic damage.

More direct interference with steps in the apoptosis pathway has also been tested in the retina using AAV vectors. XIAP, X-linked inhibitor of apoptosis (baculoviral IAP repeat-containing protein-4 BIRC4), is a potent inhibitor of caspases 3 and 8, both involved in key early steps in the apoptosis cascade. Intravitreal injection of AAV2-CBA-XIAP in rats with saline-induced ocular hypertension significantly preserved RGC axon counts relative to partner control eyes (McKinnon *et al.*, 2002). AAV2-CBA-XIAP was also evaluated in rats treated with N-methyl-N-nitrosourea (MNU) to induce rapid photoreceptor degeneration (Petrin *et al.*, 2003). Vector-pretreated eyes had significantly fewer apoptotic photoreceptors compared with AAV-GFP treated eyes 24 hours after MNU injection. Morphologically, AAV-XIAP eyes showed substantial rescue

of the photoreceptor layer at 72 hours post-MNU, whereas this layer is lost in controls. Thus the anti-apoptotic agent XIAP, when delivered by AAV, is protective against cell apoptosis both in photoreceptors and RGCs, and holds promise as a general therapeutic agent for retinal cell death.

### Treatment of neovascular retinal disease

Ocular neovascularization (NV), abnormal formation of new capillaries from pre-existing blood vessels in the eye, is a central feature of diabetic retinopathy, retinopathy of prematurity (ROP), and exudative (wet form) age-related macular degeneration (AMD). Together these conditions are the major causes of blindness in developed countries afflicting millions of people varying in age from infants to the elderly. Mechanistically, precisely how pathogenic NV occurs remains elusive, although hypoxia plays a critical role at least in retinal NV for diabetic retinopathy and ROP, and most likely also for the choroidal NV associated with AMD. Current treatments for retinal and choroidal NV such as laser photocoagulation, surgical intervention and photodynamic therapy provide only symptomatic treatment without addressing the underlying cause for the pathologic blood vessel growth. Furthermore, surgery and high energy laser treatments are often associated with significant adverse effects owing to their inherently tissue destructive nature. As a result, recurrence of symptoms is common for all current therapies, re-treatment is often necessary and appears to only slightly delay vision loss. Consequently, numerous new therapeutic interventions are being developed that require minimal surgical intervention, preserve existing vision, and provide long-term amelioration for any type of NV. Gene therapy has emerged as one of the most promising options that potentially satisfies all these requirements.

Identification of angiogenic and angiostatic factors common to both retinal and choroidal NV is the first step toward achieving an effective mechanism-based therapy. Most recent studies have focused on anti-angiogenic factors based on the presumption that ocular NV is the result of an increase in the concentrations of molecules that are known to stimulate vessel growth. This has led to the identification of several pro-angiogenic factors including vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), fibroblast growth factor (FGF), angiopoietins, platelet-derived growth factor (PDGF), tumor necrosis factor (TNF), and interleukins. Although all these factors individually or in combination have been implicated in pathogenic development of ocular NV, VEGF appears to play the pivotal role in both retinal and choroidal NV. Identified in 1982 (Chen and Chen, 1982), mechanistic confirmation that VEGF is a true angiogenic factor remained elusive until 1989 when it was purified from tumor cell-conditioned medium and shown to stimulate vascular

endothelial cell proliferation (Connolly *et al.*, 1989). Since then, expression of VEGF and its receptors, VEGFR-1 (or Flt-1) and VEGFR-2 (or KDR), have been detected in various tissues of normal eyes, including ganglion cells, RPE, and the choroids (Kim *et al.*, 1999), and have been shown to be upregulated in experimental models of either iris or retinal NV (Gao *et al.*, 2002; Pierce *et al.*, 1995; Suzuma *et al.*, 1999). Consistent with these observations, VEGF levels are increased in the retina and vitreous of diabetic patients (Aiello *et al.*, 1994; Funatsu *et al.*, 2001; Nicoletti *et al.*, 2003) and in animals with ischemia-induced retinopathy (Pierce *et al.*, 1995; Gao *et al.*, 2002), suggesting that VEGF indeed plays a major role in pathogenic ocular angiogenesis. This idea is further supported by studies in transgenic mice (Schwesinger *et al.*, 2001; Ohno-Matsui *et al.*, 2002), in which overexpression of VEGF in either photoreceptors or RPE leads to retinal and choroidal NV.

Based on these findings, most ocular anti-NV strategies have focused on neutralizing unwanted VEGF activity. Two animal models, oxygen-induced retinopathy (OIR), a neonatal rodent model of retinal NV, and laser-induced choroidal NV (CNV), have been developed and used extensively to evaluate the anti-VEGF therapeutic approaches. In the OIR model, newborn rodents are kept in a hyperoxic chamber (approximately 75 percent oxygen) for 5 days. This leads initially to central retinal capillary closure that, upon return to room air, results in a relative hypoxia and retinal ischemia that in turn results in VEGF-dependent retinal NV in 100 percent of the animals (Smith *et al.*, 1994). This model generates a reproducible and quantifiable retinal NV, thus providing a key model for the study of diabetic proliferative retinopathy and ROP. CNV can be generated by laser-rupture of Bruch membrane in many species (Miller *et al.*, 1990). In rodents the extent of CNV is usually assessed 2–4 weeks after laser treatment in choroidal flat mounts prepared from animals perfused with fluorescein-labeled dextran (Raisler *et al.*, 2002).

A variety of anti-VEGF therapeutic agents have been developed for disrupting VEGF signaling. They include anti-VEGF monoclonal antibodies (Adamis *et al.*, 1996; Krzystolik *et al.*, 2002), soluble VEGF-receptor chimeric proteins sFlt-1 (Aiello *et al.*, 1995), VEGF-antisense oligonucleotides (Robinson *et al.*, 1996), and VEGF-TRAP<sub>R1R2</sub> (Saishin *et al.*, 2003), and, as proteins or RNA, each has been shown to have therapeutic efficacy in retinal NV animals when delivered locally (intravitreally) or systematically (subcutaneously or orally). While these studies clearly confirm the validity of blocking VEGF or VEGF receptors as an anti-NV therapy, there are major limitations. The half-life of proteins or antisense oligonucleotides is short, lasting at most for several days, thus local intravitreal administration must be repeated relatively frequently. Although development of nuclease-resistant RNA aptamers to VEGF in conjunction with liposomes dramatically increase therapeutic half-life without diminishing its affinity toward a target molecule (Willis *et al.*, 1998), multiple

injections will still be required for the chronic ocular NV conditions such as AMD where sustained levels of the therapeutic agent are likely to be necessary. In contrast, systemic delivery of therapeutic agents requires that the agent crosses the blood-retina barrier. If it does, drug delivery is simplified, but serious adverse effects are possible, particularly inhibition of beneficial angiogenesis in ischemic myocardium and wound healing, since the whole body is exposed to the anti-VEGF drug. Thus, local ocular delivery is currently the favored route for anti-NV factors.

Gene-based anti-VEGF therapy offers the advantage of targeted, sustained delivery of the agent following a single ocular injection. Among potential anti-VEGF therapeutic genes, soluble Flt-1 (sFlt-1), a non-membrane-bound form of the VEGF R1 receptor, has received the most attention and shows a strong protective role during hypoxia-induced vessel loss (Shih *et al.*, 2003). Bainbridge *et al.* (2002) studied the angiostatic effect of sFlt-1 cDNA in the OIR mouse using adenovirus and AAV2 vectors. Intravitreally, adenoviral vector-mediated expression occurred primarily in the anterior chamber, the corneal endothelium, and iris endothelium, whereas AAV-mediated expression occurred primarily in ganglion cells. Despite their contrasting transduction patterns, both sFlt-1-expressing vectors resulted in a similar reduction in neovascular endothelial cells, suggesting that both are effective. This has been further supported by other studies, in which a local delivery of adenovirus-sFlt-1 resulted in a significant suppression of NV in rodent models of ocular NV (Lai *et al.*, 2001a,b; Gehlbach *et al.*, 2003b). While these data clearly demonstrate the usefulness of adenovirus as a retinal gene delivery tool, ocular administration of adenovirus is often associated with a significant cell-mediated immune response (Reichel *et al.*, 1998). This, together with its relatively short-lived expression (Reichel *et al.*, 1998; Lai *et al.*, 2001a), may limit clinical application of adenoviral vectors to acute conditions such as ROP where short-term expression is desired. In contrast, administration of AAV2-sFlt-1 either into the anterior chamber or the subretinal space of rats effectively suppressed laser-induced choroidal NV for 5 months without toxicity (Lai *et al.*, 2001a). This suggests that AAV vectors are an attractive option for chronic NV conditions involving retinal or choroidal NV.

