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Nomenclature

AAV	Adeno-associated virus
AMDV	Aleutian mink disease virus
B19V	Human parvovirus B19
Bp	Base pair
BPV	Bovine parvovirus
CPV	Canine parvovirus
Da	Dalton
DMEM	Dulbecco modified Eagle's medium
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EPC	Erythroid progenitor cell
FCS	Fetal calf serum
FPV	Feline parvovirus
GFAV	Gray fox amdovirus
GmDNV	Galleria mellonella densovirus
IF	Immunofluorescence
IP	Immunoprecipitation
ITR	Inverted terminal repeat
Kb	Kilo base
kDa	Kilodalton
mAb	Monoclonal antibody
MVM	Minute virus of mice
MVMi	Immunosuppressive strain of MVM
MVMp	Prototype strain of MVM
Nt	Nucleotide
PCR	Polymerase chain reaction
PPV	Porcine parvovirus

PstDNV	Penaeus stylirostris densovirus
qPCR	Quantitative PCR
SN	Supernatant
ssDNA	Single stranded DNA
SV40	Simian vacuolating virus 40 or Simian virus 40

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Part I

Introduction

1 Introduction

1.1 Morphology

Parvoviruses belong to the smallest of isometric viruses. They are devoid of a lipid envelope and their diameters range from 215 Å (Penaeus stylirostris densovirus, PstDNV) to 255 Å (CPV). The icosahedral nature of parvoviruses was shown unambiguously by X-ray crystallography. The capsid surface of some, particularly invertebrate, parvoviruses appears to be smooth (Galleria mellonella densovirus, GmDNV) whereas others (Adeno-associated virus-2, AAV-2) are spiky at the 3- or 5-fold symmetry axes [1, 2].

1.2 Physicochemical properties

about 75 % protein and 25 % DNA, Mr about 5.5 - 6.2 x 10^6, infectious virion buoyant density is 1.39 - 1.43 gcm^{-3}, in CsCl,mature virions are stable in the presence of lipid solvents, on exposure to pH 3-9 and for most species incubation at 56 ℃ for 60 min, inactivation occurs by treatment with formalin, β-propiolacetone, hydroxylamine, ultraviolet light, and oxidizing agentssuch as sodium hypochlorite

1.3 Taxonomy

The classification of the *Parvoviridae* family is based on morpholological and functional characteristics. Parvoviruses are common animal and insect pathogens that belong to the smallest DNA-containing viruses. Hence the prefix "parvum" which means small in Latin. The name "parvovirus" was first introduced to the literature by Carlos Brailovsky in 1966 [3]. The *Parvoviridae* family comprises all non-enveloped, isometric, small DNA viruses that contain linear single-stranded 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 palin-

dromic duplex hairpin telomers. As a consequence of such a simple genome, parvoviruses are highly dependent on their host for diverse functions in their reproduction [1, 4]. The terminal hairpins are fundamental for the unique replication strategy of the *Parvoviridae* family and hence 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 this feature, 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*. 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 and sequence homology amongst the *Parvovirinae* subfamily [5, 2].

1.3.1 The *parvovirinae* subfamily

Amdoparvovirus

The genus *Amdoparvovirus* shares most characteristics with the genera *Bocaparvovirus* and *Protoarvovirus*. Mature virions exclusively contain negative strand genomic DNA of approximately 4.8 kb in length harbouring dissimilar palindromic sequences at each end [6, 7]. 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 amdovirus (GFAV) [8]. Permissive replication is tightly restricted to Crandell feline kidney cells. In contrast to the members of the genera *Bocaparvovirus* and *Protoparvovirus*, the virion surface displays three mounds elevated around the threefold icosahedral axis of symmetry. However, several structure features 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* [9]. 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* [2].

Aveparvovirus

Copiparvovirus

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 [10, 11]. All RNA transcripts are generated from a single promoter at map unit 4.5. BPV RNA transcripts are alternatively spliced and polyadenylated either at an internal site or at the 3'-end of the genome. Noteworthy, bovine parvovirus (BPV), the main representative, encodes a 22.5 kDa nuclear phosphoprotein, NP-1, whose function still remains unknown. This protein is distinct from any other parvovirus-encoded polypeptide [12].

