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# Thesis Title

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## Erklärung

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# Nomenclature

AAV	Adeno-associated virus	HCV	Hepatitis C virus
Ad	Adenovirus	HIV	Human immunodeficiency virus
AMDV	Aleutian mink disease virus	HMG 1/2	High mobility group proteins 1 and 2
APAR	Autonomous parvovirus-associated replication	IF	Immunofluorescence
B19V	Human parvovirus B19	IP	Immunoprecipitation
BC	Basic cluster	ITR	Inverted terminal repeat
Bp	Base pair	Kb	Kilo base
BPV	Bovine parvovirus	kDa	Kilodalton
ChPV	Chicken parvovirus	KRV	Kilham rat virus
CPV	Canine parvovirus	m. u.	Map units
CRE	cAMP-responsive element	mAb	Monoclonal antibody
cRF	Closed replicative form DNA	MEV	Mink enteritis virus
CV	Column volume	mRF	Monomeric replicative form DNA
Da	Dalton	mRNA	messenger RNA
DMEM	Dulbecco modified Eagle's medium	MVM	Minute virus of mice
DNA	Deoxyribonucleic acid	MVMi	Immunosuppressive strain of MVM
DPV	Duck parvovirus	MVMp	Prototype strain of MVM
dRF	Dimeric replicative form DNA	NIH	National institutes of health
dsDNA	Double stranded DNA	NLM	Nuclear localization motif
EPC	Erythroid progenitor cell	NLS	Nuclear localization signal
FCS	Fetal calf serum	NPC	Nuclear pore complex
FPV	Feline parvovirus	NS	Non-structural (protein)
GFAV	Gray fox amovirus	Nt	Nucleotide
GmDNV	Galleria mellonella densovirus	ORF	Open reading frame
GPV	Goose parvovirus	PARV4	Parvovirus 4
H1-PV	Parvovirus H1	PCNA	Proliferating cell nuclear anti-
HBoV	Human Bocavirus		
HBV	Hepatitis B virus		

	gen	RPV	Rat parvovirus
PCR	Polymerase chain reaction	RSS	Runting-stunting syndrome
PEC	Poult enteritis complex	SAT	Small alternatively translated protein
PEMS	Poult enteritis mortality syndrome	SCID	Severe combined immunodeficiency
PIF	Parvovirus initiation factor	SN	Supernatant
PLA <sub>2</sub>	Phospholipase A2	sPLA <sub>2</sub>	Secretory PLA <sub>2</sub>
PPV	Porcine parvovirus	ssDNA	Single stranded DNA
pre-mRNA	messenger RNA precursor	SV40	Simian vacuolating virus 40 or Simian virus 40
PstDNV	Penaeus stylirostris densovirus	TuPV	Turkey parvovirus
Py	Polyomavirus	VP1	Viral protein 1
qPCR	Quantitative PCR	VP1u	VP1 unique region
RCR	Rolling circle replication	VP2	Viral protein 2
RF	Replicative form	VP3	Viral protein 3
RHR	Rolling hairpin replication		
RNA	Ribonucleic acid		
RPA	Replication protein A		

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# **Part I**

# **Introduction**



# 1 Discovery and brief history

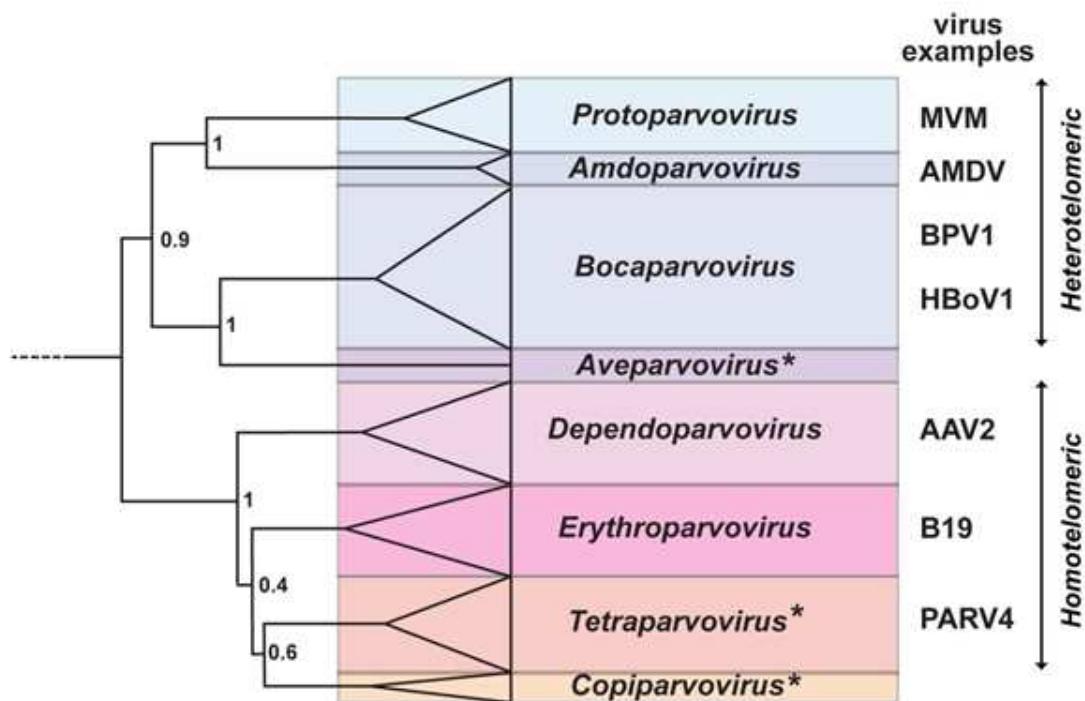
Minute virus of mice (MVM) is a small, non-enveloped autonomous replicating parvovirus. Nowadays, two variant forms of MVM, that share 96 % nucleotide sequence identity<sup>[228]</sup>, have been discovered independently. First, MVMP, the prototype strain, was isolated and characterized by Crawford in 1966. It originated from a contaminated murine adenovirus stock and was shown to replicate efficiently in mouse fibroblasts<sup>[88]</sup>. The virus was plaque purified in 1972<sup>[254]</sup> and the resulting strain was designated MVM(p) for prototype<sup>[250]</sup>. Secondly, another strain was recovered from the culture fluid of infected murine EL-4 T-cell lymphoma cells by Bonnard and colleagues in 1976<sup>[34]</sup>. This strain efficiently replicates in lymphocytes and is immunosuppressive for allogeneic mixed leukocyte cultures as it inhibits the generation of cytolytic T lymphocytes<sup>[100]</sup>. Therefore, it was referred to as immunosuppressive strain MVMi<sup>[188]</sup>. Both strains are well characterized and reciprocally restricted for growth in each other's murine host cell.

Since its discovery nearly 50 years ago, MVM served as an interesting model virus to dissect the molecular mechanisms of tissue tropism, capsid dynamics associated with endosomal trafficking, as well as viral DNA replication and packaging. Furthermore, it gained increasing interest as an important tool for cancer therapy due to its oncolytic capabilities and currently represents a commonly accepted parvovirus model.



## 2 Taxonomy

The classification of the *Parvoviridae* family is based on morphological and functional characteristics. Parvoviruses are ubiquitous pathogens that belong to the smallest DNA-containing viruses. Hence, the prefix "parvum" that means small in Latin. The name "parvovirus" was first introduced to the literature by Carlos Brailovsky, in an early attempt to establish a latinized binomial taxonomy system for viruses, in 1966<sup>[38]</sup>. The age of the *Parvoviridae* family may exceed 40 to 50 million years<sup>[25]</sup>. Apart from their ancient history, the genomes of parvoviruses were affirmed to display similar high mutation rates to RNA viruses<sup>[104,113,240,241,252,287]</sup>. Such high mutation rates in conjunction with the long history might be a reason for the vast genetic divergence and extensive diversity seen within the *Parvoviridae* family. The *Parvoviridae* family comprises of non-enveloped, isometric viruses that contain linear single-stranded DNA genomes. Indeed, parvoviruses are the only viruses in the known biosphere that have both single-stranded and linear DNA genomes. The encapsidated single genomic molecule is 4-6 kb in length and terminates in palindromic duplex hairpin telomeres. In general, there are two large open reading frames, ORF1 and ORF2, encoding for the non-structural protein(s) and the capsid protein(s), respectively. In some cases, an additional ORF3 has been identified that encodes an accessory protein, such as NP1, a non-structural protein only found in members of the genus *Bocaparvovirus* and in PPV4 a member of the genus *Copiparvovirus*<sup>[51,52,161]</sup>. As a consequence of such a simple genome, parvoviruses are highly dependent on their host for diverse functions in their reproduction<sup>[66,265]</sup>. The terminal hairpins are fundamental for the unique replication strategy of the *Parvoviridae* family and serve as an invariant hallmark for classification. Members of the family *Parvoviridae* infect a wide variety of hosts, ranging from insects to primates. Depending on their host range, the *Parvoviridae* are subdivided into *Parvovirinae* infecting vertebrates and *Densovirinae* infecting insects and other arthropods, respectively. The *Parvovirinae* subfamily is further subdivided into eight genera: *Amdoparvovirus*, *Aveparvovirus*, *Bocaparvovirus*, *Copiparvovirus*, *Dependoparvovirus*, *Erythroparvovirus*, *Protoparvovirus*, and *Tetraparvovirus* (see figure 2.1, p. 6)<sup>[87]</sup>. The subdivision into the eight genera is based on differences in transcription maps, organization of the ITRs, the ability to replicate efficiently either autonomously or with helper virus, the sense of the ssDNA that is packaged into separate virions, and sequence homology amongst the *Parvovirinae* subfamily<sup>[146,176]</sup>.



**Figure 2.1:** The *Parvovirinae* subfamily. The genera of the *Parvovirinae* subfamily are depicted in a phylogenetic tree. Phylogenetic analysis is based on the amino acid sequence of the non-structural protein, NS1. The size of the color block for each genus indicates the relative number of species currently recognized, as an indicator of its diversity. Asterisks denote the names of new genera.

## 2.1 The *Parvovirinae* subfamily

### 2.1.1 *Amdoparvovirus*

Mature virions exclusively contain negative strand genomic DNA of approximately 4.8 kb in length harbouring dissimilar palindromic sequences at each end<sup>[6,30]</sup>. A single promoter located at map unit<sup>1</sup>3 at the left end of the genome generates all mRNA transcripts of AMDV.

Polyadenylation may occur at either the proximal site or at the distal site of the genome. Thus, the transcription profile of the genus *Amdoparvovirus* most closely resembles that of the genus *Erythroparvovirus*<sup>[217]</sup>. Only two distant species have been reported. Firstly, *Carnivore amdoparvovirus 1*, which comprises only Aleutian mink disease virus (AMDV) and secondly, *Carnivore amdoparvovirus 2*, which encompasses solely gray fox amiovirus (GFAV)<sup>[164]</sup>. Permissive replication is tightly restricted to Crandell feline kidney cells. The virion surface displays three mounds elevated around the threefold icosahedral axis of symmetry. Several structure features

<sup>1</sup> Map units are commonly accepted units that relate to the position in the genome. The parvoviral genomes are arbitrarily subdivided into 100 map units (m. u.).

were ascertained to be similar to those found in B19V, CPV, FPV, and MVM. Such appearance is comparable to those observed for the genus *Dependoparvovirus*<sup>[186]</sup>. Remarkably, there is no evidence of a phospholipase 2A enzymatic core within the naturally truncated N-VP1 terminus of members belonging to the genus *Amdoparvovirus* as it is common to the other genera of the subfamily *Parvovirinae*<sup>[146]</sup>.

### 2.1.2 *Aveparvovirus*

*Aveparvovirus* is a new genus within the *Parvovirinae* subfamily that comprises of the species chicken parvovirus and turkey parvovirus. The name *Aveparvovirus* is derived from avian parvoviruses, referring to the hosts from which the members were isolated. Although these viruses were identified for years in the intestinal tracts of poultry<sup>[148,149,267]</sup>, analysis of the complete nucleotide sequence has been reported only recently. Phylogenetic study of the genomic sequences revealed that interestingly, ChPV and TuPV do not group phylogenetically with GPV and DPV, that are members of the genus *Dependoparvovirus*. It was clearly demonstrated that ChPV, along with the closely related TuPV, represents the prototype of a novel genus within the *Parvovirinae* subfamily<sup>[150,302]</sup>. Identical direct repeat sequences flank the genome at both the 3' and the 5' end. Each of which contains a 39 nt ITR that is predicted to form a hairpin structure. ChPV and TuPV feature an overall genome organization similar to that of members of the genus *Bocaparvovirus*<sup>[92]</sup>. Although it has been demonstrated that ChPV can induce clinical signs in broiler chickens that show characteristics of the runting-stunting syndrome (RSS)<sup>[147]</sup>, the role of avian parvoviruses in the aetiology of enteric diseases in poultry still remains to be demonstrated. RSS, also referred to as malabsorption syndrome, is characterized by significantly decreased egg hatchability, poorly developed hatched chickens, serious growth retardation, diarrhoea, enteritis, disturbed feathering, low vitality, and bone disorders<sup>[112,204,211]</sup>. Currently, the pathogenicity of TuPV has not been investigated yet. The predominant enteric diseases in turkeys are known as poult enteritis complex (PEC)<sup>[19]</sup> or the more drastic poult enteritis mortality syndrome (PEMS)<sup>[229]</sup>. Understanding the role of avian parvoviruses in PEMS, PEC, and RSS is of great interest due to the economic losses resulting from enteric diseases in poultry.<sup>[302]</sup>.

### 2.1.3 *Bocaparvovirus*

The name of the genus is derived from bovine and canine, referring to the two hosts of the first identified members of this genus. The genomes of members of the genus *Bocaparvovirus* are quite distinct from all other viruses in the subfamily *Parvovirinae*. As the members of the genera *Protoparvovirus* and *Amdoparvovirus* they contain non-identical imperfect palindromic sequences at both ends of their 5.5 kb genome. Mature virions contain mainly, but not exclusively, negative strand ssDNA<sup>[50,235]</sup>. All RNA transcripts are generated from a single P4 promoter at the left-hand end of the genome. The transcripts are alternatively spliced and polyadenylated either

at an internal site or at the 3'-end of the genome<sup>[218]</sup>. Noteworthy, bovine parvovirus (BPV), the main representative, encodes a 22.5 kDa nuclear phosphoprotein, NP1, whose function still remains unknown. This protein is distinct from any other parvovirus-encoded polypeptide<sup>[161]</sup>. A human bocavirus was first described in 2005, when it was detected in nasopharyngeal aspirates of young children with respiratory tract infection<sup>[7,8]</sup>. More recently, HBoV has been identified in diarrheal feces of children with gastroenteritis<sup>[278]</sup>. HBoV infection is associated with acute respiratory symptoms and is usually detected in children under 2 years of age<sup>[22,179,182]</sup>. HBoV infections have been reported world-wide and HBoV was often isolated in respiratory samples of diseased as well as asymptomatic patients sometimes long after the primary infection. Therefore, it can be frequently detected even though it is not likely acting as a pathogen, thus complicating the use of PCR in diagnostics. Furthermore, long-term persistence may explain that HBoV infection among adults was predominantly reported in association with immunosuppression or immunodeficiency<sup>[156,182]</sup>.

#### 2.1.4 *Copiparvovirus*

Based on phylogenetic analysis, the genus *Copiparvovirus* encompasses PPV4 and BPV2. PPV4 was identified in clinical samples from swine herds<sup>[29,52,133]</sup> and represents a distinct branch together with BPV2<sup>[7]</sup>. The name *Copiparvovirus* refers to cows and pigs, the hosts from which members of that genus were isolated. PPV4 is unique in that it is phylogenetically most closely related to BPV2 but the coding capacity and genome organization resemble more those of viruses of the genus *Bocaparvovirus*. While the ORF3 encoded proteins of the three recognized *Bocaparvovirus* members share amino acid identities of 43.3-47.0 % among themselves, the PPV4 ORF3 encoded protein does not display homology with any protein in the GenBank database<sup>[52,133]</sup>. Recently, two novel porcine parvoviruses, PPV5 and PPV6, were discovered<sup>[197,292]</sup>. Characterization of their nucleotide sequences revealed that their full-length genomes are approximately 6 kb in length. As a consequence of this capacious genome size, especially their capsid protein encoding genes are exceptionally large. Interestingly, the genomic organization of PPV5 and PPV6 is different from PPV4 in that they lack the extra ORF3 in the middle of the genome. Moreover, PPV5 as well as PPV6 possess the conserved putative secretory PLA<sub>2</sub> motif which is present in the capsid protein of most parvoviruses but is lacking in PPV4. In spite of considerable differences in the genomic organization between BPV2, PPV5, and PPV6 on the one hand and PPV4 on the other hand, phylogenetic analysis revealed a close evolutionary relationship of these viruses, suggesting that they share the same immediate ancestor<sup>[197,291]</sup>. Since members of the genus *Copiparvovirus* were discovered quite recently, their biological characteristics, relatedness to disease, and potential clinical manifestations are still not fully understood<sup>[52,133,197,291]</sup>. Especially, Kresse strain of porcine parvovirus belonging to the genus *Protoparvovirus* is known to be an important pathogen responsible for embryonic and

fetal death in piglets, resulting in considerable losses in the pig industry worldwide<sup>[155,190,191,276]</sup>. In order to clarify the precise role of the most recently discovered members of the genus *Copiparvovirus* as causative agents of reproductive failure in breeding animals, more comprehensive epidemiologic studies are required in the future<sup>[197]</sup>.

### 2.1.5 *Dependoparvovirus*

Positive and negative strand ssDNA is distributed indifferently among mature virions belonging to the genus *Dependoparvovirus*<sup>[27,224]</sup>. The 4.7 kb DNA molecule contains identical ITRs of 145 nt, the first 125 nt of which form a palindromic sequence<sup>[177]</sup>. Three mRNA promoters that are located at map units 5, 19, and 40 initiate transcription that can be terminated in two polyadenylation sites located at the right-hand end or alternatively, in the middle of the genome<sup>[116,178]</sup>. Common for all currently accepted replication-defective members of the genus *Dependoparvovirus* is their strict dependence upon helper adenoviruses or herpesviruses<sup>[15,43,130]</sup>. Therefore, their host range tropism strongly depends on the one of the helper virus. The only exceptions are the autonomously replicating duck and goose parvoviruses which are also comprised within the *Dependoparvovirus* genus based on phylogenetic analysis<sup>[146]</sup>. The most important members of this genus are the adeno-associated viruses (AAV). They attracted considerable interests since at least one of them, AAV-2, has been reported to integrate site-specifically into human chromosome 19<sup>[152–154,232]</sup>. This characteristic makes AAV a promising candidate for creating viral vectors for gene therapy<sup>[93,192]</sup>. As a well characterized member of the *Dependoparvoviruses* AAV-2 represents the model virus among this genus.

### 2.1.6 *Erythroparvovirus*

Equivalent numbers of positive and negative sense ssDNA are packaged into infectious virions of the genus *Erythroparvovirus*. As in the case with the genus *Dependoparvovirus*, the 5.5 kb ssDNA molecule contains identical ITRs of 383 nt in length at both the 3' and the 5' end. The first 365 nt of those secondary elements form palindromic sequences<sup>[94]</sup>. Transcription is regulated by a single mRNA promoter located at map unit 6<sup>[96]</sup>. A distal polyadenylation site for use in termination of RNA synthesis is located at the far right side. Additionally, transcripts may be terminated at an unusual internal polyadenylation site in the middle of the genome<sup>[201]</sup>. Viruses belonging to this genus are highly erythrotropic, meaning that efficient replication only occurs in rapidly dividing erythroid progenitor cells (EPCs) such as erythroblasts and megakaryocytes present in the bone marrow. B19V, a widespread human pathogen that causes fifth disease, polyarthropathia, anemic crises in children with underlying hematological diseases (e.g. sickle cell anemia or thalassemia) and intrauterine infections (with hydrops fetalis in some cases)<sup>[127]</sup> represents the model virus among the genus *Erythroparvovirus*.