Although the above studies clearly demonstrate that VEGF is a necessary stimulator for ocular NV, it has been noted that in some situations overexpression of VEGF does not necessarily lead to the progression of ocular NV. For example, intravitreous implantation of VEGF sustained-release pellets induced transient retinal NV in rabbits, but failed to induce retinal NV in primates (Ozaki *et al.*, 1997). Similarly, the retinas of patients with non-proliferative diabetic retinopathy show elevated levels of VEGF without retinal NV (Nicoletti *et al.*, 2003), suggesting that other factors, in addition to VEGF itself, may be implicated in the process of NV. These facts have led to the screening of a number of other angiostatic factors that may be implicated in ocular NV.

Pigment epithelium-derived factor (PEDF), endostatin, and angiostatin all have been shown to behave as anti-angiogenic factors in the retina, with PEDF being most potent (Dawson *et al.*, 1999). The ratio of ocular VEGF/PEDF was closely correlated with the progression of retinal NV in rats. The concept has therefore emerged that the development of NV may be related to a disrupted balance between VEGF and counteracting angiostatic factors, particularly PEDF (Stellmach *et al.*, 2001; Gao *et al.*, 2002). Consistent with this, expression of PEDF during choroidal NV development was negatively correlated to the severity of laser-induced injury (Renno *et al.*, 2002). The importance of PEDF in ocular NV was further confirmed through the finding that patients with proliferative retinopathy had decreased PEDF levels, whereas PEDF increased in patients whose retinal NV had been successfully treated by retinal scatter photocoagulation (Spranger *et al.*, 2001). These observations suggest that PEDF may be as important as VEGF in the management ocular NV.

Several groups have evaluated the therapeutic potential of PEDF for ocular NV. Either adenoviral or AAV2-PEDF vectors were effective in inhibiting both retinal and choroidal NV in rodent models (Spranger *et al.*, 2001; Auricchio *et al.*, 2002a; Mori *et al.*, 2002a,b; Raisler *et al.*, 2002; Gehlbach *et al.*, 2003a). The effectiveness of PEDF appeared to be unrelated to its intraocular site of administration (Raisler *et al.*, 2002) suggesting that secreteable agents like PEDF and sFlt-1 can effectively diffuse through retinal layers. Even after initiation of vessel proliferation in the laser CNV model, adenovirus-PEDF was effective in regressing already established NV (Mori *et al.*, 2002a), raising the possibility that PEDF gene therapy could be extended into patients with already advanced NV disease. AAV serotype 5 vectors that modulate relatively rapid passenger gene expression (4–5 days) in the retina may have usefulness in this context. In addition to its anti-NV role, intravitreally administered PEDF vector increases ganglion cell survival after ischemia-reperfusion injury of retina in a rat (Takita *et al.*, 2003), consistent with its documented role as a neurotrophin (Tombran-Tink *et al.*, 1991; Becerra, 1997; Cayouette *et al.*, 1999), as had been demonstrated earlier in rd and rds mice (Cayouette *et al.*, 1999).

## CONCLUSIONS

In summary, AAV vectors are a multidimensional tool for delivering gene-based therapies to the retina, the principal variables being the serotype, the promoter used to express the passenger cDNA, and the site of intraocular injection. Over the past few years proof-of-principle experiments in animal models have shown that properly constructed AAV vectors are likely to be clinically viable for both dominant and recessive single gene retinal diseases by gene replacement strategies or by survival factor gene therapy, and that

retinal maladies of more complex genetic etiology such as the neovascular diseases AMD and diabetic retinopathy may be equally viable targets for AAV vectored therapy. By referral to Tables 43.1 and 43.2, the reader will note that in many cases the optimal serotype-promoter combination for a given retinal disease has not yet been experimentally tested, so room for further improvement clearly exists.

## NOTE

W.W.H. and the University of Florida could be entitled to patent royalties for inventions related to this work and both own equity in a company that may commercialize some of the technology described herein.

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# Helper-independent parvoviruses as gene therapy and vaccine delivery vectors

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Autonomous parvoviruses have various properties that make them attractive for development as gene transfer vectors. In particular, some rodent parvoviruses display anti-neoplastic properties. Infection of laboratory animals with these viruses is associated with a low incidence of spontaneous and induced tumor formation or a low take of grafted tumor cells (see Chapter 25). This oncosuppressive activity, along with the oncolytic and oncotropic properties of several autonomous rodent parvoviruses, has stimulated efforts to develop antitumor vectors. Such vectors are currently being tested for antineoplastic effects in preclinical studies (Haag *et al.*, 2000; Palmer and Tattersall, 2000b; Wetzel *et al.*, 2001; Giese *et al.*, 2002).

In addition to their oncotropic and oncolytic activities, several rodent parvoviruses can persistently infect their natural hosts (Jacoby *et al.*, 1996). This property has been exploited to produce a parvoviral vector for vaccinating against Lyme disease (Palmer *et al.*, 2004). Moreover, parvoviral vectors have contributed to understanding the basic processes of the parvovirus life cycle, and notably to answering questions about cell and tissue tropism (Srivastava *et al.*, 1989; Maxwell *et al.*, 1995; Wang *et al.*, 1995; Spitzer *et al.*, 1997; Weigel-Kelley *et al.*, 2001), pseudotyping (Maxwell *et al.*, 1993b; Corsini *et al.*, 1995; Spitzer *et al.*, 1996, 1997; Ponnazhagan *et al.*, 1998; Wrzesinski *et al.*, 2003), and cell susceptibility to parvovirus-induced killing.

Although about a dozen infectious DNA clones of various autonomous parvoviruses are available, vectors have so far been developed from only a few rodent parvoviruses. The B19 genome or its promoter has been used to prepare chimeric viruses using adeno-associated virus (AAV)

inversed terminal repeats for their packaging into AAV capsids (Srivastava *et al.*, 1989; Wang *et al.*, 1995). B19 capsids were also used to package recombinant AAV genomes (Ponnazhagan *et al.*, 1998; Weigel-Kelley *et al.*, 2001).

This chapter covers the state of the art in generating parvoviral gene transfer vectors for various therapeutic purposes. Preclinical studies using parvovirus vectors are described, with emphasis on viral aspects rather than on the choice and function of the transgene. Safety is an important issue when rodent parvoviruses for human treatments are being considered, but this topic is beyond the scope of a review on vectors. Safety aspects of wild-type parvoviruses and vectors are discussed in detail in a chapter of a recently published book (Rommelaere *et al.*, 2005). Two recent reviews on parvoviral vectors provide important background information and useful suggestions for their production and titration (Palmer and Tattersall, 2000a; Maxwell *et al.*, 2002).