Dependoparvovirus

Positive and negative strand ssDNA is distributed indifferently among mature virions belonging to the genus *Dependoparvovirus* [13, 14]. The 4.7 kb DNA molecule contains identical ITRs of 145 nt, the first 125 nt of which form a palindromic sequence [15]. 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 [16, 17]. Common for all currently accepted replication-defective members of the genus *Dependoparvovirus* is their strict dependence upon helper adenoviruses or herpesviruses [18, 19, 20]. 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 [2]. 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 [21, 22, 23, 24]. This characteristic makes AAV a promising candidate for creating viral vectros for gene therapy. As a well characterized member of the *Dependoparvoviruses* AAV-2 represents the model virus among

this genus.

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 [25]. Transcription is regulated by a single mRNA promoter located at map unit 6 [26]. 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 polyadanylation site in the middle of the genome [27]. 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 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) [28] represents the model virus among the genus *Erythroparvovirus*.

Protoparvovirus

Protoparvoviruses were the first members of the subfamily *Parvovirinae* to be discovered in 1959 [29]. Some members of the genus contain positive strand DNA in variable proportions up to 50 % [30]. In mature virions of other 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 [31]. Transcription of the genome is regulated by two mRNA promoters at map units 4 and 39 [32]. 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. The genus *Protoparvovirus* is primarily represented by MVM [2, 4].

Tetraparvovirus

Genus	**Species**	**Virus or virus variants**

Amdoparvovirus	***Carnivore amdoparvovirus 1***	Aleutian mink disease virus
	Carnivore amdoparvovirus 2	Gray fox amdovirus
Aveparvovirus	***Galliform aveparvovirus 1***	Chicken parvovirus
		Turkey parvovirus
Bocaparvovirus	*Carnivore bocaparvovirus 1*	Canine minute virus
	Carnivore bocaparvovirus 2	Canine bocavirus 1
	Carnivore bocaparvovirus 3	Feline bocavirus
	Pinniped bocaparvovirus 1	California sea lion bocaviru
		California sea lion bocaviru
	Pinniped bocaparvovirus 2	California sea lion bocaviru
	Primate bocaparvovirus 1	Human bocavirus 1
		Human bocavirus 3
		Gorilla bocavirus
	Primate bocaparvovirus 2	Human bocavirus 2a
		Human bocavirus 2b
		Human bocavirus 2c
		Human bocavirus 4
	Ungulate bocaparvovirus 1	Bovine parvovirus
	Ungulate bocaparvovirus 2	Porcine bocavirus 1
		Porcine bocavirus 2
		Porcine bocavirus 6
	Ungulate bocaparvovirus 3	Porcine bocavirus 5
	Ungulate bocaparvovirus 4	Porcine bocavirus 7
	Ungulate bocaparvovirus 5	Porcine bocavirus 3
		Porcine bocavirus 4-1
		Porcine bocavirus 4-2
Copiparvovirus	***Ungulate copiparvovirus 1***	Bovine parvovirus 2
	Ungulate copiparvovirus 2	Porcine parvovirus 4
Dependoparvovirus	***Adeno-associated dependoparvovirus A***	Adeno-associated virus-1
		Adeno-associated virus-2
		Adeno-associated virus-3
		Adeno-associated virus-4
		Adeno-associated virus-6