### 2.1.7 *Protoparvovirus*

Kilham Rat virus (KRV), a member of the genus *Protoparvoviruses* was the first member of the subfamily *Parvovirinae* to be discovered in 1959<sup>[144]</sup>. Some members of the genus contain positive strand DNA in variable proportions up to 50 %<sup>[23]</sup>. However, in mature virions of most members, virtually only negative strand DNA occurs. What they have in common are their hairpin structures at both the 5' and 3' ends of the linear 5 kb ssDNA molecule that differ in both sequence and predicted structure<sup>[12]</sup>. Transcription of the genome is regulated by two mRNA promoters at map units 4 and 38<sup>[214]</sup>. There is only one polyadenylation site at the 3' end. Viral replication provokes characteristic cytopathic effects in cell culture. Many species display hemagglutination with erythrocytes of one or several species, but not enforcedly of their natural host<sup>[121]</sup>. The genus *Protoparvovirus* is primarily represented by MVM<sup>[146,255]</sup>.

### 2.1.8 *Tetraparvovirus*

The genus *Tetraparvovirus* is a new genus that arose recently. To date, six species have been discovered, which were isolated from humans<sup>[139]</sup>, chimpanzees, baboons<sup>[242]</sup>, cows, pigs<sup>[2,158,163]</sup>, as well as sheep<sup>[272]</sup>. RNA transcripts that encode the NS-proteins or the VP-proteins are generated from two promoters that are located at map units 6 and 38, respectively. Transcription can be terminated in two polyadenylation sites located at the right-hand end of the genome or alternatively, at an internal polyadenylation site. Since the full-length genome has not been sequenced yet, information of the terminal repeats is still lacking<sup>[175]</sup>. Analysis of the NS1 protein revealed a G2/M cell cycle arrest induced in NS1-expressing hematopoietic stem cells that clearly involved the predicted helicase motifs<sup>[138,193,282]</sup> of NS1. To date, no PLA<sub>2</sub>-like activity of expressed VP1u polypeptides has been demonstrated for any member of the genus *Tetraparvovirus*<sup>[175]</sup>. PARV4 is one of the only four groups of parvoviruses that is known to infect humans besides B19V, HBoV, and AAV. It was first reported in an intravenous drug user who was positive for HBV infection in 2005. The patient suffered from arthralgia, confusion, diarrhea, fatigue, neck stiffness, night sweat, pharyngitis, and vomiting. PARV4 represents a phylogenetic deeply rooted lineage between avian dependoviruses and bovine parvovirus type 3<sup>[139]</sup>. So far, most evidence about PARV4 transmission comes from patients who had engaged in high risk behaviour for blood borne viral infections, where PARV4 infection basically was observed to be strongly associated with HCV and HIV infection<sup>[183,245,296]</sup>. However, there are several reports of parenteral transmission in the absence of HIV, HBC, or HCV. PARV4 IgG has been documented independently from other blood borne viruses among injecting drug users<sup>[246]</sup>, in haemophilia patients<sup>[243]</sup>, and in patients who were subjected to intra-muscular injections in the past<sup>[160]</sup>. Currently, no definitive clinical syndrome was associated with PARV4 infection and there is no evidence for a potential pathogenicity of related members of the genus *Tetraparvovirus* in animals<sup>[158]</sup>. PARV4 viraemia appears to be asymptomatic<sup>[205]</sup> and co-existing blood borne viruses

do not increase severity<sup>[296]</sup>.

**Table 2.1:** Taxonomy for the subfamily *Parvovirinae*

<b>Genus</b>	<b>Species</b>	<b>Virus or virus variants</b>	<b>Abbr.</b>	<b>ACNO<sup>2</sup></b>
<i>Amdoparvovirus</i>	<i>Carnivore amdoparvovirus 1</i>	Aleutian mink disease virus	AMDV	JN040434
	<i>Carnivore amdoparvovirus 2</i>	Gray fox amdovirus	GFAV	JN202450
<i>Aveparvovirus</i>	<i>Galliform aveparvovirus 1</i>	Chicken parvovirus	ChPV	GU214704
		Turkey parvovirus	TuPV	GU214706
<i>Bocaparvovirus</i>	<i>Carnivore bocaparvovirus 1</i>	Canine minute virus	CnMV	FJ214110
	<i>Carnivore bocaparvovirus 2</i>	Canine bocavirus 1	CBoV	JN648103
	<i>Carnivore bocaparvovirus 3</i>	Feline bocavirus	FBoV	JQ692585
	<i>Pinniped bocaparvovirus 1</i>	California sea lion bocavirus 1	CslBoV1	JN420361
	<i>Pinniped bocaparvovirus 2</i>	California sea lion bocavirus 2	CslBoV2	JN420366
	<i>Primate bocaparvovirus 1</i>	Human bocavirus 1	HBoV1	JQ923422
		Human bocavirus 3	HBoV3	EU918736
		Gorilla bocavirus	GBoV	HM145750
	<i>Primate bocaparvovirus 2</i>	Human bocavirus 2a	HBoV2a	FJ973558
		Human bocavirus 2b	HBoV2b	FJ973560
		Human bocavirus 2c	HBoV2c	FJ170278
		Human bocavirus 4	HBoV4	FJ973561
	<i>Ungulate bocaparvovirus 1</i>	Bovine parvovirus	BPV	DQ335247
	<i>Ungulate bocaparvovirus 2</i>	Porcine bocavirus 1	PBoV1	HM053693
		Porcine bocavirus 2	PBoV2	HM053694
		Porcine bocavirus 6	PBoV6	HQ291309
	<i>Ungulate bocaparvovirus 3</i>	Porcine bocavirus 5	PBoV5	HQ223038
	<i>Ungulate bocaparvovirus 4</i>	Porcine bocavirus 7	PBoV7	HQ291308
	<i>Ungulate bocaparvovirus 5</i>	Porcine bocavirus 3	PBoV3	JF429834
		Porcine bocavirus 4-1	PBoV4-1	JF429835
		Porcine bocavirus 4-2	PBoV4-2	JF429836
<i>Copiparvovirus</i>	<i>Ungulate copiparvovirus 1</i>	Bovine parvovirus 2	BPV2	AF406966
	<i>Ungulate copiparvovirus 2</i>	Porcine parvovirus 4	PPV4	GQ387499
<i>Dependoparvovirus</i>	<i>Adeno-associated dependoparvovirus A</i>	Adeno-associated virus-1	AAV1	AF063497
		Adeno-associated virus-2	AAV2	AF043303
		Adeno-associated virus-3	AAV3	AF028705
		Adeno-associated virus-4	AAV4	U89790
		Adeno-associated virus-6	AAV6	AF028704
		Adeno-associated virus-7	AAV7	AF513851
		Adeno-associated virus-8	AAV8	AF513852
		Adeno-associated virus-9	AAV9	AX753250
		Adeno-associated virus-10	AAV10	AY631965
		Adeno-associated virus-11	AAV11	AY631966
		Adeno-associated virus-12	AAV12	DQ813647
		Adeno-associated virus-13	AAV13	EU285562
		Adeno-associated virus-S17	AAVS17	AY695376
	<i>Adeno-associated dependovirus B</i>	Adeno-associated virus-5	AAV5	AF085716
		Bovine adeno-associated virus	BAAV	AY388617
		Caprine adeno-associated virus	CapAAV	DQ335246
	<i>Anseriform dependoparvovirus 1</i>	Duck parvovirus	DPV	U22967
		Goose parvovirus-PT	GPV2	JF926695
		Goose parvovirus	GPV	U25749
	<i>Avian dependovirus 1</i>	Avian adeno-associated virus	AAAV	AY186198
	<i>Chiropteran dependoparvovirus 1</i>	Bat adeno-associated virus	BtAAV	GU226971
	<i>Pinniped dependoparvovirus 1</i>	California sea lion adeno-associated virus	CslAAV	JN420372
	<i>Squamate dependoparvovirus 1</i>	Snake adeno-associated virus	SAAV	AY349010
<i>Erythroparvovirus</i>	<i>Primate erythroparvovirus 1</i>	Human parvovirus B19-Au	B19V-Au	M13178
		Human parvovirus B19-J35	B19V-J35	AY386330
		Human parvovirus B19-Wi	B19V-Wi	M24682
		Human parvovirus B19-A6	B19V-A6	AY064475
		Human parvovirus B19-Lali	B19V-Lali	AY044266
		Human parvovirus B19-V9	B19V-V9	AJ249437
		Human parvovirus B19-D91	B19V-D91	AY083234
	<i>Primate erythroparvovirus 2</i>	Simian parvovirus	SPV	U26342
	<i>Primate erythroparvovirus 3</i>	Rhesus macaque parvovirus	RhMPV	AF221122
	<i>Primate erythroparvovirus 4</i>	Pig-tailed macaque parvovirus	PtMPV	AF221123
	<i>Rodent erythroparvovirus 1</i>	Chipmunk parvovirus	ChpPV	GQ200736
	<i>Ungulate erythroparvovirus 1</i>	Bovine parvovirus 3	BPV3	AF406967
<i>Protoparvovirus</i>	<i>Carnivore protoparvovirus 1</i>	Feline parvovirus	FPV	EU659111
		Canine parvovirus	CPV	M19296
		Mink enteritis virus	MEV	D00765
		Raccoon parvovirus	RaPV	JN867610
	<i>Primate protoparvovirus 1</i>	Bufoavirus 1a	BuPV1a	JX027296

**Table 2.1** continued

Genus	Species	Virus or virus variants	Abbr.	ACNO
<i>Rodent protoparvovirus 1</i>	Bufavirus 1b	BuPV1b	JX027295	
	Bufavirus 2	BuPV2	JX027297	
	H-1 parvovirus	H1	X01457	
	Kilham rat virus	KRV	AF321230	
	LuIII virus	LuIII	M81888	
	Minute virus of mice (prototype)	MVMP	J02275	
	Minute virus of mice (immunosuppressive)	MVMi	M12032	
	Minute virus of mice (Missouri)	MVMm	DQ196317	
	Minute virus of mice (Cutter)	MVMc	U34256	
	Mouse parvovirus 1	MPV1	U12469	
	Mouse parvovirus 2	MPV2	DQ196319	
	Mouse parvovirus 3	MPV3	DQ199631	
	Mouse parvovirus 4	MPV4	FJ440683	
	Mouse parvovirus 5	MPV5	FJ441297	
<i>Rodent protoparvovirus 2</i>	Hamster parvovirus	HaPV	U34255	
	Tumor virus X	TVX	In preparation	
	Rat minute virus 1	RMV1	AF332882	
	Rat parvovirus 1	RPV1	AF036710	
	Porcine parvovirus Kresse	PPV-Kr	U44978	
	Porcine parvovirus NADL-2	PPV-NADL2	L23427	
	Eidolon Helvum (bat) parvovirus	Ba-PARV4	JQ037753	
	Human parvovirus 4 G1	PARV4G1	AY622943	
	Human parv4 G2	PARV4G2	DQ873391	
	Human parv4 G3	PARV4G3	EU874248	
<i>Tetraparvovirus</i>	Chimpanzee parv4	Ch-PARV4	HQ113143	
	Bovine hokovirus 1	B-PARV4-1	EU200669	
	Bovine hokovirus 2	B-PARV4-2	JF504697	
	Porcine hokovirus	P-PARV4	EU200677	
<i>Ungulate tetraparvovirus 1</i>	Porcine Cn virus	CnP-PARV4	GU938300	
	Ovine hokovirus	O-PARV4	JF504699	
<i>Ungulate tetraparvovirus 2</i>				
<i>Ungulate tetraparvovirus 3</i>				
<i>Ungulate tetraparvovirus 4</i>				

The type species for each genus is indicated in bold type.<sup>[87]</sup>

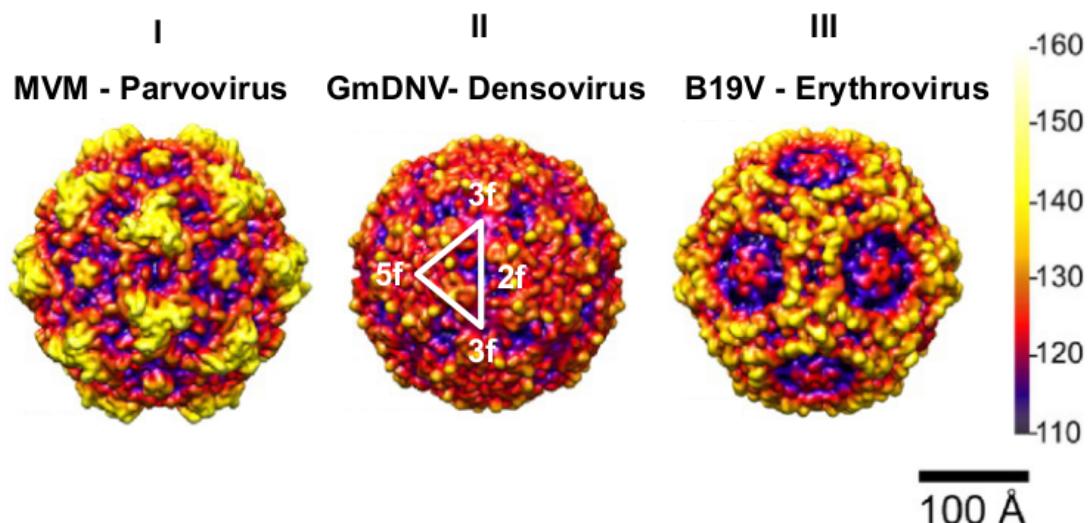
<sup>2</sup> NIH GenBank accession number



## 3 Morphology

Parvoviruses belong to the smallest of isometric viruses. A linear single-stranded DNA genome of about 5 kb is packaged into the virus capsid<sup>[28,89,224]</sup>. They are non-enveloped and their diameters range from 215 Å (*Penaeus stylirostris* densovirus, PstDNV) to 255 Å (CPV)<sup>[146,265]</sup>.

The icosahedral nature of parvoviruses was shown unambiguously by a combination of electron microscopy and, latterly, X-ray crystallography<sup>[270]</sup>. Interpretation of the structural data gave rise to three distinct types of surface topology among parvoviruses (see figure 3.1, p. 16)<sup>[203]</sup>. The icosahedral twofold axes and the protrusions surrounding the icosahedral threefold axes display profound surface topology differences between each group. Types I and III comprise members of the *Parvovirinae* subfamily described in section 2.1, see p. 6. Members of the genus *Protoparvovirus*, as for example CPV, FPV, MVM, and PPV, represent the first topology group that is characterized by a single, relatively flat, pinwheel-shaped protrusion at the icosahedral threefold axes and a wider twofold dimple. In comparison with the vertebrate parvoviruses, no large surface protrusions or depressions are present in *Densovirus* capsids that appeared to be relatively spherical and featureless, adopting a second topology group<sup>[42,247]</sup>. The third topology group encompasses the AMDV, B19V, AAV2, AAV4, and AAV5 capsids, which show three distinct mounds at a distance of ~20-26 Å from the icosahedral threefold axes. In addition, the depression at the twofold axis appears to be slightly deeper, particularly for B19V<sup>[3,119,294]</sup>.



**Figure 3.1:** Surface topology groups among members of the *Parvoviridae* family. Stereo, depth cued (blue-red-yellow-white), and space-filling capsid surface illustration of representative members of the two subfamilies of the parvoviruses. Type viruses representing the three surface topology groups (I-III) and the genus to which they belong are indicated. A viral asymmetric unit bound (white triangle) is shown by a 2-fold (2f), two 3-folds (3f) and a 5-fold (5f) axis on the GmDNV image. A horizontal scale bar (100 Å) for diameter measurement and a vertical color bar depicting color cueing as a function of particle radius in Å are shown on the right hand side. These images were computed from atomic coordinates using the UCSF-Chimera program<sup>[213]</sup>, and all are rendered at the same resolution (7.9 Å) and magnification. The figure was adapted from<sup>[119]</sup>.

## 4 The rugged virion

### 4.1 Physicochemical properties

The extracellular infectious virus entity is defined as virion. An infectious parvovirus virion only consists of two components, namely of about 75 % protein and 25 % DNA. Their molecular weight is approximately  $5.5\text{--}6.2 \times 10^6$  Da. The virion buoyant density is 1.39 to 1.43 g cm<sup>-3</sup>, measured in CsCl gradients<sup>[131,231]</sup>. Since parvoviruses are devoid of a lipid envelope, mature virions are stable in the presence of lipid solvents. In particular, animal parvoviruses show considerable heat resistance. Most species resist alcohol or ether treatment, exposure to pH 3–10, or incubation at 60 °C for 60 min<sup>[33,35,47,129,180,233]</sup>, hence they are clearly more stable compared to most other, especially enveloped, viruses. Only harsh conditions, such as treatment with formalin, β-propiolactone, hydroxylamine, ultraviolet light, and oxidizing agents as for example sodium hypochlorite, ensure effective virus inactivation<sup>[39,123,230,236]</sup>. Accordingly, the capsid effectively protects the fragile, condensed genome from detrimental biological, chemical, and physical agents. Thus it ensures efficient transmission of the virion through the extracellular environment.

### 4.2 Atomic structure

Both DNA-containing full and empty particles were crystallized in the monoclinic space group C2. Following data processing and refinement, the resulting electron density map was interpreted with respect to the amino acid sequence of MVMi. The known CPV structure was used as a phasing model since 52 % of the 587 amino acids in VP2 of MVM are identical to CPV. The polypeptide chain of the major structural protein, VP2, could be traced from residue 39 to residue 587 at the C-terminus<sup>[167]</sup>. The capsid proteins display a T=1 icosahedral symmetry. Therefore, the capsid has a 5-3-2 point group symmetry containing 31 rotational symmetry axes that intersect at the center: six fivefolds, ten threefolds, and fifteen twofolds. The common C-terminal part of the structural proteins has an eight-stranded antiparallel β-barrel topology, frequently found in viral capsid proteins<sup>[225]</sup>. Large loops between the β-strands of the β-barrel that form the principal surface features, particularly the threefold spikes, confer the surface biological properties of the capsid, such as host tropism determinants<sup>[17,208]</sup> and antigenic sites<sup>[32,210]</sup>, and were found to be quite dissimilar in different parvoviruses.

The first 38 amino acids are not visible in the electron density map. It might be highly flexible,

since the N-VP2 terminal part contains a predominantly poly-glycine conserved sequence. In virions, but not in empty capsids, there is weak density extending along the fivefold channels that was modeled as the glycine-rich N-terminal region<sup>[290,293]</sup>. These findings suggest that there is a dynamic situation at the fivefold channel. DNA packaging triggers externalization of one in five N-termini along the fivefold axes.

A substantial amount of internal electron density could be related to 10 DNA nucleotides that were previously found in the analysis of the structure of CPV<sup>[49,271]</sup>. For MVM, 19 additional DNA nucleotides were identified in a difference electron-density map with respect to the data of empty particles. Thus, 29 ordered, or partially ordered, nucleotides per icosahedral asymmetric unit imply that approximately 34 % of the total genome display icosahedral symmetry. This finding, and the conservation of base-binding sites between MVMi and CPV, identifies a DNA-recognition site on the parvoviral capsid interior<sup>[4]</sup>.