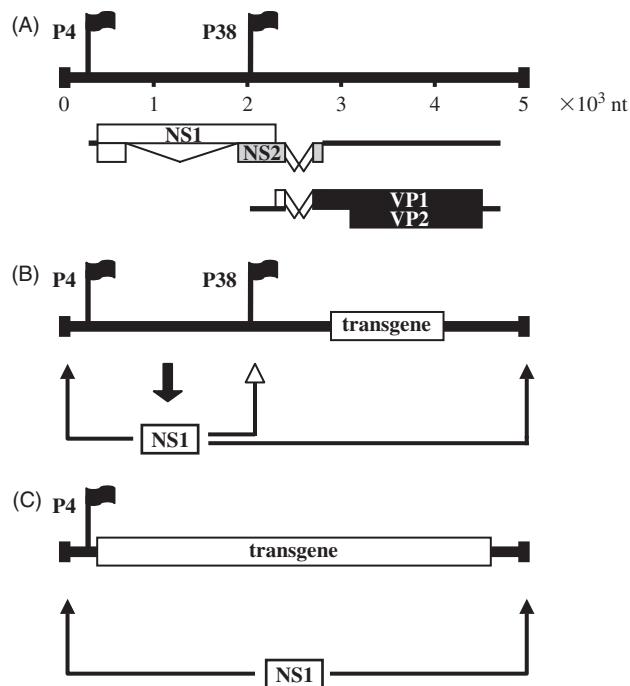
## VECTOR PRODUCTION

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### Vector design

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Three types of recombinant parvoviruses have been constructed from infectious molecular clones. These are depicted in Figure 44.1 in alignment with the genetic map of the corresponding wild-type viruses (panel A). In the first type of construct (capsid-replacement vectors), part of the parvoviral right-hand transcription unit has been replaced



**Figure 44.1** Schematic representation of the genome organization of autonomous rodent parvoviruses (A) and derived vectors (B, C). A. The viral genome (thick line) flanked by palindromic sequences at both ends is depicted on top. Messenger RNAs (thin lines) are aligned beneath the genome, with interruptions at the positions of introns. The positions of the transcription start sites for the P4 and P38 promoters are indicated by flags. The NS- and VP-coding regions in the three reading frames are indicated by boxes marked in white, gray and black. The genome terminal hairpins and RNA introns are not to scale. B and C. Inserted foreign cDNA is indicated by the open boxes labeled 'transgene'. Panels B and C depict capsid replacement vectors and complete genome replacement vectors, respectively. The residual downstream VP sequences are not translated and vary in length depending on the transgene. Filled arrowheads depict the positive effect of NSI protein on viral genome amplification. The open arrowhead depicts the ability of NSI proteins to trans-activate the P38 promoter.

with either a therapeutic transgene or a reporter gene (panel B). In the second type of vector (complete coding replacement vectors), all viral coding regions have been replaced with a transgene (panel C). In addition to these gene replacement vectors, P4-promoter-modified viruses have been designed. It can be either transgene-carrying vectors (Maxwell *et al.*, 1996; Maxwell and Maxwell, 1999) or non-defective progeny-forming viruses that only replicate in specific cells or under controlled conditions (Palmer and Tattersall, 2000a; Mallerba *et al.*, 2003). Some recombinant viruses are combinations, vectors carrying a transgene and having a P4 modification or replacement. The various vectors described to date are summarized in Table 44.1.

Parvovirus vectors retain the terminal viral sequences including all *cis*-acting determinants of genome replication, regulation, and encapsidation (Figure 44.1). Expression of the transgenes inserted in capsid-replacement vectors is driven by the genuine parvoviral P38 promoter, which is strongly *trans*-activated by the non-structural (NS) protein NS1 (Rhode, 1985) encoded by the left-hand portion of the viral genome (indicated by the open arrowhead in panel B). The filled arrowheads starting from NS1 depict the positive effect on viral DNA replication exerted by NS1 upon its binding to both ends (panels B and C). Moreover, these constructs usually encode – but not always (Dupont *et al.*, 2001) – the NS2 proteins, required for packaging and virus release (Naeger *et al.*, 1990; Cotmore *et al.*, 1997; Eichwald *et al.*, 2002; Miller and Pintel, 2002).

Complete coding replacement parvoviral vectors harbouring a firefly luciferase (Luc) or a bacterial  $\beta$ -galactosidase (*lacZ*) reporter gene (Maxwell *et al.*, 1993a,b; Corsini *et al.*, 1996; Maxwell *et al.*, 1996; Maxwell and Maxwell, 1999) have been constructed (Figure 44.1, panel C). They fail to amplify their genome unless the parvoviral NS polypeptides are provided in *trans* (indicated by the filled arrowheads). A promising strategy appears to be to develop conditionally replicating parvoviruses that depend for their replication and expression on factors expressed in the target organs (Maxwell *et al.*, 1996; Palmer and Tattersall, 2000a; Mallerba *et al.*, 2003). It should be realized that modification or replacement of the parvoviral P4 promoter may disrupt the S-phase dependence of the virus through its E2F and cyclic adenosine monophosphate (AMP) response elements (Deleu *et al.*, 1998, 1999). Moreover, a strong early promoter is required for autonomous replication of the virus (Deleu *et al.*, 1999).

Another category of vectors consists of chimeric vectors constructed in attempts to combine the advantages of two different virus systems. Several such viruses have been described. One consists of AAV capsids containing the B19 genome with its inverted terminal repeats replaced by AAV terminal repeats (Srivastava *et al.*, 1989). Another consists of AAV capsids containing B19 promoter sequences regulating the expression of the AAV2 coding sequence and inverted terminal repeats. The strong B19 promoter leads to autonomous replication of the latter virus and also confers specificity towards hematopoietic progenitor cells (Wang *et al.*, 1995). Heterologous DNA sequences cloned within AAV inverted terminal repeats could also be packaged inside B19 capsids (Ponnazhagan *et al.*, 1998). The capacity of recombinant adenoviruses to grow to high titers has been exploited to produce adenovirus vectors containing sequences from the murine parvovirus MVMP. One chimeric vector contains the MVMP P4 promoter driving expression of the  $\beta$ -galactosidase gene (*lacZ*). In another, *lacZ* gene expression is controlled by the MVMP P4-NS-P38 cassette (Raykov *et al.*, 2002). Interestingly, the latter chimeric virus could be obtained only when NS gene expression was suppressed during production. This is indicative of interference between NS polypeptides and adenovirus factors.

**Table 44.1** Vectors developed from autonomous rodent parvoviruses

Therapy	Type of vector <sup>1</sup>	Transgene/modification	Ref <sup>2</sup>
Anticancer	Capsid-replacement	IL-2, IL-4, MCP-1 IP-10, MCP-3, B7-1, B7-2 HSV-thymidine kinase $\beta$ -catenin-response element	1–4 5–6 7–8 9 10
HIV infection	P4 promoter modified	HIV-TAR element	11
Lyme disease	P4 promoter modified	Gene	12
Reporter vectors	Capsid-replacement Complete coding replacement Capsid-replacement P4 promoter modified	$\beta$ -galactosidase, luciferase GFP, luciferase, CAT Liver-specific expression GAL4 responsiveness Tetracycline responsiveness	13–16 15–23 24 24 24–25

<sup>1</sup> Vectors without any transgene (empty vectors) are not listed.

Abbreviations used: CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; HIV-TAR, human immunodeficiency virus Tat protein trans-activating response element; IL-2, interleukin 2; IP-10, interferon- $\gamma$ -inducible protein 10; MCP, monocyte chemotactic protein.

<sup>2</sup> The numbers correspond to the following references: (1) Brandenburger *et al.*, 1999; (2) Haag *et al.*, 2000; (3) Kestler *et al.*, 1999; (4) Russell *et al.*, 1992; (5) Giese *et al.*, 2002; (6) Wetzel *et al.*, 2001; (7) Gancberg *et al.*, 2004; (8) Palmer and Tattersall, 2000b; (9) Dupont *et al.*, 2001; (10) Malerba *et al.*, 2003; (11) Palmer and Tattersall, 2000a; (12) Palmer *et al.*, 2004; (13) Maxwell *et al.*, 1993a; (14) Maxwell *et al.*, 1993b; (15) Spitzer *et al.*, 1996; (16) Spitzer *et al.*, 1997; (17) Brown *et al.*, 2002; (18) Dupont *et al.*, 1994; (19) Dupont *et al.*, 2000; (20) Moehler *et al.*, 2001; (21) Moehler *et al.*, 2003; (22) Olijslagers *et al.*, 2001; (23) Wrzesinski *et al.*, 2003; (24) Maxwell *et al.*, 1996; (25) Maxwell and Maxwell, 1999.