		Adeno-associated virus-7
		Adeno-associated virus-8
		Adeno-associated virus-9
		Adeno-associated virus-10
		Adeno-associated virus-11
		Adeno-associated virus-12
		Adeno-associated virus-13
		Adeno-associated virus-S17
	Adeno-associated dependovirus B	Adeno-associated virus-5
		Bovine adeno-associated vi
		Caprine adeno-associated v
	Anseriform dependoparvovirus 1	Duck parvovirus
		Goose parvovirus-PT
		Goose parvovirus
	Avian dependovirus 1	Avian adeno-associated viru
	Chiropteran dependoparvovirus 1	Bat adeno-associated virus
	Pinniped dependoparvovirus 1	California sea lion adeno-as
	Squamate dependoparvovirus 1	Snake adeno-associated viru
Erythroparvovirus	***Primate erythroparvovirus 1***	Human parvovirus B19-Au
		Human parvovirus B19-J35
		Human parvovirus B19-Wi
		Human parvovirus B19-A6
		Human parvovirus B19-Lal
		Human parvovirus B19-V9
		Human parvovirus B19-D9
	Primate erythroparvovirus 2	Simian parvovirus
	Primate erythroparvovirus 3	Rhesus macaque parvovirus
	Primate erythroparvovirus 4	Pig-tailed macaque parvovi
	Rodent erythroparvovirus 1	Chipmunk parvovirus
	Ungulate erythroparvovirus 1	Bovine parvovirus 3
Protoparvovirus	*Carnivore protoparvovirus 1*	Feline parvovirus
		Canine parvovirus
		Mink enteritis virus

		Racoon parvovirus
	Primate protoparvovirus 1	Bufavirus 1a
		Bufavirus 1b
		Bufavirus 2
	Rodent protoparvovirus 1	H-1 parvovirus
		Kilham rat virus
		LuIII virus
		Minute virus of mice (proto
		Minute virus of mice (immu
		Minute virus of mice (Miss
		Minute virus of mice (Cutt
		Mouse parvovirus 1
		Mouse parvovirus 2
		Mouse parvovirus 3
		Mouse parvovirus 4
		Mouse parvovirus 5
		Hamster parvovirus
		Tumor virus X
		Rat minute virus 1
	Rodent protoparvovirus 2	Rat parvovirus 1
	Ungulate protoparvovirus 1	Porcine parvovirus Kresse
		Porcine parvovirus NADL-2
Tetraparvovirus	*Chiropteran tetraparvovirus 1*	Eidolon Helvum (bat) parv
	Primate tetraparvovirus 1 Human parvovirus 4 G1	PARV4G1
		Human parv4 G2
		Human parv4 G3
		Chipmanzee parv4
	Ungulate tetraparvovirus 1	Bovine hokovirus 1
		Bovine hokovirus 2
	Ungulate tetraparvovirus 2	Porcine hokovirus
	Ungulate tetraparvovirus 3	Porcine Cn virus
	Ungulate tetraparvovirus 4	Ovine hokovirus

1.4 Tropism

Most parvoviruses, such as MVM, CPV and FPV, show specific host ranges and tissue tropisms. The majority of the parvoviruses are members of those tightly controlled viruses. However, some parvoviruses, as for example many of the AAVs, infect many hosts and a variety of tissues. Understanding controls of these properties for autonomous parvoviruses show great promise for the therapeutic delivery to be controlled or modified in gene therapy applications [33].

To date, two independent strains of the parvovirus MVM have been described to occur in mice. Both strains display disparate *in vitro* tropism and *in vivo* pathogenicity despite differing by only 14 amino acids in their capsid proteins. Fist, MVMp, the prototype strain, was originally isolated from a contaminated murine adenovirus stock and was shown to replicate efficiently in mouse fibroblasts [34]. Secondly, MVMi, the immunosuppressive strain, was recovered from an infected EL-4 T-cell lymphoma culture [35]. Although MVMi infection may result in pathology of infected mice, it has been shown that the infection more likely interferes with numerous T-cell functions *in vitro*. The infection rather causes problems for the ongoing study the mice are being used for as the immune system will be activated, the activity of T-lymphocytes or B-lymphocytes will be altered and tumor formation may be suppressed [36, 37, 38].

As above-mentioned, it has been shown that the allotropic MVM strains, MVMi and MVMp, differ in their target cell tropism. In order to map the viral sequences responsible for that difference, chimeric viral genomes were constructed *in vitro* from infectious genomic clones of both strains. The differences in the cell tropism between MVMi and MVMp have been mapped to the capsid gene, in particular to the VP2 residues 317 and 321. Both residues are located at the base of the threefold spike of the virion and are involved in controlling the cell tropism of the two distinct MVM strains [39, 40, 41]. Interestingly, residue 321 aligns with residue 323 of CPV, that itself is a critical determinant for CPV host range tropism. Further residues (VP2 residues 399, 553 and 558) were identified in MVMi to be able to confer fibrotropism to single site-directed mutants. Those residues cluster around the twofold dimple-like depression [42].