### 4.3 Structural proteins

The MVM capsid is made up of 60 copies of a single polypeptide sequence. The virion contains structural proteins of three size classes (VP1-VP3) that constitute a nested set. These share the same C-terminal core structure, but differ in the sequence length on their N-termini. The capsid is assembled from about 10 copies per particle of VP1 (83 kDa), whereas VP2 (64 kDa) represents the major species<sup>[261]</sup>. In DNA containing virions, the latter of which can be cleaved post-translationally by intracellular proteolytic cleavage to generate VP3 (60 kDa), which displays a truncation of approximately 25 amino acids at its N-terminus<sup>[61,262,273,284]</sup>. The N-terminal cleavage of VP2 does not occur in empty capsids, suggesting that DNA packaging into the particle allows the N-VP2 terminus to be externalized<sup>[66,207,262]</sup>. The processing of VP2 in full virions can be mimicked *in vitro* by digestion with trypic proteases, as for instance chymotrypsin or trypsin. However, the proteolytic site *in vivo* is different to the chymotrypsin- or trypsin-sensitive site<sup>[207,262,273]</sup>. Although containing the identical amino acid sequence that is cleaved in VP2, VP1 does not appear to be cleaved at this position in either type of particle, *in vivo* or *in vitro*. VP2 is both necessary and sufficient for the assembly and encapsidation of viral ssDNA<sup>[128]</sup>. However, VP1 is required to produce an infectious particle since capsids that lack VP1 were blocked subsequent to cell binding and prior to the initiation of DNA replication, thus they are unable to fulfill a complete viral life cycle<sup>[274]</sup>. Indeed, the 142 amino acid N-terminal extension of VP1 which is referred to as VP1 unique region (VP1u) harbors several important motifs to initiate viral infection. Two of which are a PLA<sub>2</sub> motif as well as a nuclear localization signal (NLS), elaborated in section 4.4, p. 19. Since VP1u initially is sequestered in the interior of the MVM capsid, the incoming virion must undergo important structural changes *in vivo* in order to expose its functional domains on the capsid surface. By treatment of purified virions

under controlled temperature or with urea, VP1u exposure could be demonstrated *in vitro*<sup>[85,281]</sup>.

## 4.4 Functional domains

From the atomic structure of parvoviruses it can be estimated that structural proteins of 25-30 kDa theoretically suffice to constitute a capsid to protect the viral genome. However, this minimum size is generally enlarged among parvoviruses. VP1 and VP2 exceed the minimum size more than twice as much. The additional parts of the structural proteins harbor essential functional motifs that mediate a number of processes in the infectious viral life cycle. These include host cell surface receptor recognition, entry and escape from endosomes, nuclear localization, DNA packaging, nuclear export, tropism and pathogenicity determinants (see chapter 5, p. 23), immune surveillance and final maturation of particles to produce infectious virus progeny<sup>[266]</sup>.

### 4.4.1 The Phospholipase A<sub>2</sub> (PLA<sub>2</sub>) Motif

In the VP1u region of all parvoviruses, except AMDV and the members of the genus *Brevi-densovirus*, a phospholipase A<sub>2</sub> (PLA<sub>2</sub>) motif has been identified<sup>[297]</sup>. The calcium binding loop (YXGXG) and the catalytic histidine-aspartic acid dyad (HDXXY) of parvoviral phospholipases are related to Ca<sup>2+</sup>-dependent extracellular or secretory sPLA<sub>2</sub>s which are found for example in bee and snake venoms. Unlike all previously characterized sPLA<sub>2</sub>s, the viral sPLA<sub>2</sub> motifs show very weak sequence similarity and lack the characteristic multiple disulfide bonds, thus analogy is mainly restricted to the catalytic units. PLA<sub>2</sub>s specifically catalyze the hydrolysis of phospholipid substrates at the 2-acyl ester (*sn*-2) position to release free fatty acids and lysophospholipids. The viral sPLA<sub>2</sub>s hydrolyze all major classes of glycerophospholipids except phosphatidylinositol, without displaying a preference for unsaturated *versus* saturated *sn*-2 fatty acyl chains<sup>[46]</sup>. The catalytic activity of the PLA<sub>2</sub> is dependent on Ca<sup>2+</sup> in mM concentrations and reaches a maximum at a pH range 6-7, presumably associated with the deprotonation of the His residue in the catalytic dyad at such pH<sup>[24,108]</sup>.

The biological importance of the viral PLA<sub>2</sub> motif was demonstrated by mutational analyses with MVM and PPV<sup>[102,297]</sup>. Viruses lacking a functional PLA<sub>2</sub> motif were not infectious and moreover, failed to escape from endosomes<sup>[98,102,111,253]</sup>.

The cleavage sites of the different phospholipases are illustrated using phosphatidylcholine (PC), a common phospholipid, as an example. Phospholipase A<sub>1</sub>, A<sub>2</sub>, C, and D specifically cleave different ester bonds in the phospholipid. Their respective sites of attack are represented by red staggered lines.

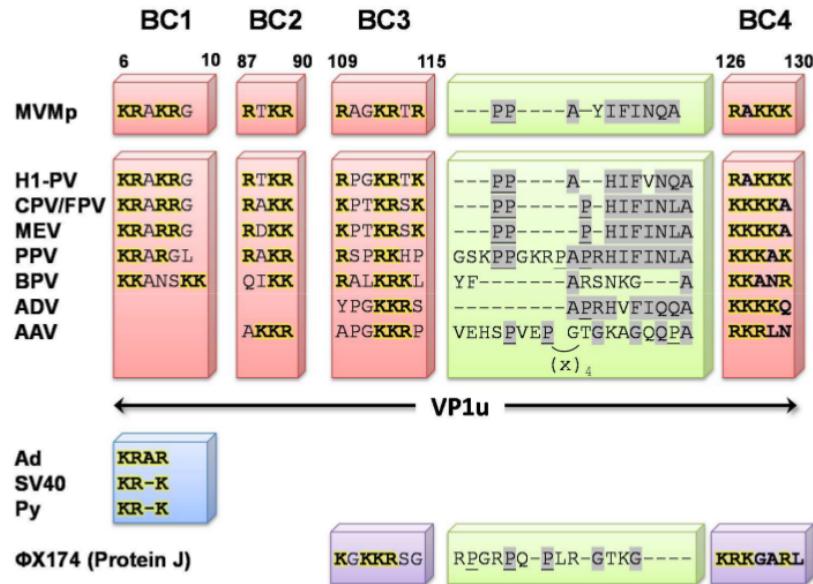
#### 4.4.2 The Nuclear Localization Signal (NLS)

Apart from the plasma membrane, the nuclear membrane constitutes a second barrier to karyophilic viruses which need to enter the nucleus in order to profit from the replication and transcription machinery of the host for their own multiplication. In fact, viral structural components enter the nucleus at two stages of their life cycle, first when the incoming virion delivers its genome and, late in infection, when viral subunits accumulate in the nucleus for self-assembly leading to the generation of virus progeny. In the absence of nuclear membrane disintegration, the transport of viral particles must proceed across the nuclear pore complex (NPC)<sup>[91,227,251]</sup>, as it is the case for cellular proteins. Interestingly, the diameter of the NPC central channel<sup>[99,206]</sup> is in the range of the 25 nm diameter of the parvovirus capsid<sup>[257]</sup>. Therefore, parvoviruses could physically traverse the NPC fully intact without partial or complete capsid uncoating, as it has been reported for more complex viruses<sup>[143,199,268,286]</sup>.

In addition to the PLA<sub>2</sub> motif<sup>[297]</sup>, the VP1u region of MVM contains four basic clusters (BCs) of amino acids, referred to as BC1 to BC4. These are highly conserved among parvoviruses and moreover, even in some other DNA viruses. BC1 and BC2 represent conventional nuclear localization signals (NLS) which are characterized by a short stretch of basic amino acids<sup>[141,142]</sup>. BC3 and BC4, which are separated by a short spacing sequence in between, may rather be arranged as a bipartite NLS domain<sup>[222]</sup>. The clustered basic amino acids interact with transport receptors of the importin/karyopherin family which mediate nuclear import<sup>[184,198,285]</sup>. Nuclear transport activity has been demonstrated for BC1 in the context of a singly expressed VP1 protein<sup>[170]</sup> and as NLS-peptide coupled to an heterologous carrier protein<sup>[279]</sup>. Furthermore, it is proven to be essential for CPV infectivity<sup>[280]</sup> and for MVM to initiate infection<sup>[170]</sup>. In contrast, BC3 and BC4 did not show such capacity to import VP1 either expressed alone<sup>[279]</sup> or in the context of the complete MVM genome<sup>[170]</sup>. Alternatively, these BCs may be involved in the tethering of the ssDNA genome to the capsid inner surface. Such function has been demonstrated for two basic, significantly homologous DNA-binding domains of the protein J of the  $\phi$ X174 bacteriophage<sup>[120]</sup>.

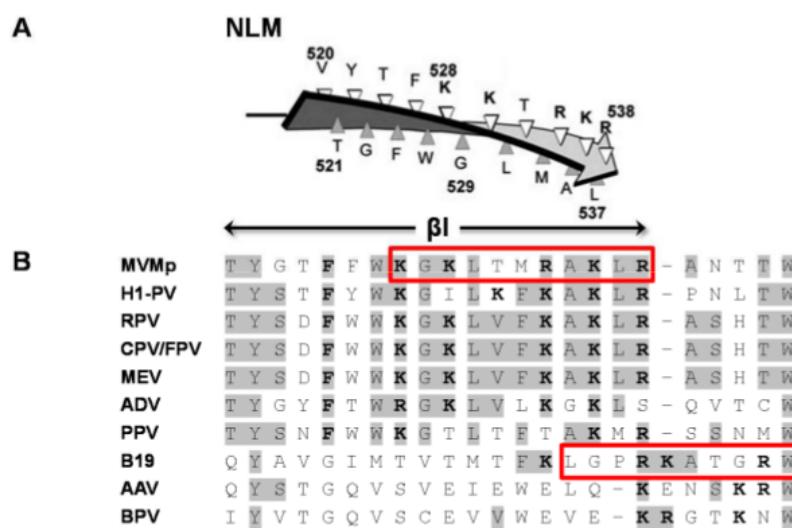
#### 4.4.3 The Nuclear Localization Motif (NLM)

Since both VP1 and VP2 singly expressed proteins efficiently target the nucleus of transfected cells<sup>[170,274]</sup> each protein must carry its own nuclear transport sequence. The common C-terminal sequence of VP1 and VP2 lacks a conventional consensus NLS. However, VP2 contains one single region which is enriched in basic amino acids (528-KGKLTMR<sub>AKLR</sub>-538) near its C-terminus. Based on the crystal structure<sup>[4,270]</sup>, analysis revealed that this sequence is structurally ordered as a  $\beta$ -sheet which forms the carboxy half of the  $\beta$ I strand (residues 520 to 538) of the eight-stranded anti-parallel  $\beta$ -barrel (ref. figure or chap). Moreover, the  $\beta$ I-strand shows marked amphiphatic characteristics, exposing all the basic amino acids to the solvent in the interior surface of the



**Figure 4.1:** VP1 nuclear targeting sequences. The alignment of basic clusters (BC1 to BC4), which are conserved in the VP1u region among parvoviruses, is boxed in red. Amino acid residues are abbreviated using the single letter code. Sequence homology between BC1 and other karyophilic dsDNA viruses is shaded in blue on the left-hand side. Conservation of BC3 and BC4 with the protein J of the ssDNA bacteriophage  $\phi$ X174 is boxed in magenta on the right-hand side. Basic residues of the BC boxes are represented in bold face and possible homologous residues in the spacing region between BC3 and BC4 are shadowed. Characteristic proline residues which are scattered along the space region are underlined. This illustration was adapted from [170,275].

capsid while the hydrophobic residues face toward the protein core. Mutational analysis revealed that the basic nature of the exposed face of  $\beta$ I, as well as the hydrophobic residues on the opposite face, conferred a nuclear localization capacity to the VP2 protein. Accordingly, this sequence in  $\beta$ I which only functions under a precise conformation, but not in a linear form, is referred to as the VP2 nuclear localization motif (NLM) [169].



**Figure 4.2:** Nuclear localization motif (NLM). (A) Schematic representation of the VP2 NLM of MVM as disposed on the  $\beta$ -strand I of the anti-parallel, eight-stranded  $\beta$ -barrel topology in the common C-terminal part of VP1 and VP2. Basic amino acids which are exposed to the solvent are represented in bold. (B) Alignment of the NLM that is conserved among parvoviruses. Homologous positions are shadowed and basic residues are in bold. Sequences with proven nuclear localization capacity are boxed in red. This illustration was adapted from<sup>[169,275]</sup>.

# 5 Host Range and Specificity

## 5.1 Tissue Tropism Determinants

Concerning their host range, most parvoviruses, such as MVM, CPV, and FPV, are tightly restricted to specific receptors of their particular hosts. However, some parvoviruses, as for example many of the AAVs, infect human cells by primary attachment to a variety of receptors.

As outlined in chapter 1 (see p. 3), two distinct strains of the parvovirus MVM have been described to occur in mice. On the one hand, MVMP, the prototype strain, replicates efficiently in mouse fibroblasts<sup>[88]</sup>. On the other hand, MVMi, the immunosuppressive strain, replicates in T lymphocytes<sup>[34]</sup>. Both strains display disparate *in vitro* tropism and *in vivo* pathogenicity despite differing by only 14 amino acids in their capsid proteins<sup>[18]</sup>. Beyond that, MVMP and MVMi are serologically indistinguishable, bind to sialic acid, and are internalized in both fibroblasts and lymphocytes<sup>[249]</sup>. Consequently, it could be demonstrated that both viruses propagate in hybrids of the two cell types<sup>[256]</sup>.

In order to map the allotropic determinants of MVM, chimeric viral genomes were constructed *in vitro* from infectious genomic clones of both strains. By mutagenesis and selective plaque assays, the major determinants for the acquisition of fibrotropism for MVMi have been mapped onto the capsid<sup>[9,18,62]</sup>, in particular to the VP2 residues 317 and 321<sup>[17,185]</sup>. Both residues are located at the base of the threefold spike of the virion<sup>[9,105,106]</sup>. Interestingly, these two VP2 residues structurally localize nearby some of the important amino acids determining CPV, FPV, and PPV host range<sup>[114,135,277]</sup>. Further residues (VP2 residues 399, 460, 553, and 558) were identified in MVMi to be able to confer fibrotropism to forward second-site mutants when either residues 317 or 321 are mutated. Those residues cluster around the twofold dimple-like depression<sup>[4]</sup>. In contrast, the switch to lymphotropism for MVMP is more complex and requires both an equivalent region of the major MVMi capsid protein gene VP2 and a segment of the non-structural protein genes<sup>[62]</sup>.

## 5.2 Pathogenicity Determinants

MVMi appears to be more pathogenic in mice than MVMP. Oronasal inoculation of MVMi in most neonatal mice resulted in lethal phenotype or severe growth-retardation in survivors<sup>[145]</sup>, as observed for other parvoviruses (see section 2.1, p. 6). MVMP infection appears to be asymptomatic.

tomatic in newborn mice<sup>[41]</sup>. In contrast, MVMi infection in neonatal mice of some inbred strains caused renal papillary hemorrhage and viral replication in endothelia<sup>[40]</sup>, hematopoietic precursors<sup>[237]</sup>, and neuroblasts<sup>[220]</sup>. Following *in utero* inoculation of MVMi or MVMP into developing embryo, a broad set of cell types were infected that partially overlapped. Nevertheless, the tissue tropism of MVMP for fibroblasts and of MVMi for endothelium, as well as the higher virulence of MVMi was preserved<sup>[137]</sup>. By reason of the complexity of MVMi pathogenesis in the neonatal mouse, a more adequate model was required to investigate the virulence of MVMi *in vivo*.

Severe combined immunodeficiency (SCID) mice<sup>[36]</sup> represent such a model since they lack an antigen-specific immune response, thus allowing the study in adult mice and circumventing the complex situation of heterogenous viral multiplication in embryonic developing tissue. MVMi infection of adult SCID mice gave rise to the suppression of long-term repopulating hemopoietic stem cells in the bone marrow<sup>[239]</sup>, leading to an acute lethal leukopenia and accelerated erythropoiesis<sup>[238]</sup>. In addition, it has been reported that MVMP evolved in intravenously inoculated SCID mice. Different variants, isolated from single plaques, carried only one of three single amino acid changes at position 325, 362, or 368 in the major VP2 capsid protein. These variants sustained their fibrotropism *in vitro*, but unlike MVMP, they propagated in mouse tissues following oronasal inoculation, eventually causing death<sup>[172,226]</sup>. Two of the three invasive fibrotropic MVMP strains, I362S and I368R, were shown to induce lethal leukopenia in oronasal inoculated SCID mice. Emerging viral populations in leukopenic mice displayed altered sequences in the MVMi genotype at position 321 and 551 of VP2 for infections with the I362S variant or changes at position 551 and 575 in the K368R virus infections. In general, a high level of genomic heterogeneity in the DNA sequence encoding the VP2 protein was observed and was found to be clustered at the twofold depression of the viral capsid<sup>[173]</sup>.

### **5.3 Comparison of Tissue Tropism and Pathogenicity Determinants to those of other Autonomous Parvoviruses**

Significantly, the amino acids dictating *in vitro* tropism (317 and 321), *in vivo* pathogenicity (325, 362, and 368), fibrotropism on MVMi (399, 460, 553, and 558), and those involved in the development of leukopenia (321, 551, and 575) were found to be located on, or near the capsid surface. Structurally, these residues cluster mainly by raised elements around the twofold axes of symmetry, in close vicinity of the sialic acid binding pocket<sup>[172,173]</sup>.

Differences in the tissue tropisms and the pathogenic phenotypes have also been mapped to the capsid proteins of Aleutian mink disease parvovirus<sup>[31]</sup>, porcine parvovirus (PPV)<sup>[26]</sup>, CPV<sup>[48,209]</sup>, and FPV<sup>[269]</sup> in a capsid region analogous to that observed for MVM (reviewed in<sup>[3]</sup>). These pronounced *in vitro* tropism and *in vivo* pathogenicity disparities between the highly homologous viruses can occur at any of the various stages of the infectious viral life cycle,

### 5.3 Comparison of Tissue Tropism and Pathogenicity Determinants to those of other Autonomous Parvoviruses

including cell receptor binding, internalization, capsid uncoating, DNA replication or transcription. Although the same structural elements of viruses are involved in mediating host and tissue tropisms, each appears to be affecting a different mechanism. Host ranges of CPV and FPV are controlled by receptor binding, whereas the cell tropisms of MVM appear to be due to restrictions of interactions with intracellular factors<sup>[4,134,249]</sup>. For MVM it was suggested that the point of restriction appeared after nuclear targeting and conversion of genomic ssDNA to RF intermediates but prior to viral genome replication. Most likely, the restraint occurs due to a block in capsid uncoating<sup>[132,216]</sup>.

As discussed in this section the functional regions among the subfamily *Parvovirinae* co-localize to similar capsid surface regions albeit three general parvovirus topology groups with characteristic local morphological surface differences emerged (see chapter 3, p. 15). A profound understanding of functional domains that are involved in fundamental steps of the viral life cycle, particularly receptor attachment, *in vitro* tropism, *in vivo* pathogenicity, and antigenicity are essential for infection and disease control. Hence, showing great promise to allow genetic engineering of parvovirus capsids for the therapeutic delivery to be controlled or modified in gene therapy applications and to develop foreign antigens<sup>[3,134]</sup>.



# 6 Genome Architecture

The MVM genome is a small, non-permuted, linear, single-stranded DNA molecule<sup>[10,37,260,283]</sup> that is 5085 nt in length for MVMi and 5149 nt for MVMp<sup>[14]</sup>. The relatively long coding sequence of approximately 4.8 kb contains two major, monosense ORFs that span most of the viral genome, with some regions having overlapping coding regions<sup>[12]</sup>. The ORFs encode a non-structural (NS) gene and a structural (VP) gene, by convention termed as occupying the "left" or the "right" half of the coding sequence, respectively. The NS gene encodes four proteins that are required for the replication of the viral genome and are referred to as NS1, NS2<sup>P</sup>, NS2<sup>Y</sup>, and NS2<sup>L</sup>. The VP gene encodes an overlapping set of capsid proteins, VP1 the minor capsid protein and VP2 the major capsid protein<sup>[65,140,214]</sup>. A representation of the genomic organization of MVM is illustrated in figure 6.1 A, p. 30.