## Vector production

To obtain a P4-promoter-modified recombinant virus, appropriate producer cells are transfected with the corresponding modified infectious molecular clone DNA. If the virus is also capable of producing progeny viruses, the virus stock produced by transfection can be used to initiate further rounds of virus replication (Deleu *et al.*, 1999; Palmer and Tattersall, 2000a; Malerba *et al.*, 2003). To produce capsid-replacement vectors and complete coding replacement vectors, cells are co-transfected with recombinant molecular clone DNA and a helper plasmid providing the genes encoding the capsid (VP) proteins, either alone (to obtain capsid-replacement vectors) or with the genes coding for the NS proteins (to obtain complete coding replacement vectors).

RCV (replication-competent viruses) are defined as viruses capable of infection and autonomous replication, emerging during production of recombinant virus stocks. Thus, RCV can produce plaques in monolayers of standard indicator cells, and their genomes hybridize with both NS- and VP-specific probes. To avoid generating RCV, overlapping DNA sequences in the molecular clone DNA and helper constructs should be minimized or, better still, completely eliminated. In the DNA constructs used to prepare the first capsid-replacement vectors, such sequences were particularly abundant both up- and downstream from the transgene (Russell *et al.*, 1992; Maxwell *et al.*, 1993a; Dupont *et al.*, 1994). Since then, a number of strategies have been developed to avoid formation of RCV. Among them are:

- construction of chimeric genomes containing sequences from two closely related viruses (Palmer and Tattersall, 2000a; Wrzesinski *et al.*, 2003) or the reduction of

homology up- and downstream from the transgene and insertion of stuffer DNA replacing residual VP sequences (Clément *et al.*, 2002);

- the use of split-helper constructs lowering the probability of recombination between co-transfecting plasmids (Maxwell *et al.*, 1995; Brown *et al.*, 2002);
- reduction of homology in the helper constructs either upstream from the NS and VP sequences, via replacement of the P4 promoter with a heterologous promoter (Maxwell *et al.*, 1993a), or downstream from the VP-coding region (Clément *et al.*, 2001) or by introducing point mutations into the VP-coding region of the helper construct, while keeping the amino-acid sequence intact (Dupont *et al.*, 2001);
- pseudotype generation, i.e. packaging of viral genomes in capsids of a related virus (Maxwell *et al.*, 1993b, 1995; Spitzer *et al.*, 1996, 1997; Wrzesinski *et al.*, 2003).

With these methods, RCV contamination of the various vector stocks has been reduced from 31–80 percent (first-generation vectors) (Russell *et al.*, 1992; Dupont *et al.*, 1994) to around 0.15 percent (Dupont *et al.*, 2001) and even to  $<2 \times 10^{-4}$  (Brown *et al.*, 2002) or  $4.3 \times 10^{-5}$  percent (Wrzesinski *et al.*, 2003; Palmer and Tattersall, 2000a). Pseudotyping is highly efficient; with the H-1 virus (H-1PV) genome and MVMp capsids and *vice versa*, it has led to obtaining RCV-free vector stocks with titers of up to  $5 \times 10^7$  replication units per ml virus suspension (Wrzesinski *et al.*, 2003). One should bear in mind that pseudotyping may alter the host and tissue tropism of the virus (Maxwell *et al.*, 1993b; Wrzesinski *et al.*, 2003). Yet pseudotyped vectors may become useful for the analysis and identification of cell factors involved in virus infection. Vector stocks devoid

of RCV are not necessarily free of VP-positive virus genomes. VP gene-positive virus populations have indeed been identified in such stocks (Dupont *et al.*, 2001; Brown *et al.*, 2002). These viruses are apparently generated through two crossings-over, but they lack sequences necessary for their autonomous propagation, as they can replicate only by complementation in the presence of NS and are lost after a few rounds of infection.

As the amount of capsid protein can be a limiting factor for the production of high titer virus stocks, some helper plasmids were supplied with an SV40 origin of DNA replication allowing their autonomous amplification in SV40-transformed producer cells such as COS, NB-E, 324K, or 293T. Whereas some investigators have found the introduction of a functional SV40 origin not to improve capsid protein expression (Cornelis *et al.*, 2000) or the vector titer (Maxwell and Maxwell, 2002, unpublished), others reported higher virus titers with this strategy (Palmer and Tattersall, 2000a). This discrepancy may be due to interference between SV40 and parvovirus replication and expression. In the experiments conducted in our laboratory, the viral NS1-transactivated P38 promoter was as potent as the constitutively strong immediate early human cytomegalovirus promoter when it comes to producing capsid proteins and vectors in 293T and NB-324K cells (unpublished). Yet for the sake of reducing homology between the plasmids, the cytomegalovirus promoter is often preferred for driving capsid protein production (Haag *et al.*, 2000; Olijslagers *et al.*, 2001; Wetzel *et al.*, 2001; Wrzesinski *et al.*, 2003). Some investigators have found vector stocks to become devoid of RCV when capsid protein expression is controlled by the early SV40 promoter, although this did not improve the vector titer (Clément *et al.*, 2002). In collaboration with Solon L. Rhode, we attempted to improve the titers of capsid-replacement vector stocks by co-infecting cells with a recombinant adenovirus delivering H-1 virus capsid proteins. This approach did not achieve higher vector titers than the co-transfection procedure (unpublished observations), possibly owing to high toxicity of the adenovirus or to adenovirus-parvovirus interference (Raykov *et al.*, 2002). An alternative split-helper strategy involves co-transfecting cells with a conventional VP-providing plasmid together with a Simbis replicon vector expressing NS proteins (Corsini *et al.*, 1996). Unfortunately, this approach did not achieve higher titers than those obtained with the usual procedure.

A different strategy for producing vectors is to use packaging cell lines. Packaging cell lines providing either NS and VP or VP proteins alone in *trans* can be infected with vector virus and should generate progeny vectors capable of infecting further packaging cells. Yet it appears difficult to develop efficient packaging cell lines (Brandenburger and Russell, 1996; El Bakkouri *et al.*, 2000). Attempts with NB-E cells (SV40-transformed human embryonic kidney cells) yielded cell lines containing VP-encoding sequences stably integrated into the cell DNA, under the control

of either an inducible mouse mammary tumor virus (MMTV) promoter (Brandenburger and Russell, 1996) or the genuine viral P38 promoter (El Bakkouri *et al.*, 2000). The latter packaging cells produced  $1 \times 10^7$  replication units of vector after 3–4 rounds of infection. The authors suggested that a further improvement of vector titers should be possible if levels of capsid genes could be enhanced. In addition, RCV were generated from the integrated P38-VP helper constructs even when the initial virus stock used for infection did not contain any detectable amount of contaminating virus, pointing to a high incidence of recombination (El Bakkouri *et al.*, 2000). Despite these shortcomings, the packaging cell lines developed provide proof of principle that such cells can be constructed at all. Further investigation of the parvovirus life cycle may uncover factors restricting the efficient functioning of packaging cells.

## Virus purification, concentration, and titration

The need for high titer recombinant AAV stocks for gene therapy has strongly prompted the development of novel methods for their purification and concentration. Autonomous parvovirus research has only marginally profited from these developments, such as the introduction of iodixanol gradients (Zolothukin *et al.*, 1999) instead of or combined with conventional centrifugation of the virus in isopycnic cesium chloride gradients (Brown *et al.*, 2002; Wrzesinski *et al.*, 2003). A rather laborious method for purifying parvovirus vectors by conventional methods has been described (Avalosse *et al.*, 1996). As in the case of AAV, immuno-, cation exchange, and sialic acid chromatography will undoubtedly be developed in the near future for purifying and concentrating parvoviruses from crude extracts. Such techniques, however, have not yet been extensively assessed.