In general, tissue tropism determining amino acids were found be located on, or near, the viral surface, mainly by raised structural elements around the threefold axes of symmetry. Differences in tropism and pathogenicity have also been mapped to the capsid proteins of Aleutian mink disease parvovirus [43], porcine parvovirus (PPV) [44],

CPV [45, 46], and FPV [47] in a capsid region analogous to that observed for MVM. Although the same structural element of viruses is 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 [42, 33].

1.5 Structure

1.5.1 Parvoviruses in general

Parvovirus capsids are devoid of a lipid envelope and have an average diameter of 18 to 26 nm. The viral capsid is made up of 60 copies of between two and four structural proteins that overlap each other. For each virus there is one major capsid protein present in the capsid structure. Minor proteins form the same core structure, but differ in the sequence length on their amino termini. The capsid proteins display a T=1 icosahedral symmetry and are variously designated VP1-VP4. Thus, the capsid has a 5-3-2 point group symmetry containing 31 rotational symmetry elements that intersect at the center: six fivefolds, ten threefolds, and fifteen twofolds. Despite the differences in protein forms and the low homology between some of the viruses, several structural elements on the capsid surface are common to most parvoviruses. These include raised cylindrical channels at the fivefold axes surrounded by depressed, canyon-like regions. Further shared surface characteristics are protrusions at the threefold axes, termed as spikes or peaks, and dimple-like depressions at the icosahedral twofold axes. A common feature of parvoviruses is their high resistance to physicochemical treatments. This stability provides an effective protection to the fragile, condensed genome in the extracellular environment ensuring transmission between their hosts. The ssDNA genome consists of approximately 5000 bases, packed as either a positive or, more usually, as a negative sense strand. At the 5' and 3' ends, the genome harbors palindromic sequences of about 120 to 250 nucleotides, that form secondary hairpin structures which are essential for the initiation of viral genome replication [48, 49, 50, 42, 51, 52, 33].

1.5.2 MVM

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 578 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 [53]. The common c-terminal part of the structural proteins has an eight-stranded antiparallel β-barrel topology, frequently found in viral capsid proteins [54]. Large loops between the β-strands of the β-barrel that form the principal surface features, particularly the threefold spikes, and determine host-range tropism were found to be quite dissimilar in MVM and CPV. The first 37 amino acids are not visible in the electron density map. Since the N-VP2 terminal part contains a predominantly poly-glycine conserved sequence, it might be highly flexible. There is density extending along the fivefold channels of the MVMi capsid that was modeled as the glycine-rich N-terminal region [55, 56]. *In vitro*, trypsin digestion of full MVM virions results in a truncated VP3 polypeptide that still contains the glycine-rich sequence. In this way, most VP2 N-termini can be cleaved. These findings suggest that there is a dynamic situation at the fivefold channel. In one model, one in five amino termini are externalized along the fivefold axes and are accessible for cleavage. Newly created, cleaved N-VP3 termini could withdraw into the virion and be replaced at the surface by an uncleaved N-VP2 terminus. [57, 42]. 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 [58, 59]. 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 [42].

1.6 Nucleic Acid

1.6.1 Genome organization

1.6.2 Transcriptome

1.7 Viral proteins

1.7.1 Structural Proteins

1.7.2 Non-structural proteins

1.8

1.8.1

1.8.2

2 Methods

2.1 Cell Cultures

A9 ouab^{r}l1 cells, a derivative from the original HGPRT^{-} L-cell line A9 represent a clone resistant to 10^{-3} M ouabain after nitrosoguanidine mutagenesis [60]. NB324K cells are a clone of SV40-transformed human newborn kidney cells [61]. The SV40 large T antigen was detected by immunofluorescent staining with monoclonal antibodies [62]. 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 ℃ in 5 % CO_2 atmosphere.