## 6.1 The MVM Left- and Right-End Telomeres

The coding sequence is bracketed by short, imperfect palindromes which form back on themselves to secondary structured duplex telomeres. Both telomeres differ considerably from each other in size, primary sequence and secondary structure<sup>[12]</sup>. Hence, they are physically and functionally disparate and vary in their resolution strategies at the two sites (see section 6.1.1, p. 28), although the molecular principles that underlie both strategies are very similar<sup>[78]</sup>.

Firstly, the MVM left-end telomere is 121 nt in length and forms into a Y-shaped configuration. The 43 bp stem region only is interrupted by a mismatched bubble sequence where a triplet GAA on the inboard arm is opposed to the dinucleotide sequence GA on the outboard arm. Additionally, an asymmetric thymidine residue is located within the stem on the outboard arm in the immediate proximity to the "ears" that are generated by small internal palindromes. These "ear"-like structures give rise to the Y-shaped configuration of the left-end terminus<sup>[10,12,13,71]</sup>. A single DNA sequence, designated the "flip" sequence, is conserved in the progeny viral left-end telomere, as is observed *in vivo*<sup>[13]</sup>. The structure of the left-end telomere is depicted in figure 6.1 B (left panel), p. 30.

Secondly, the MVM right-end telomere is 248 nt in length and is most simply depicted as an almost perfect duplex stem structure of 121 bp. The palindrome only is interrupted by a triplet of unpaired nucleotides that forms a small asymmetric bubble near the distal end of one strand, along with three unpaired bases which form the cross-link at the palindrome axis<sup>[12,13]</sup>.

As in homotelomeric parvoviruses, two distinct forms of the MVM right-end terminus, referred to as "flip" and "flop", are generated in equimolar amounts *in vivo* (see figure 6.1 D (i) and (ii), p. 30)<sup>[72,78]</sup>. These two forms are the inverted complements of one another and both give rise to viral origins, dubbed *oriR*<sup>[73,83,86]</sup>. A small internal palindrome, surrounding the three-nucleotide bubble, thermodynamically enables an alternative, asymmetric cruciform configuration of the right-end telomere (see figure 6.1 D (iii), p. 30)<sup>[11]</sup>.

### 6.1.1 Terminal Resolution *versus* Asymmetric Junction Resolution

As is the case for most of the heterotelomeric parvoviruses, MVM shows packaging bias with minus strands preferentially encapsidated to plus strands by a 10-100-fold margin<sup>[66,214]</sup>. This results from differences in the efficiency of their two DNA replication origins at both ends of their genomes, rather than any strand-specific packaging sequence. In particular, the efficient nick site of the *oriR* dictates the negative polarity of the packaged strand which is encapsidated in MVM virions<sup>[76]</sup>.

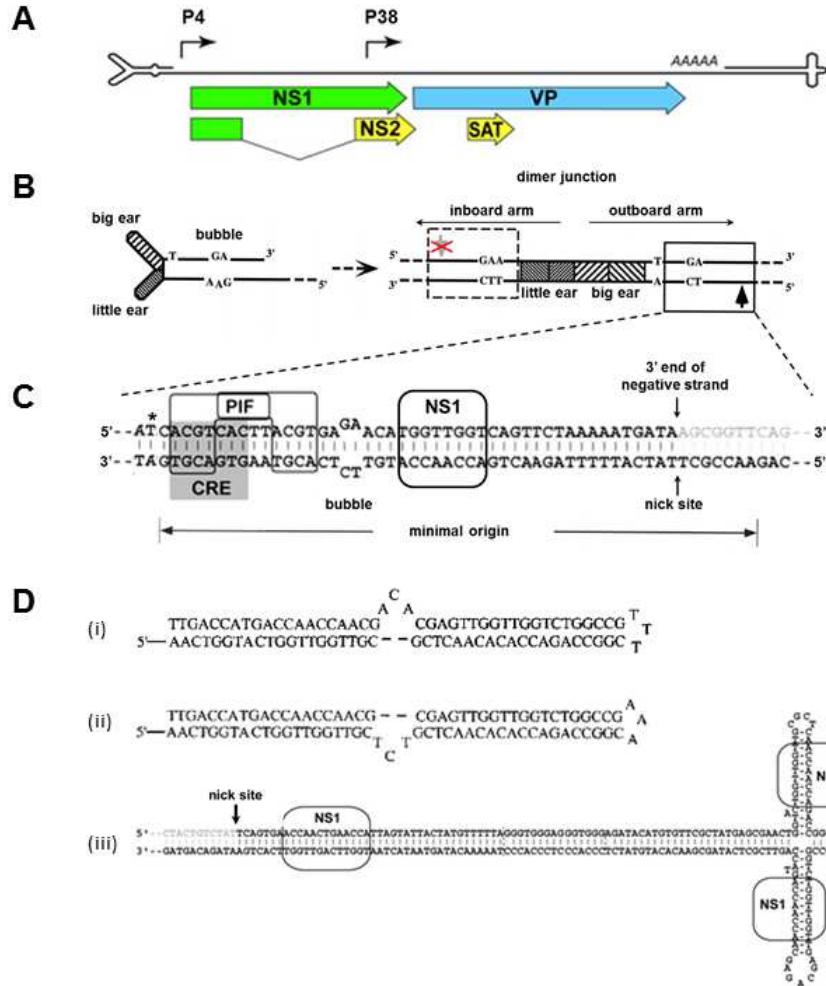
Given the fact that alike their homotelomeric cousins, the right-end hairpin of MVM exists as an equimolar mix of flip and flop sequence orientations, it is processed by a similar terminal resolution strategy. Nicking of the hairpin near the junction between palindromic and non-palindromic sequences and subsequent extension of the right-end terminus allows an efficient inversion of the palindrome (see step iii and iv in figure 7.1, p. 33). On the contrary, the MVM left-end hairpin predominates in the flip orientation, indicating its generation by an asymmetric junction resolution mechanism<sup>[75]</sup>. Briefly, the asymmetric bubble sequence in the stem of the MVM left-end telomere prevents assembly of an active nicking complex. Thus, the left-end telomere can not function as a replication origin in its hairpin conformation<sup>[16]</sup>. During rolling hairpin replication (see section 7.1, p. 31), the hairpin is unfolded, extended, and copied to form the fully basepaired, imperfect palindromic junction sequence which bridges adjacent genomes in an intermediate dimer RF (see figure 6.1 B (right panel), p. 30). It was demonstrated that such junctions can initiate DNA replication in a NS1-dependent manner<sup>[69,70]</sup>. Formation of the dimer junction effectively segregates two potential origins of DNA replication, one derived from each arm of the hairpin, on either side of the junction's symmetry axis. However, only one of these is active. The activity is regulated by the sequence of the asymmetric bubble which serves as a precise spacer between the NS1 binding site and the parvovirus initiation factor (PIF). Binding of which stabilizes the interaction of NS1 with the active (TC) origin (*OriL<sub>TC</sub>*) but not with the inactive (GAA) origin (*OriL<sub>GAA</sub>*)<sup>[57]</sup>. The minimal left-end origin of replication, dubbed *oriL*, is illustrated in figure 6.1 C, p. 30. It extends from two 5'-ACGT-3' motifs which represent binding sites for PIF<sup>[54-56]</sup>, to a 5'-(ACCA)<sub>2</sub>-3' binding site for the viral initiator nickase, NS1<sup>[84]</sup>, to the active nick site<sup>[70]</sup>. Recent studies revealed that MVM tolerates both sequence and orientation changes in its left-end hairpin. From this follows that maintaining the flip orientation

of the left-end telomere is a consequence of, but not the reason for, asymmetric dimer junction resolution. However, the same study indicated that asymmetric left-end processing is crucial for MVM replication<sup>[165]</sup>.

In summary, the heterotelomeric hairpins, together with a few adjacent nucleotides, provide all of the *cis*-acting information required for both efficient genome replication and encapsidation. In particular, these terminal nucleotides, representing less than 10 % of the entire genome, create the replication origins by providing nicking sites that will allow the DNA to be nicked and used as a primer or effectively separate unit-length genomes for DNA packaging. Additionally, they function as flexible hinge regions that are used to establish and re-orient the replication fork, allowing it to roll back and forth along the linear viral DNA<sup>[72,77,195,264]</sup>.

## 6.2 Genetic variability

When compared with cellular DNA, the genome of MVM has a relatively high GC-content (42 %), partially reflecting its high density of regulatory elements. The complexity of the viral genome is increased by transcriptional promoter sequences and various splicing signals that lie embedded within the same primary sequence, beyond the encoded proteins which are organized in multiple overlapping ORFs. Nevertheless, following inoculation of clonal populations of MVMi stocks in mice, genetically disparate antibody-escape variants emerged *in vivo*. This indicates that viral replication appears to support the generation of heterogeneity<sup>[171]</sup>. Another example concerns the emergent branch of CPV during its evolution from FPV since 1978. The substitution rate of CPV resembles that seen in rapidly evolving RNA viruses, as for example HIV-1 and human influenza A virus<sup>[241]</sup>. Remarkably, such diversity occurred despite the fact that the viral genome is multiplied by a subset of the host's DNA replication machinery, hence the mutation rates would be expected to be low. Probably, the unidirectional strand-displacement mechanism may exhibit lower fidelity compared to the bidirectional replication of eukaryotic genes. Additionally, the concatemeric duplex intermediates may allow for inter- and intramolecular recombination during replication of the viral DNA. Moreover, there are several lines of evidence that MVM exploits the DNA damage response machinery early in infection in order to enhance its replication and to ameliorate virus-induced cell cycle arrest in the S-phase<sup>[1]</sup>. Therefore, it seems possible that under such conditions the replication forks appear error-prone. Finally, environmentally induced changes in the viral DNA sequence, such as depurination or deamination, can not be corrected because virions contain ssDNA and hence do not provide a template for excision or mismatch repair systems. Nonetheless, the genetic complexity, in consequence of the constrained genome size, severely and selectively restricts the types of tolerated modifications<sup>[79]</sup>.



**Figure 6.1:** (A) The terminal hairpins, drawn to represent their predicted structures, are scaled approximately 20x relative to the rest of the genome. Major open reading frames are represented by arrowed boxes and alternative RNA splicing for NS2 is indicated. Proteins are shaded green for the major replication initiator protein (NS1), blue for the structural (VP) proteins of the capsid, and yellow for sequences unique to the ancillary non-structural proteins. The two transcriptional promoters, P4 and P38, are indicated by rightward arrows and the polyadenylation site by the AAAAA-sequence block. Abbreviations: SAT, small alternatively translated protein<sup>[80]</sup>. (B) The left-end hairpin of MVM and the dimer junction are shown in diagrammatic form. Asymmetries such as the "ear"-like structures, extra-helical T, and bubble sequence are indicated. The fully duplex, dimer junction, generated by rolling hairpin replication (see section 7.1, p. 31), is shown on the right hand side. The short, palindromic sequences derived from the hairpin ears are represented by cross-hatched boxes. The active *OriL<sub>TC</sub>* is boxed, with an arrow indicating the nick site. The equivalent sequence generated on the GAA side of the bubble is framed by a dashed box with an arrow at the potential nick site that is crossed out to indicate that *OriL<sub>GAA</sub>* is not active<sup>[44,45]</sup>. (C) Sequence details of the active left-end origin (approx. 50 bp) are shown, with an arrow indicating the active nick site. The minimal sequence required for origin activity is indicated by the double-headed arrow. Sequences of the bubble and the PIF, CRE, and NS1 binding sites are indicated. An asterisk represents the position of the extra-helical T, now base paired, and the gray box below it indicates the CRE consensus sequence<sup>[44]</sup>. (D) Alternate conformations of the right-end hairpin sequences of MVM. The right-end terminus can form a hairpin structure in either the flip (i) or flop (ii) sequence orientation or a cruciform configuration (iii). In the cruciform configuration, the binding sites for the replicator protein, NS1, are boxed and their site of nucleolytic cleavage is represented by a vertical arrow<sup>[63]</sup>.

# 7 The Parvovirus Life Cycle

## 7.1 Replication

Due to the small capsid size of an approximate maximum external radius of 140 Å<sup>[167]</sup>, the coding capacity of MVM genomic DNA is strictly limited. Consequently, the viral genes do not code for one's own DNA- and RNA polymerases and relevant accessory proteins. Thus, viral proliferation heavily depends on ancillary cellular factors that are essentially involved in viral genome replication and transcription. These factors are transiently supplied by proliferating host cells during the S-phase in the nucleus<sup>[66,95,223,249,254,258,263]</sup>. In contrast to other small, host cell depending DNA viruses, as for instance SV40<sup>[110,124]</sup>, MVM does not have the capability to stimulate resting cells and to initiate its DNA replication. Upon infection of resting host cells, MVM has to wait until infected cells enter S-phase of their own volition in order to amplify its DNA<sup>[20,59,254]</sup>.

Parvoviruses are unique among all known viruses in having a DNA genome that is both linear and single-stranded. Thus, it is not surprising that they evolutionary adapted their own one of a kind replication strategy. Their singular method to amplify the ssDNA genome resembles an ancient mechanism, known as rolling circle replication (RCR), that is utilized by many other small, circular prokaryotic and viral replicons<sup>[136,151,159,189,200]</sup>. However, in parvoviruses the RCR mechanism is modified and adapted for the replication of a linear chromosome. The parvoviral replication strategy, termed rolling hairpin replication (RHR), proceeds by a single-strand displacement mechanism, so there is no lagging-strand synthesis, and the integrity of the terminal hairpin sequences is maintained<sup>[259]</sup>. The unidirectional progression of the replication fork results in the synthesis of a single, continuous DNA strand. In addition, MVM replication forks are aphidicolin-sensitive and require the proliferating cell nuclear antigen (PCNA). Such DNA elongation mechanism argues for a DNA synthesis that is mediated by DNA polymerase δ and its accessory proteins<sup>[20,53]</sup>. Initiation of parvovirus replication provokes the reorganization of the host cell nucleus, leading to formation of distinct nuclear foci, referred to as "autonomous parvovirus-associated replication" (APAR) bodies<sup>[21,90,295]</sup>. These bodies were shown to be active sites of viral replication and to accumulate essential cellular replication proteins such as cyclin A, DNA polymerases α and δ, PCNA, and replication protein A (RPA)<sup>[20]</sup>.

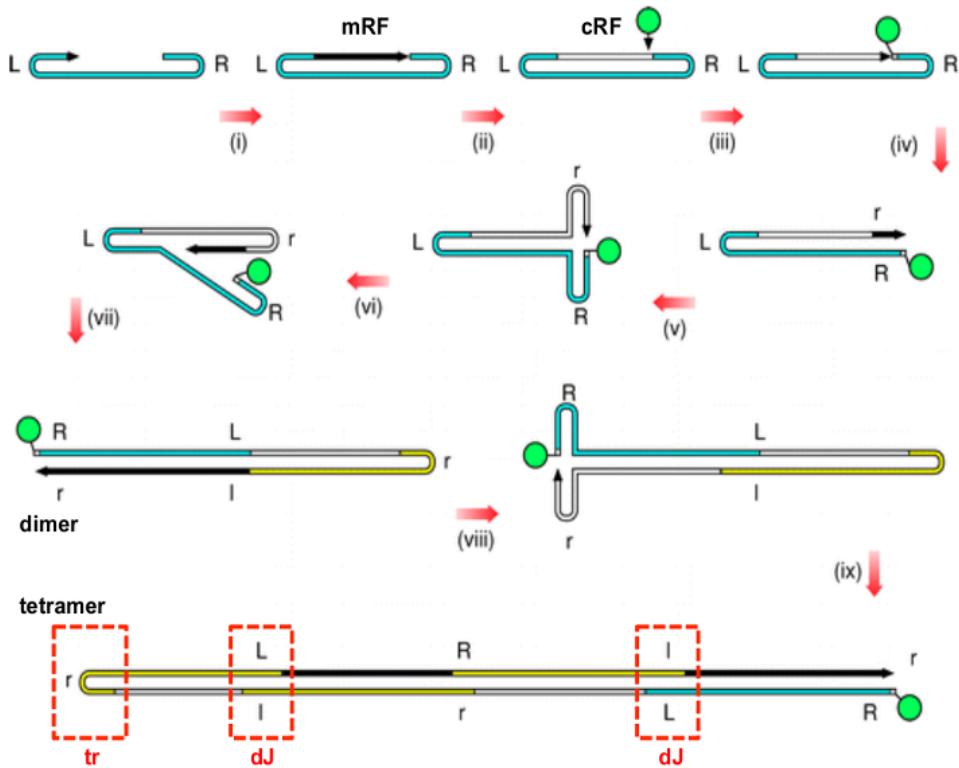
In the initial stage of the RHR, complementary strand synthesis starts from the left-end snap-back telomere, which serves as a primer for the generation of double-stranded monomeric replica-

tive form (mRF) DNA (see step (i) in figure 7.1, p. 33). Subsequently, the growing complementary strand is ligated to the flipped-back right-end telomere by a host ligase, resulting in a covalently continuous RF (cRF) species (see step (ii) in figure 7.1, p. 33)<sup>[82,168]</sup>. This monomer-length turnaround intermediate functions as a transcription template for NS1 expression. NS1 is essential for all further stages of the RHR pathway because the cellular replication machinery is unable to melt, copy, and re-orient the left-end telomere<sup>[16]</sup>. In the first instance, NS1 nicks the right-end telomere (*OriR*) of the cRF intermediate<sup>[288]</sup>, assisted by a host DNA-bending protein from the high-mobility group 1/2 (HMG 1/2) family (see step (iii) in figure 7.1, p. 33)<sup>[73]</sup>. The resulting, liberated, 3' nucleotide at the nick site serves as a platform for the assembly of a new replication fork. NS1 remains covalently attached to the 5' end of the mRF DNA, where it functions as the 3' to 5' replicative helicase<sup>[53,67,118]</sup>. The next step (see step (iv) in figure 7.1, p. 33), called "hairpin transfer", involves reopening and copying of the right-end hairpin sequence in order to generate a right-end extended duplex molecule, replacing the original sequence of the right-end telomere (R) with its inverted complement (r). The two previous steps (iii and iv) of the RHR are commonly referred to as "terminal resolution"<sup>[72]</sup>. In a NS1 dependent reaction, the extended duplex RF is melted and refolded into two hairpins, creating a "rabbit-ear" structure (see step (v) in figure 7.1, p. 33)<sup>[174,289]</sup>. In this way, the path of the replication fork is reversed effectively, redirecting it back along the internal coding sequences (see step (vi) in figure 7.1, p. 33). Finally, this results in the generation of dimeric RF (dRF) and higher-order concatemeric molecules (see steps (vii-ix) in figure 7.1, p. 33), in such a way that the viral coding sequence is replicated twice as frequently as the telomeres. Viral genomes are fused through a single palindromic junction, in either a left-end:left-end or right-end:right-end orientation. In a last step, individual, unit-length, ssDNA genomes are excised and displaced from the concatemeric RF intermediates. Initially, they feed back as new templates into the replicative pool to promote exponential DNA amplification but later they are consumed by encapsidation<sup>[76,78]</sup>.