The infectivity of recombinant virus stocks is determined by measuring the capacity of the virus to replicate its genome or to express the transgene. These measurements yield, respectively, the replication titer and the transduction titer (Russell *et al.*, 1992; Maxwell *et al.*, 1993a, 1993b; Dupont *et al.*, 1994; Brown *et al.*, 2002; Cheong *et al.*, 2003). Replication titers of whole coding replacement vectors can be determined only in cells expressing the NS1 protein. This can be achieved by co-infecting cells with wild-type virus (Maxwell and Maxwell, 1994; Maxwell *et al.*, 2002). Replication titers are determined by hybridization with an NS- or transgene-specific DNA probe (Russell *et al.*, 1992; Maxwell *et al.*, 1993a, 2002; Dupont *et al.*, 2001). Transduction titers are obtained by measuring the fraction of infected cells that are positive for transduced green fluorescent protein (GFP) (Brown *et al.*, 2002; Wrzesinski *et al.*, 2003), or IL-2 (Cheong *et al.*, 2003). For (pre)clinical assays it is also desirable to quantify the genome titer corresponding to full (DNA-containing) virus particles, as it is the total virus load, not only the infectious fraction, that

determines the immune response. The number of full particles in a vector stock has been determined by dot blot assays (Grimm *et al.*, 1999; Lang, 2003) or real time PCR (Maxwell *et al.*, 2002; Mulerba *et al.*, 2003).

## FACTORS INFLUENCING THE TITER OF INFECTIOUS PARTICLES

On the basis of their profound analysis of the initiation of parvoviral DNA replication, Cotmore and Tattersall realized that the infectious MVMp clone available at the time of the analysis contained on its left-hand side an incomplete resolution sequence (Cotmore and Tattersall, 1994). Resolution consists of specific cleavage and processing of the left telomere, which normally takes place during viral DNA replication (see Chapter 14), but which is also assumed to be involved in the excision of parvovirus DNA from infectious clone DNAs (Rhode, 1989; Li and Rhode, 1990). Repair of MVM and H-1 virus infectious clones apparently fulfills the *cis*-requirement for resolution, since it results in marked increases in parvovirus DNA replication and progeny particle formation in several transfected producer cells such as A9, NB-E, and 293T (Kestler *et al.*, 1999). These second-generation molecular clones yield infectious vector titers up to a 1000-fold higher than those obtained with the original infectious MVMp and H-1 clones (Kestler *et al.*, 1999). On the other hand, introduction of a complete left-hand origin in a LuIII clone did not result in markedly improved viral replication or vector titers (Maxwell *et al.*, 2002). The authors speculated that the original infectious LuIII clone might already contain a functional, albeit imperfect, resolution site. In another study, production of MVMp-based transducing particles was improved only 10-fold by insertion of the full origin of replication, as compared with vectors derived from the original infectious clone (Clément *et al.*, 2002). Thus, resolution may be both cell- and virus-dependent, as variable amounts of DNA replication intermediates were generated after transfection of different producer cells (Kestler *et al.*, 1999).

In a systematic study of the impact of the size of the recombinant genome on capsid replacement vector production, the highest infectious vector titers were obtained with vectors produced from infectious DNA clones deleted of 400–800 nucleotides in the VP-encoding gene (Kestler *et al.*, 1999). These titers were only three to four times lower than those achieved in parallel with the corresponding infectious H-1 virus or MVMp clone under conditions precluding wild-type virus re-infection (Cornelis *et al.*, 2000; Lang *et al.*, 2005). On the other hand, deletions of 1200 or 1600 bp in the VP gene correlated with dramatically reduced infectious titers (Kestler *et al.*, 1999). When the sole difference between two vectors carrying the same transgene was the replacement in one of residual 800 bp VP-encoding sequence by stuffer DNA of the same length, the latter recombinant virus achieved a

50-fold lower infectious titer than the vector still containing VP sequences. These data suggest, but do not prove, that DNA sequences in the VP coding region contain elements that promote the production or infectivity of the vectors (Kestler *et al.*, 1999). Similarly, vectors where the VP-coding sequence was almost completely replaced with the luciferase gene showed infectious titers more than 300-fold lower than the titers achieved with a vector having a size-matched genome retaining 800 bp of 3' end VP coding sequences (Cornelis *et al.*, 2000). All these observations corroborate the finding of Brandenburger and co-workers that the optimal recombinant genome size is 90–100 percent of the size of the wild-type genome (Brandenburger *et al.*, 1999). The introduction of stuffer DNA from various sources (mouse or a bacteriophage) can increase or reduce the vector titer (Brandenburger *et al.*, 1999; Clément *et al.*, 2002), suggesting that insertion of such DNA is not necessarily neutral for packaging of the recombinant genome or for the vector infectivity.

Titers of infectious virus dropped drastically or became undetectably low when the recombinant viral genomes exceeded by 6 percent the size of the wild-type genome (Kestler *et al.*, 1999; Brandenburger *et al.*, 1999; Maxwell *et al.*, 2002). This suggests that inefficient packaging of larger genomes could limit vector production (Brandenburger *et al.*, 1999; Kestler *et al.*, 1999). If packaging of autonomous parvoviruses proceeds like that of helper-dependent AAV, by a head-full mechanism (Dong *et al.*, 1996), the encapsidation of such large genomes might indeed considerably restrict infectious virus production. Since no genome titers were reported, it is not possible to conclude definitely that packaging is the main obstacle to obtaining high infectious vector titers.

Intracellular amplification of wild-type and vector genomes is similar after cell transfection with different molecular clones, irrespective of the removed or inserted sequences (Brandenburger *et al.*, 1999; Kestler *et al.*, 1999). Yet an element called the internal replication sequence (IRS), mapping to the extreme right end of the MVMp genome close to the terminal palindrome, has been shown to affect drastically the replication of MVM minigenomes in certain cells (Tam and Astell, 1993; Brunstein and Astell, 1997). When most of the MVMp IRS or of the homologous putative IRS in LuIII or H-1 were removed, replication of recombinant LuIII, H-1, or virus DNA is strongly reduced (Kestler *et al.*, 1999; Dupont *et al.*, 2001; Maxwell *et al.*, 2002). This reduction correlates with extremely low yields of IRS-deleted recombinant H-1 virus (Kestler *et al.*, 1999), but not of LuIII vector (Maxwell *et al.*, 2002). Thus, retention of the IRS element might not be an essential *cis*-requirement for obtaining high-titer vector stocks in certain producer cells. Further studies are required to better determine the role of the IRS region in the various viruses used, particularly as regards its cooperation with surrounding sequences, since in other constellations and host cells, its removal was found to have no effect on either viral DNA amplification or packaging (Palmer and Tattersall, 2000a).

In summary, capsid-replacement vectors with up to 800 bp of VP-coding sequences replaced with a transgene yield the highest infectious titers, whereas vectors with a larger transgene or with no or few residual VP DNA sequences consistently yield the lowest titers. Thus, both the sizes of the removed VP-encoding sequences and the recombinant genome may determine the infectious particle yield. Unfortunately, the production of vectors is very inefficient as only around  $5 \times 10^6$ – $2 \times 10^7$  replication units per  $10^6$  transfected 293T cells can be achieved with the best MVM- and H-1-derived vectors (see above). In our hands, the 293T cells were the best producers of infectious vector particles when compared with several other SV40-transformed primate cells.

## VECTORS VERSUS WILD-TYPE VIRUSES

Recombinant parvoviruses carrying reporter genes might be very useful for answering questions on parvovirus-cell interactions or tissue infection. To perform experiments focusing on such questions, one should first know to what extent vectors can substitute for wild-type viruses. In different laboratories, therefore, vector and wild-type viruses have been compared *in vitro*, for either their cytotoxicity or their ability to perform several steps of the viral life cycle (notably virus uptake by recipient cells, viral DNA replication, and viral gene expression).