2.1.1 Freezing and thawing of cells

Before use the A9 mouse fibroblasts or NB324K cells were thawed at 37 ℃ 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 ℃ over night before transfer to liquid nitrogen.

2.2 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.

2.2.1 Separation of empty and full capsids

Sucrose purified capsids were prepared as previously described in section 2.2, page 13. The virus pellet was resuspended in 10 mL PBS. Caesium chloride was added to a density of 1.38 g/mL adjusted by refractometry (η=1.371) at 4 ℃. The gradient was centrifuged to equilibrium for 24 h at 41000 rpm and 4 ℃ 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 concentrated by ultra-centrifugation when required.

2.3 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 ℃.

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

2.5 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 μL elution buffer. As templates 2 μL of the isolated viral DNA were used for the PCR reaction and were added to the following master mix:

Component	**Amount**	**Final concentration**
dH_2O, PCR grade	6 μL	-
Forward primer, 10 pM	1 μL	0.5 pM
Reverse primer, 10 pM	1 μL	0.5 pM
2x IQ™ SYBR® Green Supermix	10 μL	1x
Total volume	**18 μL**	

Table 2.1: 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:

Cycles	**Step**	**Temperature**	**Time**
1x	Initial denaturation	95 ℃	300 s
40x	Denaturation	95 ℃	15 s
	Annealing	61 ℃	15 s
	Extension	72 ℃	15 s
1x	Final denaturation	95 ℃	60 s
1x	Melting curve	65 ℃ up to 95 ℃	0.1 ℃/s

Table 2.2: 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 β-actin gene was performed. After normalization, direct comparison of the results is possible. β-actin quantification was carried out with the same PCR conditions outlined in table 2.2, 15.

2.5.1 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 μL with PBSA 1 %. The antibody was added in excess and incubated with the viral capsids for 1 h at 4 ℃ on a rotary shaker. Subsequently, 20 μL protein G-agarose beads were added. Following overnight incubation at 4 ℃ 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 ℃ until further use or immediately processed.

2.5.2

2.6

2.6.1

2.6.2

Figure 2.1 shows a photograph of D440N.

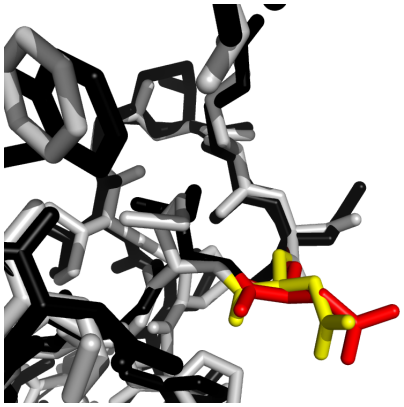

Figure 2.1: Structure of D440N

Part II

Publication

Part III

Discussion

Bibliography

[1] P. Tijssen, Molecular and structural basis of the evolution of parvovirus tropism, *Acta Vet. Hung.* **47**, 379 (1999).

[2] A. M. Q. King, M. J. Adams, E. B. Carstens, and E. J. Lefkowitz, *Virus taxonomy: Ninth report of the International Committee on Taxonomy of Viruses.*, Elsevier Academic Press, San Diego, CA, 2012.

[3] C. Brailovsky, [Research on the rat K virus (Parvovirus ratti). I. A method of titration by plaques and its application to the study of the multiplication cycle of the virus], *Ann Inst Pasteur (Paris)* **110**, 49 (1966).

[4] J. R. Kerr, M. E. Bloom, S. Cotmore, R. M. Linden, and C. R. Parrish, *Parvoviruses*, Hodder Arnold, London, UK, 2005.

[5] V. V. Lukashov and J. Goudsmit, Evolutionary relationships among parvoviruses: virus-host coevolution among autonomous primate parvoviruses and links between adeno-associated and avian parvoviruses, *J. Virol.* **75**, 2729 (2001).

[6] M. E. Bloom, R. E. Race, and J. B. Wolfinbarger, Characterization of Aleutian disease virus as a parvovirus, *J. Virol.* **35**, 836 (1980).