[13,16,66,259]

## 7.2 Transcription

Parvoviruses generally use a wide variety of alternative RNA processing strategies in order to exploit the strictly limited coding capacity of their small genomes. Alternative splicing of messenger RNA precursors (pre-mRNA) provides a powerful mechanism to generate structurally related but distinct proteins from a single gene, hence contributing to a complex, but efficient and compact genome organization<sup>[187,248]</sup>. The genome of MVM is transcribed in overlapping transcription units from two promoters located at map units 4 and 38, termed P4 and P38, respectively (see figure 7.2 A, p. 36)<sup>[214]</sup>. Products of these promoters are three major transcript classes, R1 (4.8 kb) and R2 (3.3 kb), generated from P4, as well as R3 (2.8 kb), generated from P38<sup>[157]</sup>. All



**Figure 7.1:** Modified rolling hairpin model for MVM DNA replication. The sequence of the parvoviral genome is illustrated by a continuous line, colored blue for the parental genome, yellow for progeny genomes, and black for newly synthesized DNA, the 3' end of which is capped by an arrowhead. The green sphere represents NS1, which nicks the covalently closed monomer (cRF) and remains attached to its 5' end. The letters L and R depict the palindromic sequences at each terminus, with their inverted complements represented by l and r, respectively. Red dashed boxes depict the turnaround (tr) form of the right-end and the dimer junction (dJ) form of the left-end palindrome<sup>[74]</sup>.

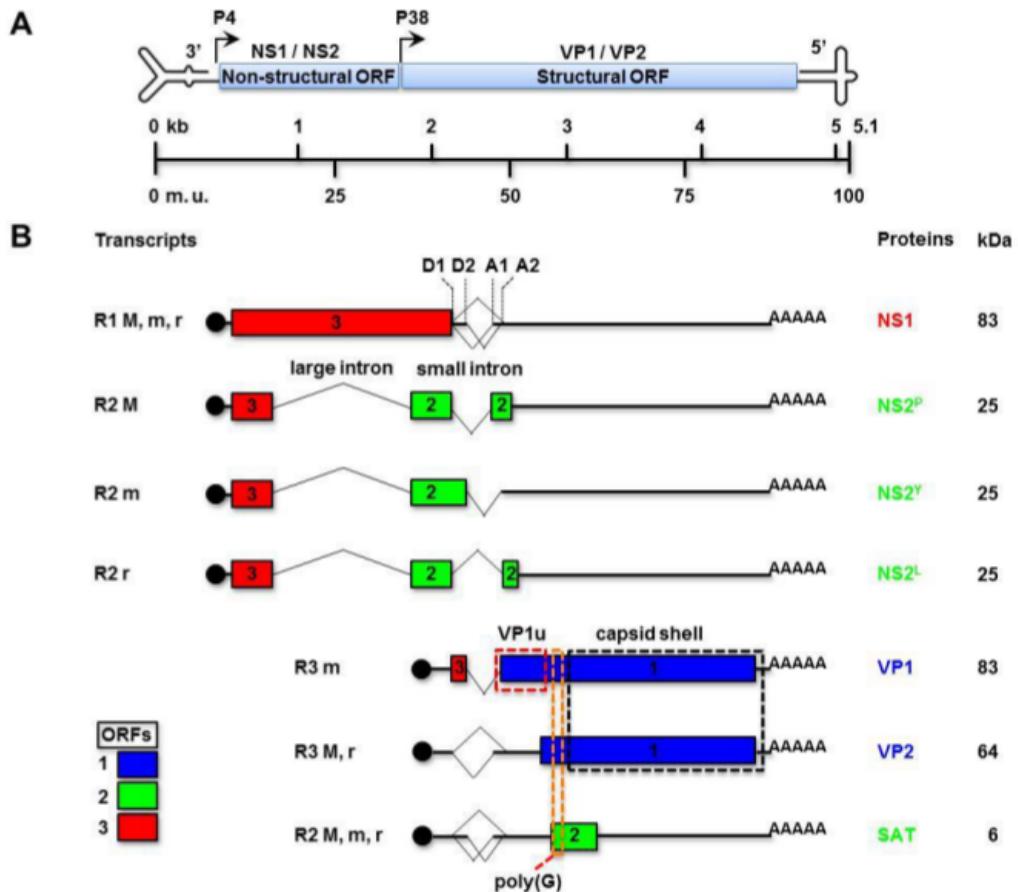
MVM mRNAs are polyadenylated at a single polyadenylation site at the far right-hand end of the genome<sup>[14,58]</sup>. On the one hand, transcripts R1 and R2 encode the viral non-structural proteins NS1 and NS2, respectively, utilizing the open reading frame in the left half of the genome<sup>[65]</sup>. On the other hand, the R3 transcripts encode the overlapping viral capsid proteins VP1 and VP2, utilizing the ORF in the right half of the genome (see figure 7.2 B, p. 36). Additionally, a small alternatively translated (SAT) protein lies embedded within the capsid genes and likewise, is expressed from the P38 promoter<sup>[298]</sup>. Transcription from the viral early and late promoters is accomplished by the host RNA polymerase II<sup>[64,214]</sup> and governed by various cellular transcription factors<sup>[5,101,107,117,215]</sup>.

All MVM pre-mRNAs contain an overlapping set of downstream small introns in the center of the genome (m. u. 44-46) that is alternatively spliced using two donor and two acceptor sites (D1, D2 and A1, A2, respectively)<sup>[60,68,140,194]</sup>. Unique to P4-generated transcripts is an upstream large intron that is located between m. u. 10 and 39. Splicing at this site is required to produce the R2 transcripts which encode the three NS2 protein isoforms<sup>[81,140,214]</sup>. Excision of the large intron is critical in determining the steady state levels of NS1 and NS2<sup>[68,234]</sup>. Because of R1 and

R2 transcripts have similar stabilities<sup>[234]</sup>, and are transported equally to the cytoplasm<sup>[196]</sup>, the ratio of accumulated levels of R1 transcripts relative to R2 directly depends upon the percentage of P4-generated R2 transcripts which lack the large intron. In this way, MVM manages to maintain the optimal balance between the crucial roles which NS1 and NS2 play in viral replication and cytotoxicity<sup>[66]</sup>. On the contrary, alternative splicing of the small intron from P4-generated pre-mRNAs leads to the production of three isoforms of NS2<sup>[60,68,194]</sup> of the one part and the two structural capsid proteins, derived from P38-generated R3 transcripts, of the other part. The joining of donor D1 to acceptor A1 [major, M (~70 %)] produces an mRNA that encodes the major capsid protein VP2, or an mRNA encoding NS2<sup>P</sup> from R3 or R2 transcripts, respectively. Alternatively, joining of D2 to A2 [minor, m (~25 %)] generates an mRNA encoding the minor capsid protein VP1, or an mRNA that encodes NS2<sup>Y</sup> from R3 or R2 transcripts, respectively. Lastly, a rare splicing pattern that joins D1 to A2 [rare, r (~5 %)] is required for the production of NS2<sup>L</sup> encoding mRNAs from R2 transcripts<sup>[14,140,157,194]</sup>. The fourth splicing pattern that joins D2 to A1 is not detected *in vivo*<sup>[194]</sup>, presumably because the distance between this sites (60 nts) is too short to enable successful excision of introns in mammalian cells<sup>[202]</sup>. To date, only a few examples of small overlapping introns with two donors and two acceptors have been described in literature<sup>[115,181,187]</sup>. For MVM, the small central intron, which is excised efficiently from all classes of MVM pre-mRNA transcripts, appears to be the center of attention for entry of the spliceosome. In addition, it dictates the relative amounts of VP1 and VP2 or of the three isoforms of NS2 produced during infection. Splicing of the large upstream intron occurs subsequent to small intron recognition and splicing. This second processing must be slowed in a way that singly spliced RNA can leave the nucleus to encode NS1. This delay most likely is ensured by the large non-consensus donors and acceptors of the splice site of the large intron<sup>[219]</sup>. However, the determinants that govern the alternative excision of the large and the small intron from MVM pre-mRNAs are complex and as yet poorly understood<sup>[109,125,126,299–301]</sup>. Nonetheless, it was demonstrated that wild-type patterns of alternative splicing of MVM pre-mRNAs are achieved exclusively by cellular splicing factors without the involvement of auxiliary viral proteins<sup>[196]</sup>. Moreover, research revealed that polyadenylation of MVM RNAs precedes splicing of the small intron in the nucleus since unspliced polyadenylated molecules can be detected. In contrast, no detectable accumulation of unspliced MVM RNAs were observed in the cytoplasm of infected cells<sup>[59]</sup>. This does not apply for the large intron which is only spliced in a proportion of the pre-mRNAs prior to its export from the nucleus. Once in the cytoplasm, R1 transcripts are prevented from further splicing to R2 transcripts. The determinants that govern export of R1 versus its nuclear retention and further splicing to R2 remain obscure<sup>[219]</sup>. All aforementioned splicing patterns are exemplified in figure 7.2 B, p. 36.

Although viral proteins are not participating in the regulation of alternative splicing, they are indispensable for controlling transcription, besides further relevant cellular transcription factors

and viral *cis*-acting sequences. Interestingly, there is a chronological order to the production of MVM RNA transcripts. It was demonstrated that R1 and R2, the P4-generated pre-mRNAs, precede the P38-generated R3 transcripts during synchronous infection<sup>[59]</sup>. This temporal phasing is the result of NS1-dependent up-regulation of transcription from the P38 promoter<sup>[97,221]</sup>. The acidic C-terminal domain of NS1 acts as a classical transcriptional activator that can potentiate P38 transcription approximately 100 fold<sup>[162]</sup>. In this way, the non-structural proteins, particularly NS1 that is essential for MVM DNA replication (see section 7.1, p. 31), are available prior to the structural capsid proteins in order to initiate early events in parvoviral infection and to stimulate the transcription of the VP and SAT genes under the control of the late P38 promoter. An example for viral *cis*-acting sequences that regulate infection represents the left-end hairpin, where both transcription and replication factors compete for specific recognition elements distal to the bubble sequence. Binding of CRE to this sequence has been shown to contribute to basal levels of P4 activity and to the up-regulation of P4 activity in transformed cells<sup>[103,212]</sup>. The latter is believed to be one of the dominant mechanisms allowing MVM-mediated oncolysis (ref.....Oncolysis??). CRE binding overlaps with the distal of the two 5'-ACGT-3' half sites needed to bind PIF (see figure 6.1 C, p. 30) which is essential for stabilizing NS1 binding to the active left-end origin (*OriLTC*) for replication initiation (see section 6, p. 27)<sup>[53]</sup>. Therefore, these two processes, replication and transcription, are in competition with each other in order to inter-coordinate viral infection.



**Figure 7.2:** Transcription map of MVM. (A) The single-stranded, negative-sense DNA genome of MVM is illustrated by a single line terminating in dissimilar hairpin telomeres. The two major ORFs are boxed in light blue and the proteins which they encode are indicated above. The two viral promoters, P4 and P38 are shown by rightward arrows. Below, arbitrary map units are diagrammed relative to the 5.1 kb genome. (B) The three major cytoplasmic transcript classes R1, R2, and R3 are displayed. A black sphere indicates the capped 5' ends and (AAAAA) denotes their polyadenylated tails near the far right-hand end of the genome. ORFs encoding the viral proteins, named on the right, are displayed in different coloring according to their reading phase. Their spliced-out large or small introns are indicated by thin-lined caretts. The small intron is excised from each transcript class by the alternative use of three different splicing patterns, denoted M (major), m (minor), and r (rare). Splice donor and acceptor sites for splicing of the small intron are denoted D1, D2 and A1, A2, respectively. On the one hand, alternative splicing of the small intron generates the R3 transcripts encoding VP1 and VP2, the two structural capsid proteins, and the R2 transcripts encoding three C-terminally distinct isoforms of NS2, referred to as NS2<sup>P</sup>, NS2<sup>Y</sup>, and NS2<sup>L</sup>. On the other hand, excision of the large intron is critical in determining the steady state levels of NS1 and NS2 transcripts. The N-terminal protein sequence boxed in red represents VP1u which harbors the PLA<sub>2</sub> motif that is involved in entry functions. Sequences boxed in black, comprising the C-terminal region common to all VP polypeptides, assemble to form the capsid shell. Poly(G), boxed in orange, identifies a short glycine-rich region present in all VPs that can be modeled into X-ray density occupying the fivefold pores in virions. This figure was adapted from [80].

## **Part II**

# **Methods**



# 8 Methods

## 8.1 Cell Cultures

A9 ouab<sup>r</sup>11 cells, a derivative from the original HGPRT<sup>-</sup> L-cell line A9 represent a clone resistant to 10<sup>-3</sup> M ouabain after nitrosoguanidine mutagenesis<sup>[166]</sup>. NB324K cells are a clone of SV40-transformed human newborn kidney cells<sup>[244]</sup>. The SV40 large T antigen was detected by immunofluorescent staining with monoclonal antibodies<sup>[122]</sup>. However, NB324K cells do not produce infectious SV40 spontaneously. Both cell lines, A9 mouse fibroblasts and NB324K cells, were routinely propagated under a minimal number of passages in DMEM supplemented with 5 % of heat inactivated fetal bovine serum at 37 °C in 5 % CO<sub>2</sub> atmosphere.

### 8.1.1 Freezing and thawing of cells

Before use the A9 mouse fibroblasts or NB324K cells were thawed at 37 °C and cultured in 5 mL of pre-warmed DMEM supplemented with 5 % FCS. The medium was replaced every 3 to 4 days. In order to freeze the cells for long storage in liquid nitrogen they were passed the day before, to ensure exponential growth. Subsequently, 7.5 % DMSO was added and the cells were frozen slowly at -70 °C over night before transfer to liquid nitrogen.

## 8.2 Virus Stocks

Stocks of MVM without detectable levels of VP3 were propagated on A9 mouse fibroblast monolayers. As soon as the cytopathic effect became evident, the supernatant was collected and pre-cleared from cell debris by low-speed centrifugation. Thereby, intracellular, VP3 rich capsids were discarded. In order to remove low-molecular contaminants, virus containing SN was pelleted through 20 % sucrose cushion in PBS by ultra-centrifugation. Virus titers were determined by qPCR as DNA-packaged particles per microliter.

### 8.2.1 Separation of empty and full capsids

Sucrose purified capsids were prepared as previously described in section 8.2, page 39. The virus pellet was resuspended in 10 mL PBS. Caesium chloride was added to a density of 1.38 g/mL adjusted by refractometry ( $\eta=1.371$ ) at 4 °C. The gradient was centrifuged to equilibrium for 24

h at 41000 rpm and 4 °C in a Beckmann SW-41 Ti rotor. Gradients were fractionated and tested for intact capsids by dot blot analysis using B7 mAb. CsCl was depleted from the corresponding fractions by size-exclusion chromatography through PD-10 desalting columns and the capsids were concentrated in Amicon® centrifugal filter devices when required.

### 8.3 Freezing bacteria stocks in glycerol

Bacteria were frozen in dry ice. A volume of 700 µL of the bacteria culture that was grown over night in LB-medium was mixed with 300 µL of 50 % glycerol in a cryotube. In order to mix well the glycerol the cryotube was vortexed intensively. Following snap-freeze in dry ice the bacteria were stored at -70 °C.

### 8.4 Anion-exchange chromatography

A Mono Q HR 5/5 (Pharmacia) column (5 x 50 mm) was used to analyse viral samples. The Mono Q column was connected to the ÄKTAmicro chromatography system (GE Healthcare) that was operated by the UNICORN control software. The Mono Q column was equilibrated with six column volumes (CV) starting buffer (20 mM Tris-HCl, 1 mM EDTA, pH 7.2). Samples (1 mL) containing at least  $10^{10}$  virus particles in 10 mM Tris-HCl, 1 mM EDTA, pH 8 were applied to the Mono Q column trough a 2 mL loop. After eluting the protein, which did not bind to the column in the starting buffer, a linear salt gradient (0-2 M NaCl) in 20 mM Tris-HCl, 1 mM EDTA, pH 7.2, was applied. Fractions of 0.185 mL were collected in 96-well plates. Viral genomes in each fraction were quantified by qPCR.

Occasionally, the Mono Q column needed to be washed. Increased back-pressure, colour change at the top of the column, decreased sample recoveries, or loss of resolution indicates that the column matrix requires regeneration. In order to circumvent such problems, the column was washed every tenth run. To elute contaminants that tightly stick to the column the following harsh conditions were applied to the reversed (bottom to top) Mono Q column. 500 µL 2 M NaCl solution was injected and subsequently, the column was rinsed with water. Then, 500 µL 2 M NaOH solution was injected and the column was rinsed with water. Finally, 500 µL 75 % acetic acid was injected before the column was re-equilibrated with starting buffer.

All buffers were filtered and degassed before application to the Mono Q column.

### 8.5 Quantitative PCR

Amplification of MVM DNA and real-time detection of PCR products were performed by using BioRad CFX96 technology with SYBR green supermix. PCR was carried out by using the hot-start iTaq™ DNA polymerase (Bio-Rad Laboratories) following the manufacturer's guide-lines.

Viral DNA was isolated using DNeasy blood and tissue kit. Elution of the purified vDNA was carried out using 100  $\mu$ L elution buffer. As templates 2  $\mu$ L of the isolated viral DNA were used for the PCR reaction and were added to the following master mix:

<b>Component</b>	<b>Amount</b>	<b>Final concentration</b>
dH <sub>2</sub> O, PCR grade	6 $\mu$ L	-
Forward primer, 10 pM	1 $\mu$ L	0.5 pM
Reverse primer, 10 pM	1 $\mu$ L	0.5 pM
2x IQ™ SYBR® Green Supermix	10 $\mu$ L	1x
<b>Total volume</b>	<b>18 <math>\mu</math>L</b>	

**Table 8.1:** Master mix for quantitative PCR. In order to minimize pipetting errors a master mix was prepared. Following preparation the master mix was distributed across the 96 well plates. The master mix contains all the ingredients which are required for the DNA amplification except the initial DNA template that differs among the samples.

To ensure accurate quantification, the 96-well plates containing master mix and template DNA were shortly spun and transferred into the BioRad CFX96 unit. The following PCR program was used for quantification of viral DNA:

<b>Cycles</b>	<b>Step</b>	<b>Temperature</b>	<b>Time</b>
1x	Initial denaturation	95 °C	300 s
40x	Denaturation	95 °C	15 s
	Annealing	61 °C	15 s
	Extension	72 °C	15 s
1x	Final denaturation	95 °C	60 s
1x	Melting curve	65 °C up to 95 °C	0.1 °C/s

**Table 8.2:** PCR conditions for the amplification and real-time detection of MVM DNA.

To provide standards for sample quantification, serially diluted plasmids containing the entire MVM genomic DNA were used. For cell number variations that may exist between the samples, the number of applied cells per PCR reaction needed to be quantified for normalization as well. For this purpose quantification of cellular  $\beta$ -actin gene was performed. After normalization, direct comparison of the results is possible.  $\beta$ -actin quantification was carried out with the same PCR conditions outlined in table 8.2, 41.

## 8.6 Immunoprecipitation

Either *in vitro* treated viruses or viruses from cell extracts were transferred to LoBind tubes that were pre-blocked with PBS containing 1 % bovine serum albumin (PBSA 1 %). The volume was adjusted to 200  $\mu$ L with PBSA 1 %. The antibody was added in excess and incubated with the viral capsids for 1 h at 4 °C on a rotary shaker. Subsequently, 20  $\mu$ L protein G-agarose beads

were added. Following overnight incubation at 4 °C and centrifugation at 2500 rpm for 5 min the supernatant was discarded. The beads were washed 4 times with PBSA 1 %. To remove the BSA an additional wash step was carried out with PBS. Finally, the beads were frozen at -20 °C until further use or immediately processed.