### Virus DNA replication and gene expression

In experiments where commonly used cell lines (A9, 324K, HeLa) were infected in parallel with wild-type (H-1 virus or MVMP) and various derived capsid-replacement vectors (at the same low infectious virus input multiplicity ranging from 1–10 replication units/cell), the measured NS1 and NS2 levels were the same for the different viruses (Haag *et al.*, 2000; Lang *et al.*, 2005). Likewise, similar amounts of monomer-length replication intermediates were obtained from Hirt supernatants of A9 cells infected with vectors or wild-type viruses (Lang *et al.*, 2005), when the number of infectious input virus particles was the same. Altogether, these data clearly show that the infectious virus titer is a meaningful parameter of infectivity (Shabram and Aguilar-Cordova, 2000), since various tumor cells appear to sense the same differences in infectivity as the indicator cells on which the stocks were tittered. Thus capsid-replacement vectors do not seem to be affected in their DNA replication and expression capacity as compared with the wild-type viruses from which they derive.

### Oncotropism

Parvoviral oncotropism is defined as the preferential replication/expression of a parvovirus in tumor-derived or

*in vitro*-transformed cells as compared with corresponding normal or untransformed parental cells (see Chapter 25). On the basis of current knowledge of parvovirus-host cell interactions, one should expect capsid-replacement vectors to retain the oncotropic properties of the parental viruses. To check this assumption, several investigators have compared normal non-established cells and *in vitro*-transformed parental or tumor-derived cells for vector gene expression. In all such experiments, higher levels of NS1 or transgene expression were observed in cultures of transformed/tumor cells than in cultures of the corresponding normal cells. This suggests that transformation-dependent elements are retained (Russell *et al.*, 1992; Dupont *et al.*, 1994, 2000; Gancberg *et al.*, 2000). At the single-cell level, interestingly, fewer normal than transformed cells were positive for NS1 expression after wild-type H-1 virus infection (Dinsart *et al.*, 1996). Likewise, more transformed than normal human fibroblasts were positive for expression of a reporter gene encoding GFP (Dupont *et al.*, 2000). Yet the intensity of gene expression could be as high in positive normal cells as in transformed cells. This argues in favor of ‘all-or-nothing’ expression of the viral genes, rather than an overall decrease in the capacity of cells to express the viral genes. Whether complete gene replacement vectors retain the oncotropic properties of the parental viruses has not been reported.

### Killing effects and oncolysis

As NS protein expression in transformed cells correlates with the failure of these cells to form visible colonies, it was initially proposed that NS1, possibly in cooperation with NS2, might be responsible for the cytotoxic effect of parvoviruses (Brandenburger *et al.*, 1990; Caillet-Fauquet *et al.*, 1990; Li and Rhode, 1990; Momoeda *et al.*, 1994). It was therefore highly surprising to find that capsid-replacement vectors turned out to be less toxic than the corresponding wild-type MVMP and H-1 viruses towards all cell lines tested to date for this property, when infected with the same number of infectious virions (Lang *et al.*, 2005; our unpublished results). Thus, the inability of a vector virus to produce capsid proteins or to assemble capsids may reduce its cytotoxicity. The fact that equal levels of NS1 (and of NS2) are produced from vector and wild-type viruses is apparently not sufficient to promote similar cytolytic activities. It appears that the greater killing capacity of wild-type viruses is not due to the cells’ ability to produce infectious viruses, since very susceptible cells unable to produce infectious virions also show different sensitivities to killing by wild-type and vector viruses.

### Vector infectivity

The ratio of the genome titer (the total number of full virus particles) of a virus stock to its infectious titer gives the

so-called particle-to-infectivity ratio, which is a relative measure of the capacity of the virus to infect a standard indicator cell. Particle-to-infectivity ratios of some autonomous rodent parvoviruses were reported to range from 200 to 1000 (Tattersall, 1972; Linser *et al.*, 1977; Paradiso, 1981; Maxwell *et al.*, 2002). The particle-to-infectivity ratios of various MVMp-derived capsid-replacement vector stocks prepared from transfected 293T cells and purified in CsCl or iodixanol gradients and titrated on A9 mouse fibroblasts could be two orders of magnitude or more higher than that of wild-type MVMp stocks conventionally prepared by infection of A9 cells. The lower infectivity of vectors versus wild-type virus appears to depend both from the producer cell, production method and the type of vector (Lang *et al.*, 2005). Others have made similar observations of a lower, infectivity of vectors versus wild-type viruses (Maxwell *et al.*, 2002). Whether the structure of the packaged transgene contributes to the vector yields, as was suggested by some authors (Brandenburger *et al.*, 1999; Maxwell *et al.*, 2002) remains to be proven. An argument in favor of this idea is that foreign DNA of different origin but with the same size appeared to positively or negatively influence vector production (Brandenburger *et al.*, 1999; Clément *et al.*, 2002). Only a systematic comparison of vector titers obtained from newly prepared DNA clones containing different size-matched transgenes would provide a sound basis for establishing an effect of the transgene on vector production and infectivity. Such experiments require determining both the infectious and the genome titer. As the transgenes of complete coding replacement vectors can be larger than those of capsid replacement vectors, the former vectors are probably the better tools for answering questions regarding the impact of the transgene DNA on packaging. The genome of canine parvovirus was reported to make contacts with amino acids in the capsid interior (Chapman and Rossmann, 1995). Conceivably, shorter genomes and/or the presence of transgenes might disrupt or modify such contacts, and this might cause changes at the capsid exterior. The latter modifications in their turn may disrupt interactions with intracellular receptors crucial for the infection process and so reduce the vector's infectivity. This hypothesis is, however, purely speculative and needs to be supported by experimental data.

## THE USE OF VECTORS FOR EXPERIMENTAL AND THERAPEUTIC PURPOSES

### Transduction of transgenes and host range

As parvoviral genomes are small and cannot accommodate longer genomes than the authentic ones (Brandenburger *et al.*, 1999; Kestler *et al.*, 1999), the transgenes to be inserted cannot exceed 4.6 and 1.8 kbp in complete coding and capsid-replacement vectors respectively. Parvovirus vectors

transducing small reporter proteins (the firefly luciferase enzyme, GFP, the chloramphenicol acetyl transferase enzyme) have been used to determine the susceptibility of various cells to parvovirus replication (Maxwell *et al.*, 1993b; Dupont *et al.*, 1994; Spitzer *et al.*, 1997; Giese *et al.*, 2000; Moehler *et al.*, 2001, 2003; Olijslagers *et al.*, 2001; Wrzesinski *et al.*, 2003). So far, these analyses gave a reliable impression of the cellular infectability as the expression of the transgenes reflected gene expression from wild-type virus infections.

The expression of NS and transgenes from vectors in cell cultures is transient (Russell *et al.*, 1992; Maxwell *et al.*, 1993a; Haag *et al.*, 2000; Wetzel *et al.*, 2001). Yet high levels of cytokine/chemokine (from 1–10 µg/10<sup>6</sup> infected cells) can be produced, possibly owing to the strong NS1-trans-activated P38 promoter and to the amplification potential of capsid-replacement vectors (Haag *et al.*, 2000; Wetzel *et al.*, 2001, and unpublished results).

Maxwell and coworkers were the first to show that recombinant LuIII genomes can promote their own packaging into capsids of the closely related H-1 and MVM viruses (Maxwell *et al.*, 1993b, 1995) or of the more distant FPV and CPV viruses (Spitzer *et al.*, 1996). Pseudotyping has confirmed the finding that the fibrotropism and lymphotropism of the MVMp and MVMi strains, respectively, of MVM are mediated by these strains' respective capsids (Maxwell *et al.*, 1995). It was also found that tropism was in particular determined by the VP2 component of the capsids, although VP1 is required for vector infectivity (Maxwell *et al.*, 1995; Spitzer *et al.*, 1997).

Srivastava and co-workers demonstrated that recombinant AAV genomes can be packaged in capsids from the not closely related autonomous parvovirus B19 and that these chimeric viruses can efficiently transduce transgenes into primary human erythroid cells (Ponnazhagan *et al.*, 1998). Moreover, they found that these chimeric viruses infect certain non-erythroid human cells such as endothelial cells and fibroblasts (Weigel-Kelley *et al.*, 2001) and confirmed the role of the blood group P antigen as the primary receptor for binding of B19 (Weigel-Kelley *et al.*, 2001). The latter studies clearly showed that P antigen is necessary but not sufficient to support infection of human hematopoietic cells by B19 virus (Weigel-Kelley *et al.*, 2001). These observations thus confirm and extend the findings that the cell and tissue tropism of autonomous parvoviruses is mainly determined by the capsids (Tattersall and Bratton, 1983; Tijssen *et al.*, 1995; Truyen and Parrish, 1995), although the viral genome may also play a role (Colomar *et al.*, 1998; Wrzesinski *et al.*, 2003).