[7] S. Alexandersen, M. E. Bloom, and S. Perryman, Detailed transcription map of Aleutian mink disease parvovirus, *J. Virol.* **62**, 3684 (1988).

[8] L. Li, P. A. Pesavento, L. Woods, D. L. Clifford, J. Luff, C. Wang, and E. Delwart, Novel amdovirus in gray foxes, *Emerging Infect. Dis.* **17**, 1876 (2011).

[9] R. McKenna, N. H. Olson, P. R. Chipman, T. S. Baker, T. F. Booth, J. Christensen, B. Aasted, J. M. Fox, M. E. Bloom, J. B. Wolfinbarger, and M. Agbandje-McKenna, Three-dimensional structure of Aleutian mink disease parvovirus: implications for disease pathogenicity, *J. Virol.* **73**, 6882 (1999).

[10] K. C. Chen, B. C. Shull, E. A. Moses, M. Lederman, E. R. Stout, and R. C. Bates, Complete nucleotide sequence and genome organization of bovine parvovirus, *J. Virol.* **60**, 1085 (1986).

[11] D. Schwartz, B. Green, L. E. Carmichael, and C. R. Parrish, The canine minute virus (minute virus of canines) is a distinct parvovirus that is most similar to bovine parvovirus, *Virology* **302**, 219 (2002).

[12] M. Lederman, J. T. Patton, E. R. Stout, and R. C. Bates, Virally coded noncapsid protein associated with bovine parvovirus infection, *J. Virol.* **49**, 315 (1984).

[13] K. I. Berns and S. Adler, Separation of two types of adeno-associated virus particles containing complementary polynucleotide chains, *J. Virol.* **9**, 394 (1972).

[14] J. A. Rose, K. I. Berns, M. D. Hoggan, and F. J. Koczot, Evidence for a single-stranded adenovirus-associated virus genome: formation of a DNA density hybrid on release of viral DNA, *Proc. Natl. Acad. Sci. U.S.A.* **64**, 863 (1969).

[15] E. Lusby, K. H. Fife, and K. I. Berns, Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA, *J. Virol.* **34**, 402 (1980).

[16] M. R. Green and R. G. Roeder, Definition of a novel promoter for the major adenovirus-associated virus mRNA, *Cell* **22**, 231 (1980).

[17] E. W. Lusby and K. I. Berns, Mapping of the 5' termini of two adeno-associated virus 2 RNAs in the left half of the genome, *J. Virol.* **41**, 518 (1982).

[18] R. W. Atchison, The role of herpesviruses in adenovirus-associated virus replication in vitro, *Virology* **42**, 155 (1970).

[19] R. M. Buller, J. E. Janik, E. D. Sebring, and J. A. Rose, Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication, *J. Virol.* **40**, 241 (1981).

[20] M. D. Hoggan, N. R. Blacklow, and W. P. Rowe, Studies of small DNA viruses found in various adenovirus preparations: physical, biological, and immunological characteristics, *Proc. Natl. Acad. Sci. U.S.A.* **55**, 1467 (1966).

[21] R. M. Kotin, M. Siniscalco, R. J. Samulski, X. D. Zhu, L. Hunter, C. A. Laughlin, S. McLaughlin, N. Muzyczka, M. Rocchi, and K. I. Berns, Site-specific integration by adeno-associated virus, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2211 (1990).

[22] R. M. Kotin, J. C. Menninger, D. C. Ward, and K. I. Berns, Mapping and direct visualization of a region-specific viral DNA integration site on chromosome 19q13-qter, *Genomics* **10**, 831 (1991).

[23] R. M. Kotin, R. M. Linden, and K. I. Berns, Characterization of a preferred site on human chromosome 19q for integration of adeno-associated virus DNA by non-homologous recombination, *EMBO J.* **11**, 5071 (1992).

[24] R. J. Samulski, X. Zhu, X. Xiao, J. D. Brook, D. E. Housman, N. Epstein, and L. A. Hunter, Targeted integration of adeno-associated virus (AAV) into human chromosome 19, *EMBO J.* **10**, 3941 (1991).