## 8.7 Dot Blot

Viruses ( $10^8$  in 2  $\mu\text{L}$ ) were spotted on a nitrocellulose membrane. The membrane was blocked for 20 min with TBST containing 5 % milk. The primary antibody was diluted in TBST supplemented with 1 % milk and incubated for 30 min at room temperature. Unbound antibody was removed by washing the membrane 3 times for 5 min with TBST containing 1 % milk. The HRP-coupled secondary antibody was diluted 1:20000 in TBST supplemented with 1 % milk and added to the membrane for 30 min. Excess secondary antibody was removed by the same procedure as aforementioned for the primary antibody. The membrane was developed by exposure to photo films.

## 8.8 SDS-PAGE and Western blotting

Immunoprecipitated capsids were dissolved in 20  $\mu\text{L}$  protein loading buffer (reference) containing 2 % SDS and 10 % glycerol. The samples were boiled at 96 °C for 8 min. Viral proteins were separated through a NuPAGE® 10 % Bis-Tris Gel (Invitrogen). The XCell Sure Lock™ Electrophoresis Cell (Invitrogen) was used to separate the proteins. The gel was first run at 30 V for 10 min to stack the proteins. In this way, sharper bands could be achieved. Separation of the different proteins was accomplished at 200 V. Following separation, the proteins were blotted on a methanol activated, porous, 0.2  $\mu\text{m}$  polyvinylidene fluoride (PVDF) Immobilon® Transfer Membrane (EMD Millipore). Blotting was carried out at 30 V for 1 h 10 min using XCell II™ Blot Module (Invitrogen). The membrane was blocked in TBS-T buffer (reference) supplemented with 5 % milk overnight at 4 °C. Subsequently, the membrane was probed with a polyclonal rabbit antibody against linear MVM-VP epitopes that was diluted 1:2000 in 3 mL TBS-T containing 1 % milk. The first antibody (reference) was incubated for 1 h at RT. The PVDF membrane was washed in TBS-T for a total 90 min with many buffer replacements. Subsequently, the horseradish peroxidise conjugated secondary antibody (goat  $\alpha$ -rabbit-HRP, reference) was added for 1 h at RT. This secondary goat anti-rabbit antibody was diluted 1:20000 in TBS-T supplemented with 1 % milk. To deplete remaining antibodies, the membrane was washed in the same way as described above except for a final wash step with TBS (reference). VP1, VP2, and possibly VP3 were visualized by a chemiluminescence system (SuperSignal West Dura Extended Duration Substrate, reference) following the manufacturer's instructions. Following this treatment, the PVDF membrane was exposed to a film (Amersham Hyperfilm™ ECL, reference). Finally, the

film was developed using Anatomix Developer Replenisher Solution and Fixer and Replenisher Solution (reference).

## **8.9 Chymotrypsin treatment**

Virus particles were incubated with 0.5 mg/mL chymotrypsin (Sigma) in PBS for 1.5 h at 37 °C. The reaction was stopped by adding 100 µM chymostatin (Sigma). Negative controls were incubated in the same buffer for the same time.

### **8.9.1**

### **8.10**

#### **8.10.1**

#### **8.10.2**



# **Part III**

# **Publication**



# **1 Wolfisberg et al., Journal of Virological Methods, 2013**

**Impaired genome encapsidation restricts the *in vitro* propagation of human parvovirus B19.**

Raphael Wolfisberg, Nico Ruprecht, Christoph Kempf and Carlos Ros



## Impaired genome encapsidation restricts the *in vitro* propagation of human parvovirus B19



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### ABSTRACT

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The lack of a permissive cell culture system hampers the study of human parvovirus B19 (B19V). UT7/Epo is one of the few established cell lines that can be infected with B19V but generates none or few infectious progeny. Recently, hypoxic conditions or the use of primary CD36+ erythroid progenitor cells (CD36+ EPCs) have been shown to improve the infection. These novel approaches were evaluated in infection and transfection experiments. Hypoxic conditions or the use of CD36+ EPCs resulted in a significant acceleration of the infection/transfection and a modest increase in the yield of capsid progeny. However, under all tested conditions, genome encapsidation was impaired seriously. Further analysis of the cell culture virus progeny revealed that differently to the wild-type virus, the VP1 unique region (VP1u) was exposed partially and was unable to become further externalized upon heat treatment. The fivefold axes pore, which is used for VP1u externalization and genome encapsidation, might be constricted by the atypical VP1u conformation explaining the packaging failure. Although CD36+ EPCs and hypoxia facilitate B19V infection, large quantities of infectious progeny cannot be generated due to a failure in genome encapsidation, which arises as a major limiting factor for the *in vitro* propagation of B19V.

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### 1. Introduction

Human parvovirus B19 (B19V) is spread worldwide and typically causes a mild self-limiting infection in children known as *erythema infectiosum*. B19V has also been associated to myocarditis, acute and chronic arthropathies in adults, transient aplastic crisis and chronic anemia in individuals with altered immunologic or hematologic conditions, hydrops fetalis and intrauterine fetal death (Heegaard and Hornsleth, 1995; Heegaard and Brown, 2002; Survey et al., 2007).

Considering its worldwide distribution, prevalence and associated disorders, B19V is regarded as a prominent human pathogen and the only parvovirus undoubtedly linked to human disease. However, the experimental research with B19V is hampered seriously due to the lack of an appropriate and sufficiently permissive cell system to propagate the virus and study its biology. The reason for this is the rigorous replication requirements of the virus. B19V has an extraordinary tropism for erythroid progenitor cells in the bone marrow at a particular differentiation stage corresponding to BFU-E and CFU-E (Takahashi et al., 1990; Ozawa et al.,

1986, 1987). The narrow tropism of B19V is mediated, at least in part, by its particular uptake mechanism. B19V utilizes globoside (Gb4Cer) as a primary attachment receptor, which is expressed in few cell types (Brown et al., 1993) and a co-receptor (Weigel-Kelley et al., 2003) to initiate the internalization process. However, cells expressing the required receptors and co-receptors are not always permissive, suggesting that the selective replication of B19V is determined by additional intracellular factors restricted to erythroid cells (Pallier et al., 1997; Bruneck et al., 2000; Gallinella et al., 2000; Guan et al., 2008; Chen et al., 2010; Luo et al., 2011). The high viremia that is typically associated to B19V acute infections, exceeding occasionally  $10^{13}$  genome equivalents (geq) per ml of plasma (Kooistra et al., 2011), suggests that the virus can replicate efficiently in the target cells when all the required elements are present. However, despite continuous efforts, the specific cellular factors that control B19V infection in the natural target cells have not yet been reproduced adequately in an established cell line. Some erythropoietin-dependent leukemic cell lines, notably UT7/Epo (Shimomura et al., 1992) and KU812Ep6 (Miyagawa et al., 1999), have been shown to be semi-permissive to B19V infection, producing in general none or minor amounts of infectious progeny. The permissivity of non-erythroid cells, such as HepG2 cells has produced contradictory results (Caillet-Fauquet et al., 2004a; Bonvicini et al., 2008). Considering all these limitations, highly viremic donors without B19V neutralizing antibodies remain the only source of infectious B19V. Thus, the need to develop

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a cell culture method capable of producing large amounts of infectious B19V remains a major challenge.

Recently, the use of cells cultured under hypoxic conditions has been described as a promising method to produce high quantities of infectious particles (Caillet-Fauquet et al., 2004b; Pillet et al., 2004; Chen et al., 2011). Similarly, the use of *ex vivo* expanded CD36+ primary human erythroid progenitor cells (CD36+ EPCs), previous CD34+ *in vitro* preselection (Pillet et al., 2008; Wong et al., 2008), has also been described as a highly permissive system, based on the expression of B19V non-structural and capsid proteins. A simplified approach to generate CD36+ EPCs directly from ordinary blood samples, without *ex vivo* stem cell mobilization has been reported (Filippone et al., 2010). The combination of both approaches, primary CD36+ EPCs cultured under hypoxic conditions, has been shown to enhance remarkably B19V infection (Chen et al., 2011). Hypoxia, which mimics the oxygen microenvironment in the bone marrow, seems to promote B19V infection by the direct stimulating effect of HIF1 $\alpha$  on the B19V p6 promoter (Pillet et al., 2004). However, an alternative HIF1 $\alpha$ -independent mechanism based on STAT5A and MEK signaling has been proposed recently (Chen et al., 2011).

These novel approaches based on hypoxia and primary CD36+ EPCs have been compared systematically in infection and transfection experiments with the established erythroid cell line UT7/Epo. In all cases, a substantial amount of capsid progeny was obtained. The use of the novel approaches resulted in a significant acceleration of the infection and the augmentation in the number of infected cells resulting in a modest but noticeable increase in virus progeny production. However, in all tested cells and under all conditions genome encapsidation was impaired seriously generating an empty non-infectious virus progeny. Differently to the wild-type virus, the VP1 unique region (VP1u) of the virus progeny was exposed partially and upon heat treatment did not undergo the expected conformational change that renders VP1u fully externalized. The abnormal configuration and rigidity of VP1u, which utilizes the genome encapsidation portal for its externalization, might constrict the fivefold axes channel impeding the translocation of the viral genome into the pre-assembled capsid.

## 2. Materials and methods

### 2.1. Cells and viruses

UT7/Epo cells were maintained in Eagle's minimum essential medium (MEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) and 2 U/ml of recombinant human erythropoietin (Epo; Janssen-Cilag, Midrand, South Africa) at 37 °C with 5% CO<sub>2</sub>. For hypoxic conditions the oxygen tension was lowered to 1%. Cells with adherent phenotype were selected by removing the non-adherent cells in every passage. CD36+ erythroid progenitor cells (CD36+ EPCs) were obtained from ordinary blood samples and cultured as described previously (Filippone et al., 2010). A B19V-infected plasma sample (Genotype 1; CSL Behring AG, Charlotte, NC), without detectable B19V-specific IgM or IgG antibodies, was used as a source of native infectious virus. The virus was pelleted by ultracentrifugation through 20% (w/v) sucrose and the concentration of virions was determined by quantitative PCR (qPCR).

### 2.2. Antibodies and chemicals

Two human monoclonal antibodies (mAb), one directed to a conformational epitope in the major capsid protein VP2 (mAb 860-55D), which detects exclusively intact capsids, and the other against the N-terminal region of VP1, also known as VP1 unique region (VP1u) (mAb 1418), were provided by S. Modrow

(Regensburg, Germany). These antibodies were produced from peripheral blood mononuclear cells of normal, healthy individuals with high titers of serum antibodies against B19 virus proteins (Gigler et al., 1999). A rabbit antibody against the C-terminal region of VP1u was described earlier (Bönsch et al., 2008). A mouse mAb against B19V capsids (mAb 521-5D) was purchased from Millipore (Billerica, MA). A globoside-specific mouse IgM mAb (AME-2) was provided by J. de Jong (The Netherlands Red Cross, Amsterdam, Netherlands). Mouse IgG mAb against Ku80 and CD49e were purchased from BD Biosciences (San Jose, CA). A mouse antibody against B19V proteins was obtained from US biologicals (Swampscott, MA). Chloroquine diphosphate (CQ) was purchased from Sigma (St. Louis, MO) and dissolved in water.

### 2.3. Flow cytometry

The presence of B19V receptors and co-receptors on the cell surface of UT7/Epo cells was analyzed quantitatively by flow cytometry. UT7/Epo cells were incubated with either an anti-Ku80 or an anti-Gb4Cer antibody at 4 °C for 1 h in PBS containing 2% fetal calf serum, followed by incubation with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgG or IgM, respectively (BD Biosciences). Additionally, UT7/Epo cells were stained with R-phycocerythrin conjugated anti-human CD49e (BD Biosciences). The cells were analyzed on a BD FacsCanto II (Becton Dickinson, San Jose, CA). Data acquisition and analysis were conducted with a software (BD FacsDivा, BD Biosciences).

### 2.4. Infection

UT7/Epo and primary CD36+ EPCs ( $3 \times 10^5$ ) cultured under normoxia or hypoxia (1% O<sub>2</sub>) during 2 days, were infected with B19V at  $10^4$  geq per cell for 1 h at 4 °C. The cells were washed to remove unbound viruses and further incubated at 37 °C. At different post-infection (p.i.) times, cells and supernatants were collected. The cells were washed and processed for immunofluorescence (IF), immunoprecipitation (IP), as well as DNA and RNA extraction. The supernatant was used for IP and DNA extraction.

### 2.5. Transfection

A total of  $5 \times 10^6$  UT7/Epo cells, cultured under normoxia or hypoxia (1% O<sub>2</sub>) during 2 days, were transfected using the AMAXA nucleofector™ II device (Lonza, Cologne, Germany) following the manufacturer's instructions. Transfection was carried out with 5 µg of the B19V genome excised from a B19V infectious clone (pB19-M20) (Zhi et al., 2004) or with 2 µg of a GFP-control plasmid, using the T-20 program. As a transfection reagent, AMAXA™ Cell Line Nucleofector™ Kit R (Lonza) was used. After transfection, the cells were maintained in 20 ml of pre-warmed culture medium. A volume of 5 ml of fresh MEM culture medium supplemented with 5% FCS and Epo (2 U/ml) was added to the cells 24 h post-transfection (p.t.). At increasing times p.t., the cells and supernatant were collected for further analysis.

### 2.6. Quantitation of B19V DNA and NS1 mRNA

Total DNA was extracted from cells or from the supernatant by using the DNeasy blood and tissue kit (Qiagen, Valencia, CA). For the isolation of total mRNA, cells were transferred to RNase-free tubes (Safe-Lock Tubes 1.5 ml, Eppendorf Biopur) and washed twice with PBS. Total poly-A-mRNA was isolated with the Dynabeads mRNA direct kit (Roche Diagnostics, Mannheim, Germany). The RNA preparations were used for reverse transcription as described previously (Bönsch et al., 2010a). Amplification of DNA or cDNA and real-time detection of PCR products were performed by qPCR

with the iQ SYBR Green Supermix and the CFX96 device (Bio-Rad, Cressier, Switzerland). Primers used for amplification were described elsewhere (Bönsch et al., 2010a).

### 2.7. Immunoprecipitation of B19V particles and quantitation of virions

Viral particles were immunoprecipitated from cell extracts or from the supernatant of infected cells with a human mAb against intact capsids (860-55D) (Gigler et al., 1999). As reference control, a known amount of virions was added to the uninfected cell extracts or to the supernatant. After overnight incubation at 4 °C in the presence of 20 µl of protein G agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany) the supernatant was discarded and the beads were washed four times with PBSA. Immunoprecipitated viral capsids were detected by SDS-PAGE. To verify the presence of the viral genome, DNA was extracted from the immunoprecipitated virions by using the DNeasy blood and tissue kit (Qiagen) and quantified as specified above.

### 2.8. Immunofluorescence

Cells or purified viruses were fixed on coverslips by using acetone/methanol (1:1 [v/v]) solution at -20 °C for 4 min. Following blocking with goat serum diluted in PBS (20% [v/v]), the samples were incubated with the primary antibodies in PBS containing 2% goat serum for 1 h at room temperature (RT). The samples were washed and the appropriate fluorescently labeled secondary antibody in 2% goat serum was added for 1 h at RT. Nuclei was stained with 4',6-diamidino-2-phenylindole (DAPI). Mowiol supplemented with 2.5% 1,4-Diazabicyclo[2.2.2]octan (DABCO) was used to maintain the fluorescent signal. Samples were examined by confocal laser scanning microscopy (Axiovert 200M, Carl Zeiss A.G., Feldbach, Switzerland).

### 2.9. Fluorescence *in situ* DNA hybridization

The presence of newly replicated viral genomes in the infected cells was examined by fluorescence *in situ* DNA hybridization (FISH). Biotinylated probes specific for B19V DNA were generated from PCR products by nick translation (Roche), according to the manufacturer's instructions. The size of the hybridization probes was 200–500 nucleotides in length, as confirmed by agarose gel electrophoresis. Cells were fixed and immunostained with mAb 860-55D against capsids and incubated in a humid chamber at 37 °C for 18 h with a volume of 20 µl hybridization mix (5 ng/µl biotinylated probe in 60% deionised formamide, 300 mM NaCl, 20 mM sodium citrate, 10 mM EDTA, 25 mM NaH<sub>2</sub>PO<sub>4</sub> pH 7.4, 5% dextran sulfate and 250 ng/µl sheared salmon sperm DNA). Subsequently, the cells were washed (50% deionized formamide, 25 mM NaCl and 2.5 mM sodium citrate pH 7.4) three times for 5 min at RT and once at 37 °C. The samples were blocked for 30 min with 1% blocking solution (Roche) in 150 mM NaCl, 100 mM Tris-HCl pH 7.4. Biotin was detected with avidin-rhodamine (Roche) 1:500 in blocking solution for 45 min. Finally, the cells were washed three times 10 min (200 mM Tris-HCl pH 7.4, 1.5 M NaCl and 0.05% Tween-20), mounted with mowiol supplemented with DABCO and examined by confocal laser scanning microscopy.

## 3. Results

### 3.1. General profile of B19V infection in UT7/Epo cells

UT7/Epo cells have been used extensively to study B19V infection. However, intracellular factors restrict severely the infection of B19V in these and other cells, resulting in the production of

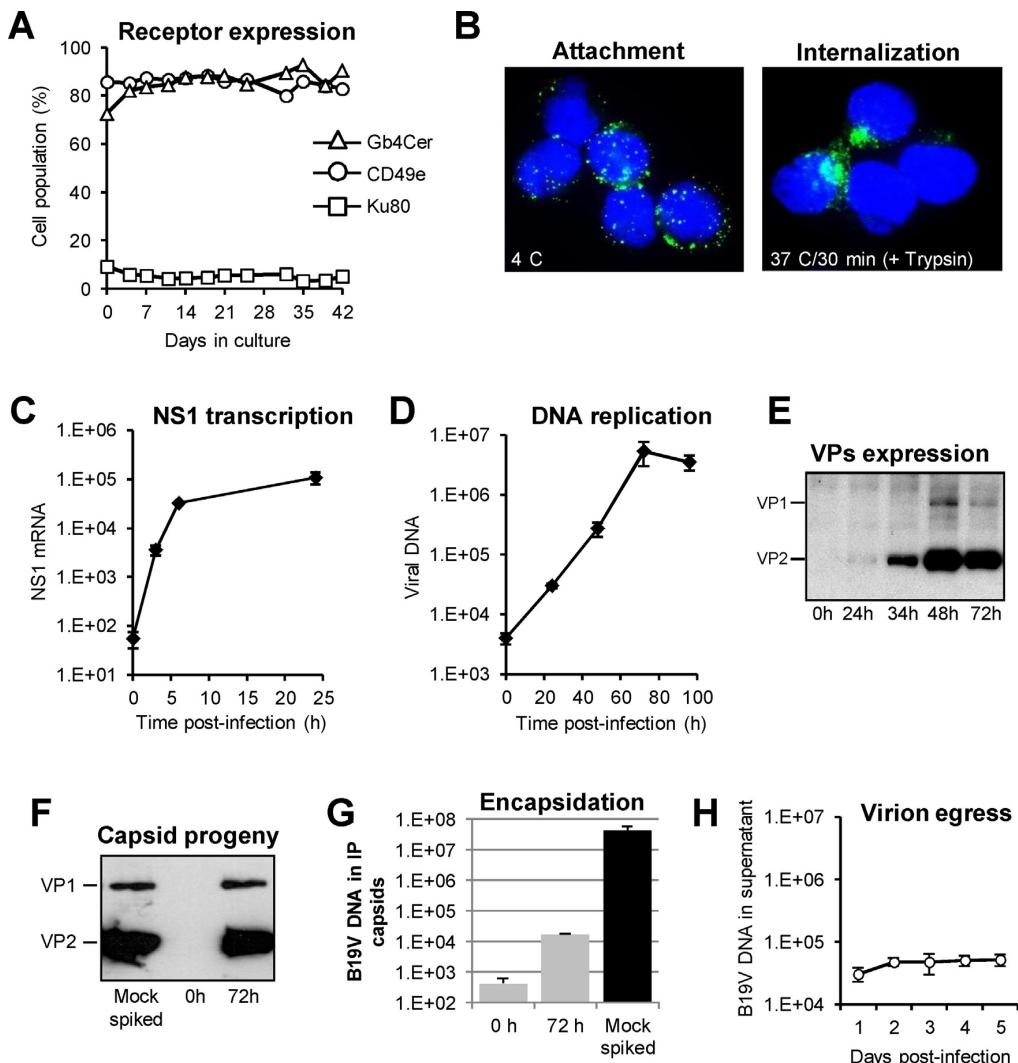
none or few infectious progeny (Pallier et al., 1997; Brunstein et al., 2000; Gallinella et al., 2000; Guan et al., 2008). In order to better identify which steps of the infection are deficient, different parameters of B19V infection in UT7/Epo cells have been analyzed. Analysis of the expression profile of B19V receptor and co-receptors over a period of six weeks showed a high and stable expression of Gb4Cer and CD49e along the specified period. In contrast, expression of Ku80, which may have a similar role to Gb4Cer in certain cells (Munakata et al., 2005), was not significant (Fig. 1A). IF microscopy examination of infected cells confirmed that B19V can attach and internalize cells, adopting the typical intracellular distribution around the microtubule organizing center (MTOC) observed in other parvovirus infections (Fig. 1B). The kinetics of viral transcription and replication were analyzed quantitatively. The synthesis of viral RNA (NS1 mRNA) was already detectable by 3 h p.i. and reached a plateau by 24 h p.i. (Fig. 1C). Viral replication started later and reached a plateau by the third day p.i. (Fig. 1D). Expression of viral proteins became detectable after 24 h and reached a plateau after 2 days (Fig. 1E). Immunoprecipitation at 3 days p.i. with an antibody against intact viral particles (mAb 860-55D) (Gigler et al., 1999) demonstrated that virus assembly occurred and that a significant amount of capsid progeny was produced (Fig. 1F). Quantitative determination of the viral DNA from the immunoprecipitated capsids revealed that the virus progeny was essentially empty (Fig. 1G). Mature virion progeny was not either detected in the supernatant of the infected cells (Fig. 1H). These results together indicate that despite the substantial amount of capsid progeny produced, deficiencies in genome packaging and capsid egress limit the progression of B19V infection in UT7/Epo cells.

