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Einari Niskanen

On Dynamics of Parvoviral Replication Protein NS1

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ABSTRACT

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Members of the family *Parvoviridae* are small, non-enveloped viruses. Their DNA genome is unique among the viruses in being both single-stranded and linear. Viruses of the genus *Parvovirus*, belonging to the vertebrate infecting subfamily *Parvovirinae*, have only two genes in their genome: one for the capsid proteins (VP1 and VP2) and the other for non-structural proteins (NS1 and NS2). Their small protein capsids are formed from 60 copies of VP proteins. The capsid protects the viral genome outside the cell and enables cellular entry. Parvoviruses replicate in the nucleus of the host cell, exploiting the host replication machinery. However, the genome replication is dependent on the function of NS1 – a multifunctional nuclear phosphoprotein with helicase, ATPase, transcriptional regulation, sequence-specific DNA recognition, and site- and strand-specific nicking functions. In this thesis, the canine parvovirus (CPV), a member of the genus *Parvovirus*, was used to investigate changes that occur in nuclear organization during parvovirus infection and to elucidate the mechanisms behind the dynamics of the replication protein NS1. Most of the studies were done using fluorescent fusion proteins in arguably the most relevant context: living cells. In case of the multifunctional NS1 protein, this setup has the advantage of obviating the need to know all interacting partners in order to study its dynamics. At the same time, however, the unambiguous interpretation of the results is challenging. CPV infection was observed to induce massive reorganization in the host nucleus, including marginalization of the chromatin. In addition, the dynamics of the NS1 protein was concluded to depend on its ability to bind and hydrolyze ATP and on the integrity of the predicted DNA-binding regions in the N-terminal and helicase domains. Moreover, NS1 was hypothesized to utilizing both the N-terminal and helicase domains in non-specific binding of a double-stranded DNA. This suggests a functional similarity between NS1 and the papillomavirus replication proteins.

Keywords: ATPases; ATP binding; canine parvovirus; fluorescent recovery after photobleaching; helicases; NS1; protein dynamics.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by following Roman numerals (I-III):

I Ihalainen, T. O., Niskanen, E. A., Jylhävä, J., Paloheimo, O., Dross, N., Smolander, H., Langowski, J., Timonen, J. & Vihinen-Ranta, M., 2009. Parvovirus Induced Alterations in Nuclear Architecture and Dynamics. PLoS ONE 4(6): e5948.

II Niskanen, E. A., Ihalainen, T. O., Kalliolinna, O., Vuokko, M. & Vihinen-Ranta, M., 2010. Effect of ATP binding and hydrolysis on dynamics of CPV NS1. Journal of Virology 84(10): 5391-403.

III Niskanen, E. A., Kalliolinna, O.*, Ihalainen, T. O.*, Vuokko, M. & Vihinen-Ranta, M., 2010. The dynamics of the canine parvovirus NS1 protein depends equally on N-terminal and helicase domains. Manuscript.
* equal contribution

RESPONSIBILITIES OF EINARI NISKANEN IN THE THESIS ARTICLES

Article I I was responsible for the cloning, and took part in FISH and BrdU experiments. Teemu Ihalainen was responsible for the planning of the experiments and also conducted most of the experiments. Juulia Jylhävä was mainly responsible for FISH experiments and assisted in cloning of the PAGFP-VP2. Outi Paloheimo took part in the PAGFP-VP2 characterization. Jussi Timonen assisted in FRAP data analysis. Nicolas Dross assisted in the FFM and in the FFM data analysis. Hanna Smolander was responsible for the nuclear volume calculations. Teemu Ihalainen produced article figures and wrote the article together with Maija Vihinen-Ranta while I took part in finalizing it.

Article II I was responsible for the planning of the article and the experiments. I conducted most of the experiments including protein modeling, FRAP and ivFRAP. Teemu Ihalainen planned and conducted virtual cell simulations. I cloned the constructs with Olli Kalliolinna and Milla Häkkinen. I did the infectivity and P38 transactivation studies together with Olli Kalliolinna. I analyzed the data, produced the article figures, and wrote the article while Maija Vihinen-Ranta and Teemu Ihalainen took part in finalizing it.

Article III I was responsible for the planning of the article and the experiments. I conducted most of the experiments including protein modeling, analysis of the protein alignments, FRAP and ivFRAP. Teemu Ihalainen planned and conducted virtual cell simulations. I cloned the constructs with Olli Kalliolinna and Milla Häkkinen. I did the infectivity studies together with Olli Kalliolinna. I analyzed the data, produced the article figures, and wrote the article.

Studies for articles I, II and III were carried out under the supervision of Docent Maija Vihinen-Ranta. Article I was done in collaboration with Professor Jussi Timonen and Professor Jörg Langowski.