Some autonomous parvoviruses encapsidate both strands (i.e. LuIII), while most of them (i.e. MVM, H-1) package mainly the minus strand. Maxwell and collaborators seized the opportunity offered by the pseudotyping technique to investigate the packaging mechanism (Corsini *et al.*, 1995). They first showed that it is not the capsid but the genome of LuIII that is responsible for symmetric

encapsidation, since encapsidation remained symmetric when LuIII genomes were packaged into H-1 or MVM capsids. Second, the LuIII genome was symmetrically packaged whether a unique A-rich region at its right-hand terminus, postulated to be involved in symmetric packaging, was present or not. Recent research has provided evidence that genome packaging is controlled by the efficiency of the right-end nick site (Cotmore and Tattersall, 2005).

## Vector targeting

Most cells in culture tested so far can take up parvoviruses, and in an experiment where mice were injected with recombinant MVMP, all organs of these animals assessed became positive for vector DNA, as was shown with the sensitive PCR technique (Giese *et al.*, 2000). Consequently, both systemic administration of virus and intralesional virus application are likely to lead to the loss or unintended expression of many input particles in non-target tissues. In order to improve delivery of the therapeutic transgene to a specific organ, attempts have been made to target the vector. In one type of approach (internal targeting), the P4 promoter was replaced with a heterologous promoter or modified to be active only in some tissues. Alternatively, small peptides were displayed on vector capsids for binding to specific cell receptors (external targeting).

The first approach was used in an experiment where most of the authentic LuIII promoter was replaced with liver-specific enhancer elements from the alpha 1-protease-inhibitor gene (Table 44.1). The heterologous promoter permitted transgene transduction into human liver cells in culture (Maxwell *et al.*, 1996). These data demonstrate that parvoviruses can be engineered to display an altered host range, without becoming defective. In another experiment, a complete coding replacement vector was constructed where the expression could be stopped by addition of the drug tetracycline, owing to a tetracycline-responsive element inserted into a minimal P4 promoter in cells expressing the transactivator (Maxwell *et al.*, 1996). Possibly owing to expression of the NS proteins, transgene expression from a capsid-replacement vector could not be regulated in a similar way. Instead, transgene expression from this type of vector could be achieved using a tetracycline-response *trans* repressor (Maxwell and Maxwell, 1999). Similarly, a conditionally replication-proficient H-1 virus was designed to selectively infect and kill tumor cells with a constitutively activated Wnt signaling pathway (Malerba *et al.*, 2003). This was achieved by inserting into the P4 promoter a binding site for the transcription factor  $\beta$ -catenin/Tcf. Human colon tumor cells with an activated Wnt signaling pathway, but not ones with a silent Wnt signaling pathway, could be killed as efficiently by such a conditionally replicating virus as by the parental wild-type H-1 virus (Malerba *et al.*, 2003). To the same class of vectors belongs the recombinant virus that was developed to selectively kill HIV-infected lymphocytes (see below).

In another study aiming to target autonomous parvoviruses to human tumor cells, a short peptide known to bind to  $\alpha\beta$  integrin was included in the capsid proteins of FPV, in the hope that the outside location of the inserted peptide would promote virus uptake and internalization by human cells (Maxwell *et al.*, 2001). The same authors showed that wild-type FPV is unable to infect human cells (Spitzer *et al.*, 1996). Unfortunately, only one cell among many tested human cells could be infected with the altered FPV, suggesting that particle binding to  $\alpha\beta$  integrin is not sufficient to promote successful parvovirus particle uptake in human cells. Despite this failure, this preliminary study showed the feasibility of this approach.

Taking advantage of the oncotropic and cytotoxic activities of the H-1 virus, an antimetastasis strategy, using wild-type-virus-infected carrier cells known to home to the lungs, was developed in rats. Cell-mediated delivery of the H-1 virus strongly reduced the number of small pre-established lung metastases, although systemic administration of the virus also had antitumor effects (Raykov *et al.*, 2004). From the greater antitumor effect of vectors transducing cytokines/chemokines as compared with the wild-type virus (see below), one may hope that targeted delivery of vector virus by appropriate carrier cells might be even more efficient.

## Antitumor vectors

Among the potential therapeutic transgene products for cancer therapy are toxins or prodrug-activating enzymes that can kill vector-infected cancer cells (Table 44.1). Ideally, these suicide gene products should also elicit a bystander effect causing the death of neighboring cells not primarily hit by the virus. Approaches aimed at reducing tumor volume are important as such but may have far greater impact if tumor-cell death can trigger immune responses that stimulate the uptake of tumor antigens by dendritic cells (DC) and the generation of cytotoxic antitumor T lymphocytes (Ronchetti *et al.*, 1999; Somersan *et al.*, 2001). Interestingly, wild-type H-1 virus infection of human melanoma cells has been shown to promote the release of inducible heat-shock protein 70 (HSP70), one of the main players in antigen uptake by and maturation of DC (Hoehler *et al.*, 2003). Suicide genes encoding the herpes simplex virus thymidine kinase and the chicken anemia viral protein Apoptin have been introduced into parvovirus-based vectors, enabling the vectors to kill cultures of target tumor cells (Olijslagers *et al.*, 2001; Dupont *et al.*, 2000). It is noteworthy that the lytic effect of Apoptin could only be demonstrated in cells that were relatively resistant to the wild-type virus, showing that the cell killing activity of vectors can be enhanced by transduction of a cytotoxic product.

In various laboratories, vectors capable of transducing immunostimulatory molecules have been produced, the ultimate aim being to achieve tumor rejection and establish long-term immunity. Immunostimulatory molecules can

be co-stimulatory factors such as B7–1 and B7–2 or secreted immunomodulators, such as cytokines and chemokines. The latter can stimulate host defense mechanisms or recruit specific leukocyte populations to the site of their expression. Because cytokine cDNAs are usually short (approximately 400 bp), the size of the recombinant genome will not limit production of these vectors (Kestler *et al.*, 1999; Haag *et al.*, 2000; Wetzel *et al.*, 2001; Giese *et al.*, 2002). Certain cytokines (i.e. interferon  $\gamma$ -inducible protein 10, IP-10) may also inhibit neo-angiogenesis and hence tumor progression (Arenberg *et al.*, 1996). Recombinant MVMP and H-1 viruses have been created that transduce the cytokines/chemokines interleukin (IL)-2 (Russell *et al.*, 1992; Brandenburger *et al.*, 1999; El Bakkouri *et al.*, 2005; Haag *et al.*, 2000), IL-4 (Russell *et al.*, 1992), monocyte chemotactic protein (MCP)-1 (Kestler *et al.*, 1999), MCP-2 (unpublished), MCP-3 (Wetzel, 2000, 2001), IP-10 (Giese *et al.*, 2002), and tumor necrosis factor alpha (unpublished). Moreover, the co-stimulatory ligands B7–1 and B7–2 involved in T-cell activation, have also been transduced by parvovirus vectors (Gancberg *et al.*, 2000; Palmer and Tattersall, 2000b).