### 3.2. B19V infection of UT7/Epo cells, under normoxia or hypoxia, generates mostly empty capsids

Infected cells were collected at progressive days, washed and lysed. Viral particles were immunoprecipitated from the cell lysate with the antibody 860-55D, against assembled capsids. The results confirmed that under hypoxic conditions, the capsid progeny was more abundant but also appeared earlier (after 48 h p.i. under normoxia and after 24 h p.i. under hypoxia) (Fig. 2A and B). These results confirmed previous observations indicating that hypoxia enhances B19V infection (Cailliet-Fauquet et al., 2004b; Pillet et al., 2004; Chen et al., 2011). The virus progeny generated under hypoxic or normoxic conditions was further characterized. The amount of viral genomes in the immunoprecipitated viral particles from the experiment shown in Fig. 2A and B was analyzed quantitatively. The results revealed that independently of the oxygen environment, a limited number of progeny capsids (<1% of the reference control) contained the viral DNA (Fig. 2C and D). Quantitation of the viral DNA in the supernatant of the infected cells showed no increase over the background signal (day 0 p.i.) under normoxia and modestly under hypoxic conditions (Fig. 2E and F). Capsid proteins in the supernatant were undetectable by IP and Western blot (data not shown). These results indicate that although hypoxic conditions result in the acceleration of the infection and an augmented capsid production, the improvement of the genome encapsidation step was not significant.

### 3.3. Hypoxia enhances significantly the transfection efficiency, however genome packaging and egress remained restricted

In a control transfection experiment in UT7/Epo cells, the oxygen level did not influence the transfection efficiency with a control plasmid expressing green fluorescent protein (GFP) (Fig. 3A). However, the transfection efficiency increased drastically under hypoxic conditions with an infectious clone of B19V (pB19-M20)



**Fig. 1.** Characterization of B19V infection in UT7/Epo cells. (A) Expression of B19V-related receptors in UT7/Epo cells. The presence of B19V receptors and co-receptors on the cell surface of UT7/Epo cells was quantitatively analyzed by flow cytometry during a period of 6 weeks. (B) Binding and internalization of B19V in UT7/Epo cells. B19V was added to the cells at 4 °C for 2 h, washed, fixed and stained with an antibody against intact capsids. For internalization, the cells were further incubated for 30 min at 37 °C, washed and trypsinized to remove uninternalized particles. (C) Kinetics of NS1 mRNA synthesis in infected cells. At increasing times p.i., total mRNA was isolated and NS1 mRNA quantified. Samples taken 10 min p.i. served as background controls. (D) Kinetics of viral DNA replication. At increasing times p.i., total DNA was isolated and viral DNA quantified. Samples taken prior to virus internalization served as background controls. (E) Kinetics of B19V capsid proteins expression. (F) Production of assembled capsid progeny in UT7/Epo cells. B19V capsids were immunoprecipitated from cell extracts with mAb 860-55D against intact capsids. As a reference control, B19V ( $4 \times 10^{10}$ ) was added to mock-infected cell extracts. (G) B19V capsids were immunoprecipitated and B19V DNA was quantified. As a reference control, B19V ( $4 \times 10^{10}$  virions) was added to mock-infected cell extracts. (H) Quantitation of virus egress. B19V DNA was quantified from the supernatant of the infected cells.

(Fig. 3B). Immunoprecipitation experiments confirmed that assembled capsids were generated (Fig. 3C) and similarly to the infection experiments, progeny capsids were slightly more abundant and appeared earlier under hypoxic conditions.

As shown in Fig. 3D, at progressive times p.t. no viral DNA above the input signal was detected in the immunoprecipitated capsids. Additionally, virions were not detectable in the supernatant of the transfected cells (Fig. 3E).

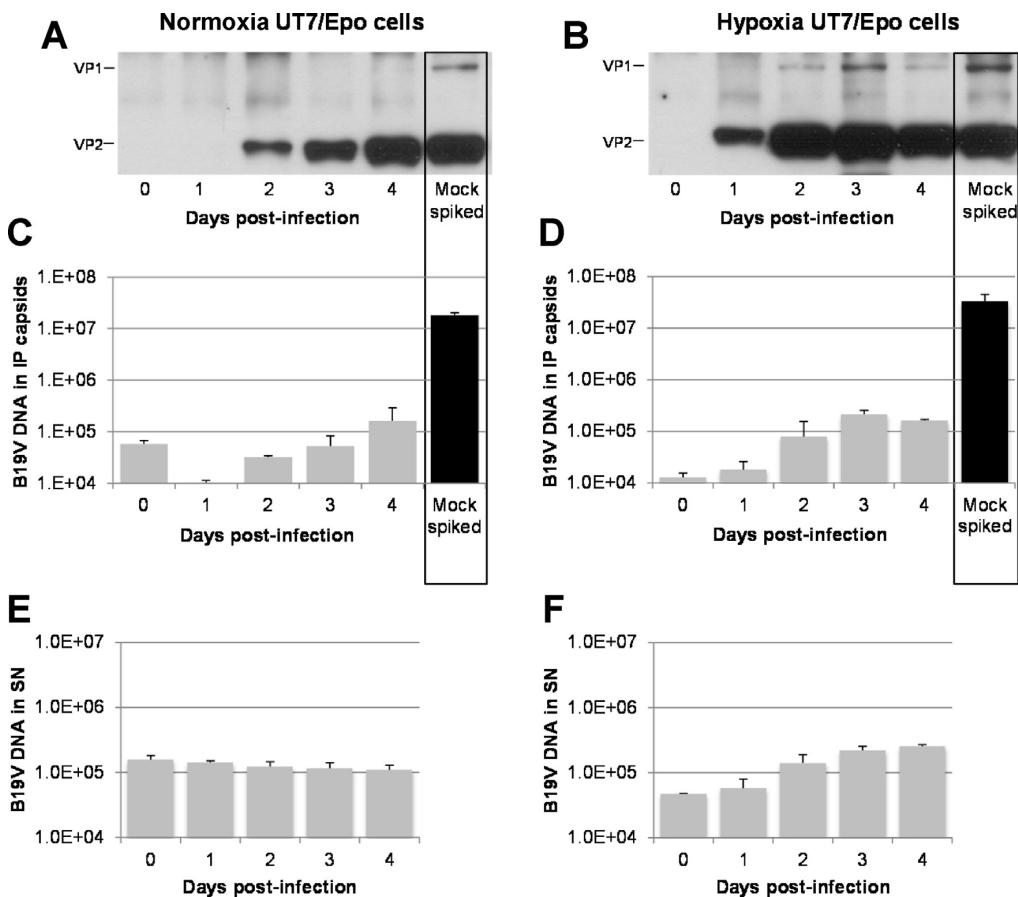
### 3.4. Chloroquine enhances B19V infection in UT7/Epo cells but has no influence in genome encapsidation and egress

It has been shown previously that chloroquine (CQ) enhances B19V infection. In the presence of CQ, an increased production of

viral DNA, RNA and proteins was observed and the infection was accelerated (Bönsch et al., 2010b). The production of mature virions in CQ-treated UT7/Epo cells was examined. The results confirmed, that in the presence of CQ, an increased amount of assembled capsids was produced (Fig. 4A). However, similar to untreated cells, most of the progeny capsids remained empty (Fig. 4B). Viral DNA or capsid proteins were not detected in the supernatant of infected cells (data not shown).

### 3.5. B19V infection is enhanced in CD36+ EPCs, in particular under hypoxia, but genome encapsidation remains restricted

Immunofluorescence microscopy examination of infected primary CD36+ EPCs confirmed that B19V can attach and internalize



**Fig. 2.** Capsid progeny and quantitation of virions in UT7/Epo cells. Cells ( $3 \times 10^5$ ) were infected with B19V under normoxia or hypoxia. At progressive times p.i., the supernatant was collected and the cells were lysed. (A and B) B19V capsids were immunoprecipitated from the cell extracts with mAb 860-55D. As a reference control, B19V ( $4 \times 10^{10}$ ) was added to mock-infected cell extracts. (C and D) B19V DNA was quantified from the immunoprecipitated capsids. As a reference control, B19V ( $4 \times 10^{10}$  virions) was added to mock-infected cell extracts. (E and F) B19V DNA was quantified from the supernatant of the infected cells. Data are the mean  $\pm$  SD of two independent experiments.

EPCs without noticeable differences to UT7/Epo cells or between normoxic and hypoxic conditions (Fig. 5A). However, the oxygen environment had an important influence in the number of cells infected by B19V. By 2 days p.i., the number of UT7/Epo cells with detectable capsid progeny was 1–5% and 15–20% under normoxia and hypoxia, respectively. In CD36+ EPCs, the number of infected cells increased to approximately 25% under normoxia and above 70% under hypoxic conditions (Fig. 5B).

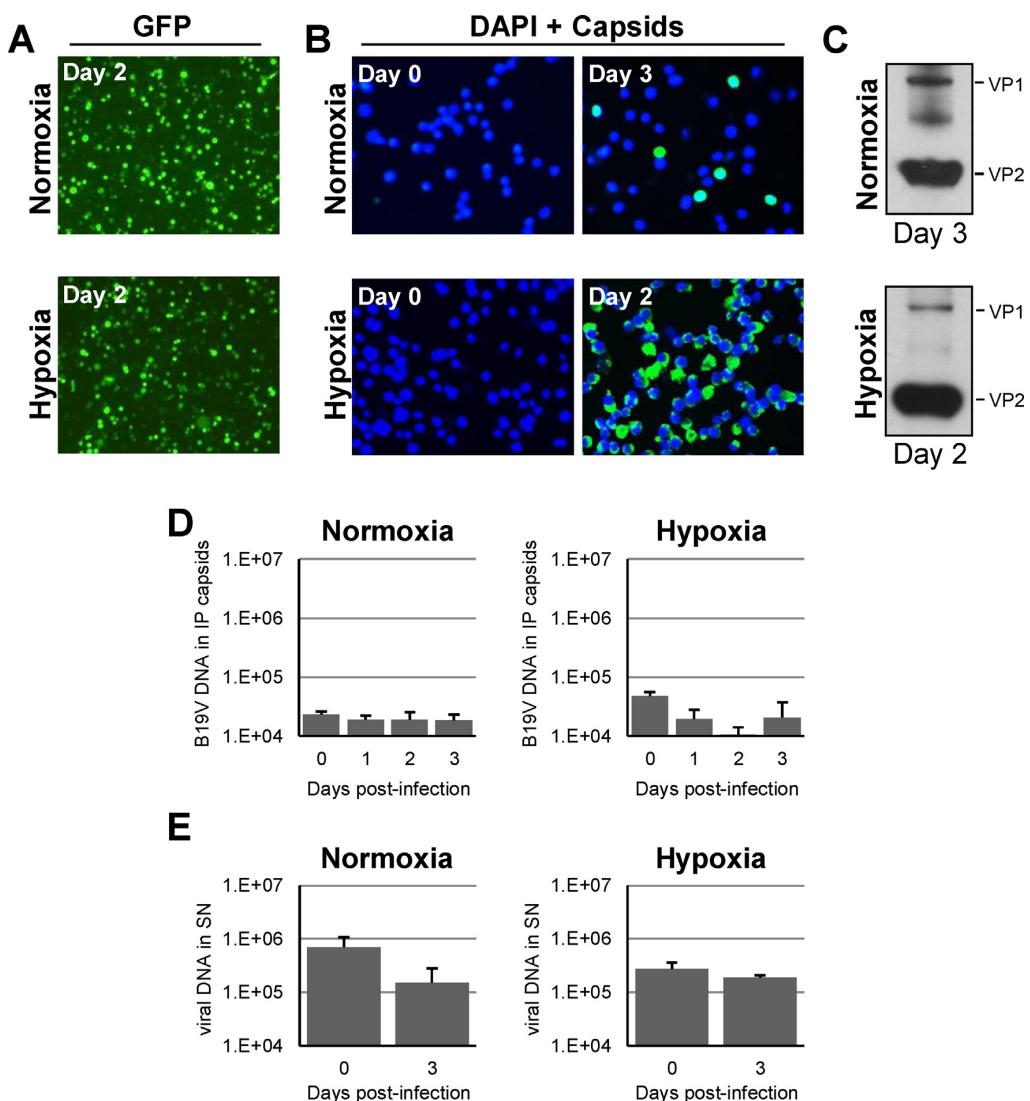
Immunoprecipitation experiments with the antibody 860-55D (against intact capsids) at progressive days p.i. showed, that regardless the oxygen conditions, progeny capsids appeared earlier in CD36+ EPCs than in UT7/Epo cells. While in UT7/Epo cells, capsid progeny production reached a plateau on day 4 under normoxia and on day 2–3 under hypoxia, in CD36+ EPCs, maximal capsid progeny was observed already after 24 h p.i. (compare Fig. 6A and B and Fig. 2A and B). The amount of viral DNA in the immunoprecipitated samples from the experiment shown in Fig. 6A and B was analyzed quantitatively. The results revealed that a limited number of capsids containing the viral DNA were produced after 24 h p.i. and did not increase subsequently (Fig. 6C and D). The presence of viral DNA in the supernatant increased and reached similarly a plateau already after 24 h p.i. (Fig. 6E and F).

Capsid progeny was detectable in the supernatant of infected EPCs, in particular under hypoxic conditions (Fig. 7A and B).

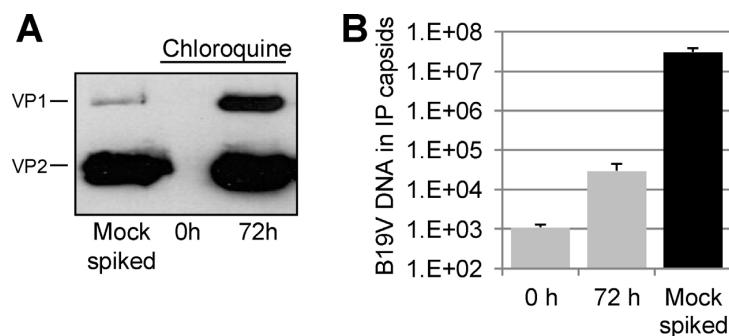
However, quantitation of their DNA content and comparison with the reference control revealed that only a modest proportion of the particles represented mature infectious virions (Fig. 7C and D). The IP of capsid-associated DNA increased and reached a plateau by 24 h p.i. At this time, the capsid progeny was undetectable under normoxia and hardly detectable under hypoxia (Fig. 7A and B). Therefore, the increase of capsid progeny observed in the following days represented essentially empty particles. These results indicate that despite the augmented and earlier production of virus progeny, the deficient packaging step remains the limiting factor for the propagation of B19V in CD36+ EPCs.

### 3.6. Intracellular distribution of viral genomes and capsids

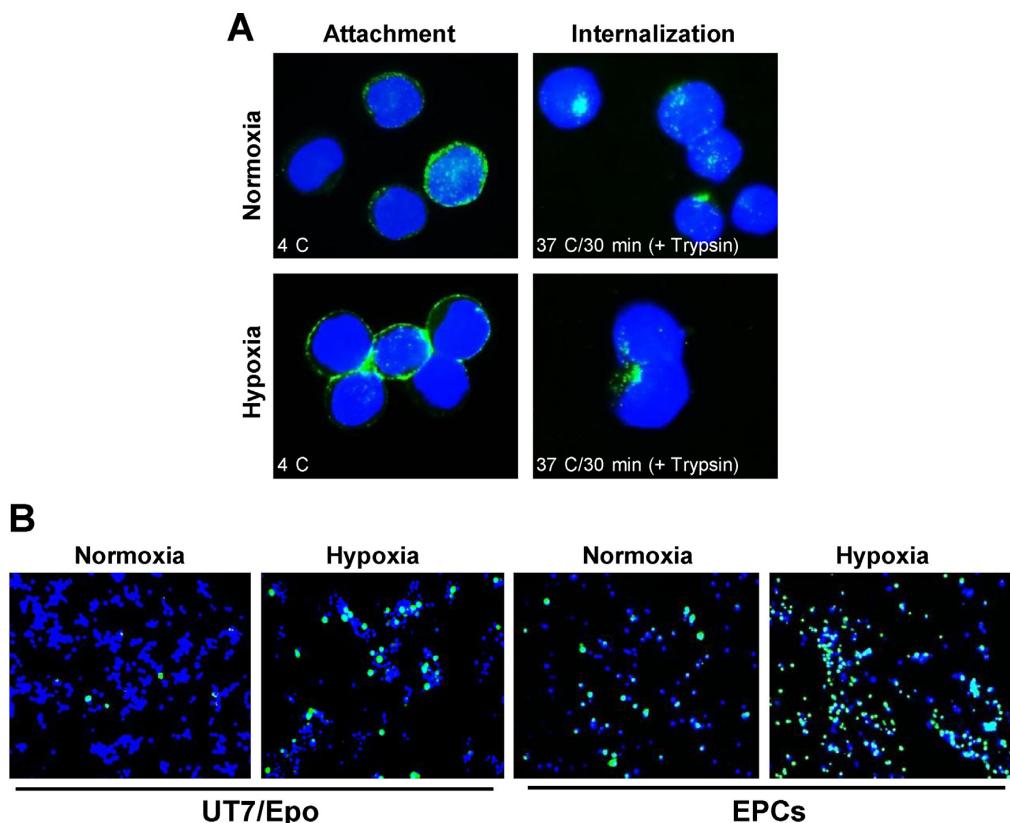
The presence and distribution of the viral genomes and capsids in the infected UT7/Epo cells was examined by FISH. In some cells, assembled capsids and viral genomes colocalized within large intranuclear clusters (Fig. 8A, panel i) resembling the nuclear compartments described earlier in AAV, containing non-structural proteins, capsids, and viral genomes and where presumably encapsidation takes place (Hunter and Samulski, 1992; Wistuba et al., 1997). However, in a larger proportion of cells the viral genomes appeared isolated in the nucleus, while the assembled capsids were detected in the cytoplasm (Fig. 8A, panel ii).