[25] V. Deiss, J. D. Tratschin, M. Weitz, and G. Siegl, Cloning of the human parvovirus B19 genome and structural analysis of its palindromic termini, *Virology* **175**, 247 (1990).

[26] C. Doerig, P. Beard, and B. Hirt, A transcriptional promoter of the human parvovirus B19 active in vitro and in vivo, *Virology* **157**, 539 (1987).

[27] K. Ozawa, J. Ayub, Y. S. Hao, G. Kurtzman, T. Shimada, and N. Young, Novel transcription map for the B19 (human) pathogenic parvovirus, *J. Virol.* **61**, 2395 (1987).

[28] E. D. Heegaard and K. E. Brown, Human parvovirus B19, *Clin. Microbiol. Rev.* **15**, 485 (2002).

[29] L. KILHAM and L. J. OLIVIER, A latent virus of rats isolated in tissue culture, *Virology* **7**, 428 (1959).

[30] R. C. Bates, C. E. Snyder, P. T. Banerjee, and S. Mitra, Autonomous parvovirus LuIII encapsidates equal amounts of plus and minus DNA strands, *J. Virol.* **49**, 319 (1984).

[31] C. R. Astell, M. Thomson, M. Merchlinsky, and D. C. Ward, The complete DNA sequence of minute virus of mice, an autonomous parvovirus, *Nucleic Acids Res.* **11**, 999 (1983).

[32] D. Pintel, D. Dadachanji, C. R. Astell, and D. C. Ward, The genome of minute virus of mice, an autonomous parvovirus, encodes two overlapping transcription units, *Nucleic Acids Res.* **11**, 1019 (1983).

[33] K. Hueffer and C. R. Parrish, Parvovirus host range, cell tropism and evolution, *Curr. Opin. Microbiol.* **6**, 392 (2003).

[34] L. V. Crawford, A minute virus of mice, *Virology* **29**, 605 (1966).

[35] G. D. Bonnard, E. K. Manders, D. A. Campbell, R. B. Herberman, and M. J. Collins, Immunosuppressive activity of a subline of the mouse EL-4 lymphoma. Evidence for minute virus of mice causing the inhibition, *J. Exp. Med.* **143**, 187 (1976).

[36] H. D. Engers, J. A. Louis, R. H. Zubler, and B. Hirt, Inhibition of T cell-mediated functions by MVM(i), a parvovirus closely related to minute virus of mice, *J. Immunol.* **127**, 2280 (1981).

[37] G. K. McMaster, P. Beard, H. D. Engers, and B. Hirt, Characterization of an immunosuppressive parvovirus related to the minute virus of mice, *J. Virol.* **38**, 317 (1981).

[38] D. G. Baker, Future Directions in Rodent Pathogen Control, *ILAR J* **39**, 312 (1998).

[39] E. M. Gardiner and P. Tattersall, Evidence that developmentally regulated control of gene expression by a parvoviral allotropic determinant is particle mediated, *J. Virol.* **62**, 1713 (1988).

[40] E. M. Gardiner and P. Tattersall, Mapping of the fibrotropic and lymphotropic host range determinants of the parvovirus minute virus of mice, *J. Virol.* **62**, 2605 (1988).

[41] J. P. Antonietti, R. Sahli, P. Beard, and B. Hirt, Characterization of the cell type-specific determinant in the genome of minute virus of mice, *J. Virol.* **62** (1988).

[42] M. Agbandje-McKenna, A. L. Llamas-Saiz, F. Wang, P. Tattersall, and M. G. Rossmann, Functional implications of the structure of the murine parvovirus, minute virus of mice, *Structure* **6**, 1369 (1998).

[43] M. E. Bloom, B. D. Berry, W. Wei, S. Perryman, and J. B. Wolfinbarger, Characterization of chimeric full-length molecular clones of Aleutian mink disease parvovirus (ADV): identification of a determinant governing replication of ADV in cell culture, *J. Virol.* **67**, 5976 (1993).

[44] J. Bergeron, B. Hebert, and P. Tijssen, Genome organization of the Kresse strain of porcine parvovirus: identification of the allotropic determinant and comparison with those of NADL-2 and field isolates, *J. Virol.* **70**, 2508 (1996).