Data obtained after direct injection of vectors transducing cyto/chemokines into tumor lesions, or after tumor cell infection *in vitro* followed by subcutaneous grafting of the infected tumor cells in mice (the *ex vivo* approach), show that capsid-replacement vectors transducing various cytokines or the co-stimulatory molecule B7–1 are, with a few exceptions, effective antitumor agents in various animal models (Table 44.2). In these experiments the antitumor effects could be ascribed largely to expression of the transgene. Thus, MVMP- or H-1-based vectors transducing B7–1, IL-2, MCP-3, or IP-10 cDNA exerted significant

antineoplastic activity in various animal tumor models (El Bakkouri *et al.*, 2005; Haag *et al.*, 2000; Palmer *et al.*, 2000b; Wetzel *et al.*, 2001; Giese *et al.*, 2002). When no therapeutic benefit was observed, this could not be ascribed to poor transgene expression but to the chosen cytokine/tumor cell combination (Haag *et al.*, 2000; Giese *et al.*, 2002). The therapeutic vectors exhibited greater antitumor effects than corresponding control viruses (wild-type virus, empty vectors, or vectors expressing a reporter transgene). All of these control viruses achieved only weak tumor suppression in the most susceptible tumor models (Giese *et al.*, 2000; Haag *et al.*, 2000; Wetzel *et al.*, 2001; our unpublished results). Despite the limited number of tumor models tested so far, it seems that tumor-cell susceptibility to parvovirus infection is a prerequisite to successful treatment with vector viruses.

An attempt to treat established tumors by intratumoral injection of virus proved less effective than expected on the basis of the *ex vivo* data obtained with the same vector (Giese *et al.*, 2000). This was undoubtedly due to poor intratumoral virus spread, to the presence of non-tumor cells, and to generation of antiviral antibodies. Yet strong antineoplastic effects were obtained with MCP3-transducing MVMP virus in an immunocompetent mouse melanoma tumor model (Wetzel *et al.*, 2000), even though the tumor cells were rather poor virus recipients (Giese *et al.*, 2000). Successful intratumoral delivery of vector DNA was monitored by reverse transcription PCR and immunohistochemical techniques through its expression in mouse tumors (Giese *et al.*, 2000, 2002; Wetzel *et al.*, 2001; Haag *et al.*, 2000). The same methods revealed a correlation between antitumor effects and the intratumoral infiltration and activation of lymphocytes, NK cells, and macrophages in nude and immunocompetent mice.

**Table 44.2** Antitumor effects of vectors carrying therapeutic genes<sup>1</sup>

Virus/transgene <sup>1</sup>	Infection <sup>2</sup>	Tumor cells <sup>3</sup>	Mice <sup>4</sup>	Antitumor effect <sup>5</sup>	Ref <sup>6</sup>
MVMP/IL-2	<i>in vitro</i>	K1735	syn	+	1
MVMP/IL-2	<i>in vivo</i>	H5V	syn	—	2
MVMP/IP-10	<i>in vivo</i>	H5V	syn	+	2
MVMP/MCP-3	<i>in vivo</i>	B78/H1	syn	+	3
MCMp/MCP-3	<i>in vitro</i>	B78/H1	syn	+	3
MVMi/B7-1	<i>in vivo</i>	VL4	syn	+	4
H-1/IL-2	<i>ex vivo</i>	HeLa	nude	+	5
H-1/MCP-1	<i>ex vivo</i>	HeLa	nude	—	5
H-1/MCP-3	<i>ex vivo</i>	HeLa	nude	+	6

<sup>1</sup>Transgene products: B7–costimulatory molecule B7–1; IL-2, interleukin 2; IP-10, Interferon- $\gamma$ -inducible protein 10; MCP-1 and MCP-3, monocyte chemotactic proteins 1 and 3.

<sup>2</sup>*Ex vivo*: tumor cells were infected in culture prior to their subcutaneous implantation. *In vivo*: animals bearing established tumors were treated with vector or vector was injected into the tumor.

<sup>3</sup>Tumor cells used: B78/H1 are cells derived from a mouse B16 melanoma subclone; H5V cells are mouse endothelioma cells causing metastatic hemangiosarcomas; EL4 is a mouse thymoma cell line; HeLa cells derive from a human cervical carcinoma.

<sup>4</sup>H-1-virus-derived vectors were used against human tumor cells implanted in immunodeficient (nude) mice, while MVM-based vectors were used against mouse tumor cells implanted in syngeneic (syn) mice.

<sup>5</sup>Antineoplastic effects of recombinant viruses comprise: longer life expectancy, tumor growth inhibition, regression of established tumors, and prevention of tumor appearance. +: stronger effect with the vector than with an appropriate control (wild-type virus, empty vector, or vector carrying a marker transgene); —: no improvement of the antineoplastic effect with the recombinant virus with respect to a control virus.

<sup>6</sup>The numbers correspond to the following references: (1) El Bakkouri *et al.*, 2005; (2) Giese *et al.*, 2000; (3) Wetzel, 2000; (4) Palmer and Tattersall, 2000b; (5) Haag *et al.*, 2000; (6) Wetzel *et al.*, 2001.

## Vectors designed to treat infectious diseases

In addition to their potential use as anticancer vectors, recombinant parvoviruses have been developed with a view to treating humans infected with HIV or suffering from Lyme disease (Palmer and Tattersall, 2000a; Palmer *et al.*, 2004). Owing to the human immunodeficiency virus Tat protein response element inserted into a modified MVMi P4 promoter, a conditionally replication-proficient infectious virus was produced that is able to selectively infect and kill mouse and human cells expressing the Tat protein (Palmer and Tattersall, 2000a). Whether such a virus can be used to treat HIV-infected persons remains to be proven. With a view to developing a vaccine vector against Lyme disease, an MVM-based capsid-replacement vector has been produced, containing the sequence of the *Borrelia burgdorferi* outer surface protein A (Palmer *et al.*, 2004). High titers of antibodies against the transgene product were obtained in mice after a single vector injection, this being sufficient to protect the animals against a challenge with live bacteria. These promising results should stimulate efforts to further exploit the potential of parvoviruses to persistently infect animals to develop parvoviral vectors that generate life-long protection against viral or bacterial infections.

This review will not be complete without mentioning the intriguing use of empty virus particles as safe antigen platforms for vaccination against infections with the corresponding live virus or against heterologous infectious agents (for a recent review see Boisgérault *et al.*, 2002). Vaccination against heterologous viruses has been achieved by displaying peptides containing heterologous epitopes on the outside of empty virus particles, so as to generate a strong immune response against infection by the heterologous pathogen. For instance, vaccination of mice with porcine parvovirus empty capsids that are tagged with an epitope from the lymphocytic choriomeningitis virus has been shown to stimulate production of helper T cells and cytolytic T-cells (CTL), and to induce neutralizing antibody responses against lymphocytic choriomeningitis virus infection. Moreover, such particles were capable of inducing protective immunity against challenges with live choriomeningitis virus (Sedlik *et al.*, 1997). As these parvovirus-based particles are not gene-transfer vehicles in the proper sense, we shall not further discuss these data. Yet these studies have considerably contributed to our knowledge of the immune responses elicited by parvoviruses and of the sites that can be used to insert peptides for targeting. It might be possible to design vectors exposing an antigen on their capsids and carrying a transgene to boost a humoral or cellular immune response against it.

## PROSPECTS

The data summarized in this review show that autonomous parvoviruses have potential as vectors for short-term

expression of therapeutic genes. In particular, parvoviral vectors may be promising gene transfer vehicles for human cancer therapy. The possible application of parvoviral vectors for somatic gene therapy have not been explored so far. Indeed, the possibility that autonomous parvovirus vectors might support, like AAV vectors, long-term expression in certain tissues such as the muscle, should be tested. In particular, prolonged gene expression is required when vectors are used as vaccines to induce sustained immune responses against infectious agents.

A promising strategy seems the development of B19-based vectors to correct acquired and inherited diseases that affect cells of the erythroid lineage such as sickle cell anemia. A further intriguing avenue for further research is the development of parvovirus vectors as powerful anti-cancer vaccines, since preliminary results have shown that HSP70 can be activated and released from wild-type H-1-infected tumor cells and that vectors carrying certain cyto/chemokine genes can elicit strong antitumor activity in mice injected with vector-infected tumor cells. Autologous tumor cells or DC infected with such vectors might be used as vaccines.

The low yield of infectious vectors constitutes the major obstacle to performing preclinical studies. Therefore, a high priority is to identify the cell and viral factors that promote generation of infectious virus. If efforts to produce more infectious particles per cell are successful, this will be helpful towards achieving the high titer vector stocks needed for the treatment of humans.

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