**Fig. 3.** Transfection of UT7/Epo cells with a B19V infectious clone under normoxia and hypoxia. (A) Transfection of UT7/Epo cells with a control plasmid expressing GFP is not influenced by normoxia or hypoxia. (B) Detection of B19V capsids by IF following transfection with a B19V infectious clone (pB19-M20). (C) Detection of B19V capsids by IP with mAb 860-55D from pB19-M20 transfected cells. (D) At progressive days p.i. B19V capsids were immunoprecipitated from cell lysates and B19V DNA was quantified. (E) B19V DNA was quantified from the supernatant of the infected cells. Data are the mean  $\pm$  SD for two independent experiments.



**Fig. 4.** Effect of chloroquine (CQ) in B19V infection in UT7/Epo cells. (A) Production of capsid progeny in UT7/Epo cells treated with CQ (25  $\mu$ M). B19V capsids were immunoprecipitated from the cell extracts with mAb 860-55D. As a reference control, B19V ( $4 \times 10^{10}$ ) was added to mock-infected cell extracts. The production of capsid progeny in untreated UT7/Epo cells is shown in Fig. 1F. (B) B19V DNA was quantified from the immunoprecipitated capsids. As a reference control, B19V ( $4 \times 10^{10}$  virions) was added to mock-infected cell extracts. Data are the mean  $\pm$  SD for two independent experiments.



**Fig. 5.** Attachment, internalization and infection of B19V in EPCs under normoxia and hypoxia. Cells ( $3 \times 10^5$ ) were infected with B19V under normoxia or hypoxia. (A) Binding and internalization of B19V in EPCs. B19V was added to the cells at 4 °C for 1 h, washed, fixed and stained with an antibody against intact capsids. For internalization, the cells were further incubated for 30 min at 37 °C, washed and trypsinized to remove uninternalized particles. (B) Detection of virus progeny by IF 2 days p.i. in UT7/Epo cells and EPCs cultured under normoxic and hypoxic conditions.

### 3.7. VP1u conformation in the virus progeny differs from that of wild-type virus

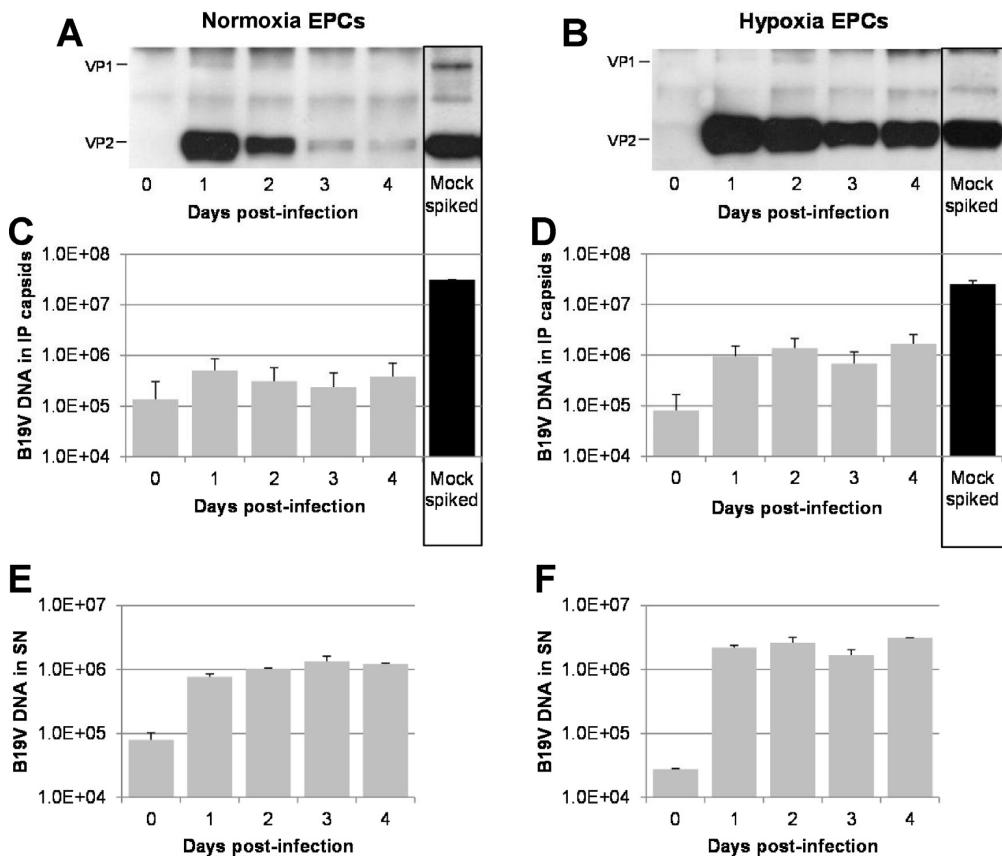
The pores at the fivefold symmetry axis are the portals for the encapsidation of the viral genome but also for the externalization of VP1u. The fivefold cylinder is narrow and constrictions of the channel impair the encapsidation of the viral genome and the externalization of VP1u (Farr and Tattersall, 2004; Bleker et al., 2005, 2006; Plevka et al., 2011). Examination of the VP1u conformation in the mostly empty virus progeny revealed, that differently to the wild-type virus, VP1u was partially exposed. The most N-terminal part was accessible to antibodies, while the C-terminal region remained internal and inaccessible (Fig. 8B and C). Similar to other parvoviruses (Cotmore et al., 1999; Viihinen-Ranta et al., 2002), exposure to mild temperature triggers the externalization of the N-terminal and C-terminal regions of VP1u from B19V without capsid disassembly (Ros et al., 2006). In clear contrast to the wild-type virus, heat treatment did not trigger the externalization of the C-terminal region of VP1u from the capsid progeny generated under normoxia and only discretely from capsids generated under hypoxia (Fig. 8D). Therefore, the failure to encapsidate the viral genome is possibly due to the constriction of the fivefold axis channel by a partially exposed and inflexible VP1u.

## 4. Discussion

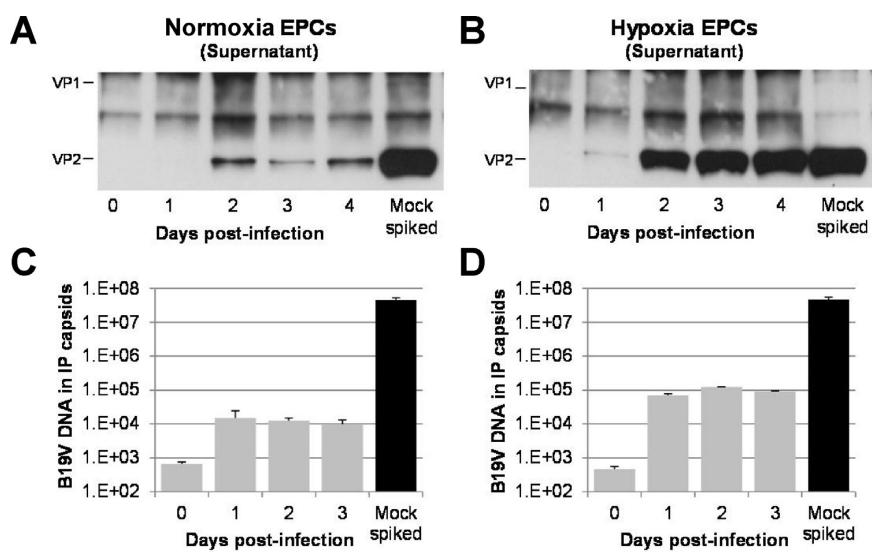
Discovered in 1975 (Cossart et al., 1975), today B19V is recognized as a major human pathogen involved in multiple syndromes.

However, the lack of a suitable cell culture system or an animal model restricts the availability of infectious virus and hampers seriously the studies with B19V. The virus has an extraordinary tropism for human erythroid progenitor cells (EPCs) in the bone marrow (Mortimer et al., 1983) where it can infect cells at the BFU-E and CFU-E stages of differentiation (Takahashi et al., 1990). During a natural infection B19V is able to replicate efficiently in the target cells, as judged by the typical high viremia observed in the infected individuals. However, the efficient B19V replication *in vivo* has not yet been mimicked *in vitro* with an established cell line, indicating the existence of highly restricted and still poorly understood cellular factors required for B19V replication. Some erythroleukemia cell lines, such as UT7/Epo (Shimomura et al., 1992) and KU812Ep6 (Miyagawa et al., 1999), have been shown to support B19V replication to a certain level, but none of them can produce significant quantities of infectious progeny. The human megakaryoblastic cell line UT7/Epo, has been shown to be the most permissive system for the *in vitro* replication of B19V (Wong and Brown, 2006) and it is used widely to study B19V infection.

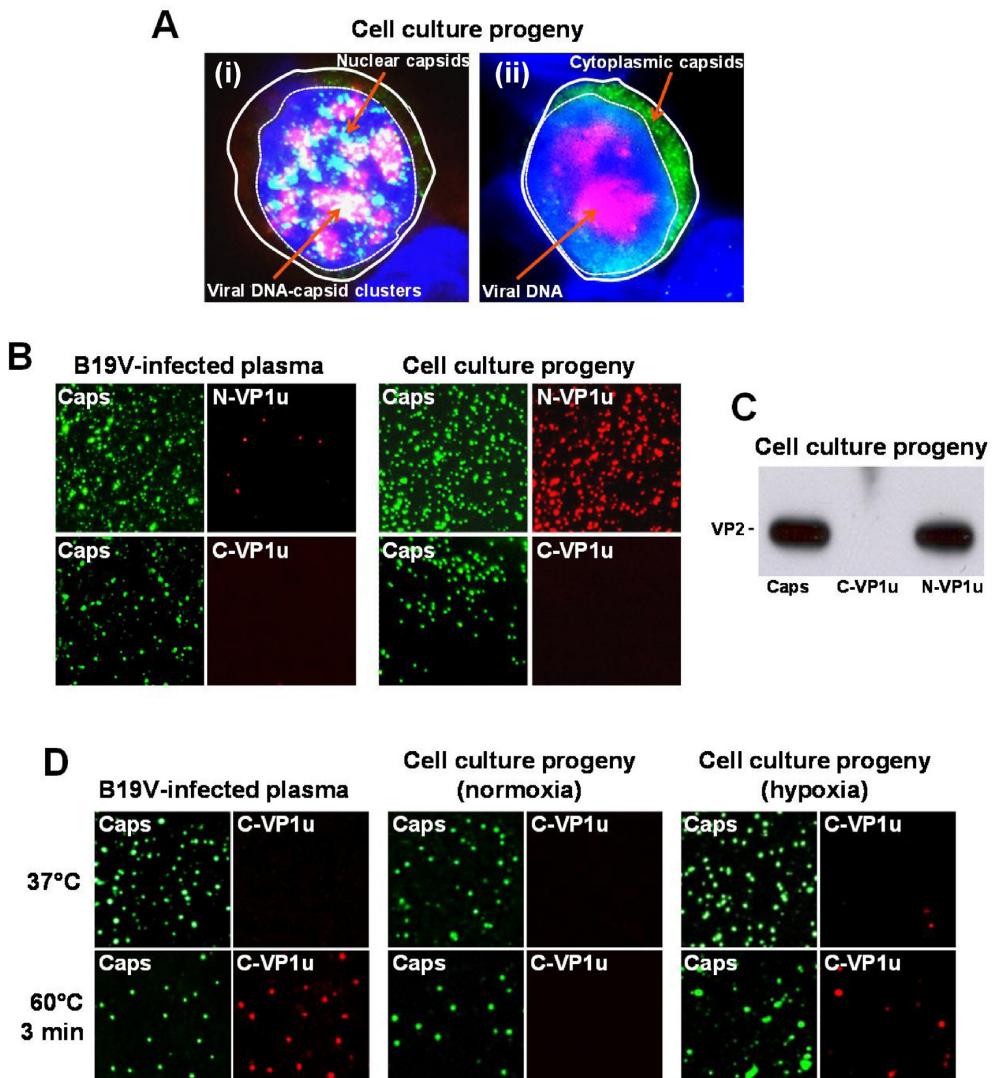
The reason for the defective replication of B19V in these cells has been shown to be multifactorial. Restrictions occur already at the cell surface, by the variable and limited expression of receptors and co-receptors required for binding and internalization of B19V (Brown et al., 1993; Munakata et al., 2005; Weigel-Kelley et al., 2003), but also by required intracellular factors restricted mainly to the erythroid lineage. Those intracellular factors can operate at the level of transcription, controlling the generation of sufficient full-length capsid-encoding transcripts (Guan et al., 2008; Liu et al.,



**Fig. 6.** Capsid progeny and quantitation of virions in EPCs. Cells ( $3 \times 10^5$ ) were infected with B19V under normoxia or hypoxia. At progressive times p.i., the supernatant was collected and the cells were lysed. (A and B) B19V capsids were immunoprecipitated from the cell extracts with mAb 860-55D, against intact capsids. As a reference control, B19V ( $4 \times 10^{10}$ ) was added to mock-infected cell extracts. (C and D) B19V DNA was quantified from the immunoprecipitated capsids. As a reference control, B19V ( $4 \times 10^{10}$  virions) was added to mock-infected cell extracts. (E and F) B19V DNA was quantified from the supernatant of the infected cells. Data are the mean  $\pm$  SD of two independent experiments.



**Fig. 7.** Virus egress in EPCs. Cells ( $3 \times 10^5$ ) were infected with B19V under normoxia or hypoxia. (A and B) At progressive times p.i., B19V capsids were immunoprecipitated from the cell supernatant with mAb 860-55D. As a reference control, B19V ( $4 \times 10^{10}$ ) was added to mock-infected cell supernatant. (C and D) B19V capsids were immunoprecipitated and B19V DNA was quantified. As a reference control, B19V ( $4 \times 10^{10}$  virions) was added to mock-infected cell supernatant. Data are the mean  $\pm$  SD of two independent experiments.



**Fig. 8.** Intracellular distribution of capsids and viral genomes and VP1u conformation in the capsid progeny. (A) Simultaneous detection of viral genomes and capsids in infected UT7Epo cells by FISH. Two representative cells are shown. (i) In some cells, B19V genomes and capsids were detectable in large clusters in the nucleus. (ii) In a larger proportion of cells, viral genomes were detected isolated in the nucleus while capsids were detected in the cytoplasm. (B) VP1u conformation in the plasma-derived virus differs from that of the cell culture progeny (UT7/Epo cells). Plasma-derived virus and cell culture progeny were concentrated by sucrose cushion centrifugation, spotted onto coverslips, fixed and detected by IF with mAb 860-55D or mAb 521-5D (Caps) and antibodies against the N-terminal and C-terminal regions of VP1u. (C) Immunoprecipitation of the cell culture progeny (UT7/Epo cells, 3 days p.i.) with mAb 860-55D (Capsids) and antibodies against the N-terminus and C-terminus of VP1u. (D) Flexibility of VP1u in the plasma-derived virus and cell culture progeny obtained under normoxia or hypoxia. Viruses were untreated (37 °C) or heat-treated (60 °C for 3 min) to trigger the exposure of VP1u and detected by IF with the indicated antibodies.

1992). In non-permissive cells the majority of viral mRNAs encode for NS1, with only limited production of the capsid-encoding transcripts. NS1 causes cell death by its cytotoxic or apoptotic characteristics (Moffatt et al., 1998). In contrast, more B19V RNAs are read through the multiple polyadenylation sites in permissive cells, which results in sufficient full-length capsid-encoding mRNAs (Liu et al., 1992). Studies have also shown that B19V replication and transcription were restricted to a small subset of cells but without production of capsid proteins, while in other cells, the single-stranded viral DNA was not converted to the double-stranded form (Gallinella et al., 2000). All the described restrictions at early (receptor/co-receptor) and late (replication/transcription) stages of the infection result in none or limited production of virus progeny.

Recently, two novel approaches based on hypoxic conditions (Caillet-Fauquet et al., 2004b; Pillet et al., 2004) and the use of *ex vivo* expanded CD36+ primary human erythroid progenitor cells (CD36+ EPCs), previous CD34+ *in vitro* preselection (Pillet et al., 2008; Wong et al., 2008), or directly from unselected peripheral blood mononuclear cells (Filippone et al., 2010), have been shown to improve B19V infection. The obtained results are in agreement with previous observations, which showed that B19V replicates better in CD36+ EPCs, in particular under hypoxia (Chen et al., 2011). However, despite these improvements, the final genome encapsidation step was still insufficient, producing abundant but mostly non-infectious empty capsids. In the study by Chen et al. (2011), the use of EPCs under hypoxia was shown to improve B19V infection, however large quantities of infectious virus were not

recovered from the supernatant of the infected cells, as it should be expected for a lytic virus. Therefore, CD36+ EPCs cannot yet be considered as a highly permissive cell culture system to propagate B19V and a robust source of infectious virus. Moreover, compared to UT7/Epo cells, the generation of primary CD36+ EPCs remains time-consuming, requires large quantities of expensive growth factors and the permissivity to B19V is limited within a narrow and variable time-frame when B19V receptor and co-receptors are expressed in concert with a favorable intracellular microenvironment (Wong et al., 2008).

Parvoviruses pack their single-stranded, linear DNA genome into the pre-assembled capsids in the nucleus (Cotmore and Tattersall, 2005; King et al., 2001; Timpe et al., 2005). The helicase activity of the parvovirus nonstructural protein, which is present in the encapsidation complexes, functions as a molecular motor to translocate the viral genome into the empty capsid through the fivefold symmetry axes pore, a process that is also mediated by the terminal telomeric structures of the viral genome (Cotmore and Tattersall, 2005; King et al., 2001). Besides genome encapsidation, the channels at the fivefold symmetry axis are also used for the externalization of VP1u during the infection process (Bleker et al., 2005, 2006; Cotmore and Tattersall, 2012; Farr and Tattersall, 2004; Plevka et al., 2011). The channel is narrow and minor modifications of its diameter result in defective genome encapsidation and VP1u externalization (Bleker et al., 2005; Cotmore and Tattersall, 2012). Therefore, specific capsid and genome conformations play a critical role in the packaging step. VP1u from parvoviruses is not accessible, but can become exposed *in vitro* by mild heat or low pH treatments and *in vivo* during the intracellular trafficking of the virus (Cotmore et al., 1999; Kronenberg et al., 2005; Mani et al., 2006; Ros et al., 2006; Viñinen-Ranta et al., 2002) or upon receptor binding in the case of B19V (Bönsch et al., 2010a). In clear contrast to natural plasma-derived virus, VP1u was exposed partially in the capsid progeny. While the most N-terminal region was externalized and accessible to antibodies, the C-terminal region remained internal (Fig. 8). This particular conformation was irreversible and did not change upon heat treatment. The aberrant conformation and rigidity of VP1u might explain the encapsidation failure in semi-permissive cell systems. Further studies will elucidate whether the VP1u conformation in the virus progeny is due to an aberrant assembly or the lack of a final maturation step.

## 5. Conclusions

When compared to UT7/Epo cells and normoxia, hypoxic conditions or the use of CD36+ EPCs resulted in a significant acceleration of the infection/transfection, an increase in the number of infected cells and a modest increase in the yield of capsid progeny. However, despite these improvements, genome encapsidation was impaired seriously under all tested conditions and cells. The fivefold axes channel might be constricted in the virus progeny by the atypical partial exposure of VP1u hindering the packaging step, which arises as a major limiting factor for the *in vitro* propagation of B19V.

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## **Part IV**

# **Discussion**



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