[45] C. R. Parrish, C. F. Aquadro, and L. E. Carmichael, Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus and related feline, mink, and raccoon parvoviruses, *Virology* **166**, 293 (1988).

[46] S. F. Chang, J. Y. Sgro, and C. R. Parrish, Multiple amino acids in the capsid structure of canine parvovirus coordinately determine the canine host range and specific antigenic and hemagglutination properties, *J. Virol.* **66**, 6858 (1992).

[47] U. Truyen, M. Agbandje, and C. R. Parrish, Characterization of the feline host range and a specific epitope of feline panleukopenia virus, *Virology* **200**, 494 (1994).

[48] C. Ros, C. Baltzer, B. Mani, and C. Kempf, Parvovirus uncoating in vitro reveals a mechanism of DNA release without capsid disassembly and striking differences in encapsidated DNA stability, *Virology* **345**, 137 (2006).

[49] M. Agbandje, C. Parrish, and M. Rossmann, The structure of parvoviruses, *Seminars in Virology* **6**, 299 (1995).

[50] M. Agbandje, R. McKenna, M. G. Rossmann, M. L. Strassheim, and C. R. Parrish, Structure determination of feline panleukopenia virus empty particles, *Proteins* **16**, 155 (1993).

[51] A. A. Simpson, B. Hebert, G. M. Sullivan, C. R. Parrish, Z. Zadori, P. Tijssen, and M. G. Rossmann, The structure of porcine parvovirus: comparison with related viruses, *J. Mol. Biol.* **315**, 1189 (2002).

[52] J. Tsao, M. S. Chapman, M. Agbandje, W. Keller, K. Smith, H. Wu, M. Luo, T. J. Smith, M. G. Rossmann, and R. W. Compans, The three-dimensional structure of canine parvovirus and its functional implications, *Science* **251**, 1456 (1991).

[53] A. L. Llamas-Saiz, M. Agbandje-McKenna, W. R. Wikoff, J. Bratton, P. Tattersall, and M. G. Rossmann, Structure determination of minute virus of mice, *Acta Crystallogr. D Biol. Crystallogr.* **53**, 93 (1997).

[54] M. G. Rossmann and J. E. Johnson, Icosahedral RNA virus structure, *Annu. Rev. Biochem.* **58**, 533 (1989).

[55] H. Wu, W. Keller, and M. G. Rossmann, Determination and refinement of the canine parvovirus empty-capsid structure, *Acta Crystallogr. D Biol. Crystallogr.* **49**, 572 (1993).

[56] Q. Xie and M. S. Chapman, Canine parvovirus capsid structure, analyzed at 2.9 A resolution, *J. Mol. Biol.* **264**, 497 (1996).

[57] M. S. Chapman and M. G. Rossmann, Structure, sequence, and function correlations among parvoviruses, *Virology* **194**, 491 (1993).

[58] M. S. Chapman and M. G. Rossmann, Single-stranded DNA-protein interactions in canine parvovirus, *Structure* **3**, 151 (1995).

[59] J. Tsao, M. S. Chapman, H. Wu, M. Agbandje, W. Keller, and M. G. Rossmann, Structure determination of monoclinic canine parvovirus, *Acta Crystallogr., B* **48 (Pt 1)**, 75 (1992).

[60] J. W. LITTLEFIELD, THREE DEGREES OF GUANYLIC ACID–INOSINIC ACID PYROPHOSPHORYLASE DEFICIENCY IN MOUSE FIBROBLASTS, *Nature* **203**, 1142 (1964).

[61] H. M. SHEIN and J. F. ENDERS, Multiplication and cytopathogenicity of Simian vacuolating virus 40 in cultures of human tissues, *Proc. Soc. Exp. Biol. Med.* **109**, 495 (1962).

[62] E. Harlow, L. V. Crawford, D. C. Pim, and N. M. Williamson, Monoclonal antibodies specific for simian virus 40 tumor antigens, *J. Virol.* **39**, 861 (1981).