ABBREVIATIONS

AAA+	ATPases associated with various cellular activities
AAV	Adeno-associated virus
Ab	Rabbit IgG antibody
APAR	Autonomous parvovirus replication body
BPV	Bovine papillomavirus
BrdU	5-bromo-2′-deoxyuridine
CPV	Canine parvovirus
D	Diffusion coefficient
E1	Initiator protein of papillomaviruses
EGFP	Enhanced green fluorescent protein
EYFP	Enhanced yellow fluorescent protein
FPV	Feline panleukopenia virus
FRAP	Fluorescence recovery after photobleaching
GFP	Green fluorescent protein
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
HSV-1	Herpes simplex virus-1
LTag	Large tumor antigen protein of Simian virus 40
Mab	Mouse IgG antibody
MVM	Minute virus of mice
MSD	Mean square displacement
NS1	Non-structural protein 1
NS2	Non-structural protein 2
ORI	Origin of replication
OriL	Left-hand side origin of replication
OriR	Right-hand side origin of replication
PCNA	Proliferating cell nuclear antigen
Rep	Replication protein of AAV
RBS	Rep binding sequence of AAV
SF1-6	Helicase superfamilies I-VI
SV40	Simian virus 40
Tf	Transferrin
TfR	Transferrin receptor 1
VP1	Viral capsid protein 1
VP2	Viral capsid protein 2

1 INTRODUCTION

The cell is the fundamental unit of life. Cell division leads to new entities, in the case of unicellular organisms, or increases cell number in multi-cellular organisms, and it is the only way to create life. Cells adapt to the changes in their surrounding environment. The accumulating gradual changes have lead to different life forms through a process known as evolution. In addition, there are small non-living factors that widely affect life and evolution: viruses. These obligatory parasites take advantage of their host organisms in multiple ways in order to prevail and reproduce. Most viruses are very selective of their host organisms and cell types therein. Even though viruses are usually harmful to the host, they are also recognized as a major driving force in evolution. Indeed, the diversity of life would be greatly diminished in a world without viruses.

Viruses come in all sizes and shapes. Some, such as mimivirus, have large genomes with hundreds of genes, while others are more modest. Parvoviruses can be considered minimalists, as they only have two genes: one for producing protein capsid and the other for proteins that assist in the replication of their genome. Irrespective of their size, all viruses utilize their host organisms in multiple ways and thus can be used to investigate the function of a cell. This approach has been very successful in discovering fundamental cellular processes. As tools in cell biology, viruses are truly invaluable.

In this thesis, canine parvovirus (CPV) infection was studied in cell culture conditions. This variant of a cat virus, feline panleukopenia virus (FPV), emerged in late 1970s, when it gained the ability to enter dog cells. At the cellular level, the infection starts when CPV binds cell surface receptors and is internalized by clathrin-mediated endocytosis. Virus particles escape from the endosomes and continue their journey to the cell nucleus, where the viral genome is released. CPV has a linear single-stranded DNA genome with two genes: the hallmark of parvoviruses. The first gene produces replication proteins. The second gene produces proteins for capsid, which protects the viral genome and enables entry into the cell. The replication protein NS1 has evolved to be multifunctional, perhaps to compensate for the genomic size limits that are imposed by the small capsid. This thesis gives insight into the function and

intranuclear dynamics of the NS1 protein. In addition, it covers some of the drastic changes that take place inside the cell nucleus during CPV infection.

2 REVIEW OF THE LITERATURE

2.1 Family Parvoviridae

The first virus that we now recognize as a member of the family *Parvoviridae* was discovered in 1959 from an artificial rat tumor (Kilham & Olivier 1959). In the following years, similar viruses were found in other vertebrates (e.g., LuIII, H-1, FPV) and also later in invertebrates (Meynardier et al. 1964). The name parvovirus (latin *parvo*, meaning small) was first suggested in 1966 (Brailovsky 1966), and the taxonomic family name *Parvoviridae* was accepted in the 1970s by International Committee on the Taxonomy of Viruses (ICTV). Members of this family are defined as non-enveloped, small, icosahedral viruses with linear single-stranded DNA (ssDNA) genomes. Today, this family is divided in two subfamilies based on the host range, *Parvovirinae* and *Densovirinae* (ICTV). The former subfamily contains viruses that infect vertebrates, and the latter viruses that infect invertebrates. The subfamily *Parvovirinae* is further divided into five genera (*Parvovirus*, *Erythrovirus*, *Dependovirus*, *Amdovirus* and *Bocavirus*; Fig. 1) and *Densovirinae* is divided into four genera (*Densovirus*, *Iteravirus*, *Brevidensovirus* and *Pefudensovirus*), and together, they contain 37 species (Fauquet et al. 2005). Most members of the *Dependovirus* genus need a helper virus to undergo replicative infection, but without a helper, they can integrate into host genome to establish latency (Atchison et al. 1965, Berns et al. 1975). Other members of *Parvovirinae* are able to infect host cells independently of helper virus. These autonomous parvoviruses are, however, dependent on S-phase-specific cellular factors for their DNA replication, limiting infection to dividing cells (Siegl et al. 1985, Tattersall & Bratton 1983, Tennant et al. 1969).

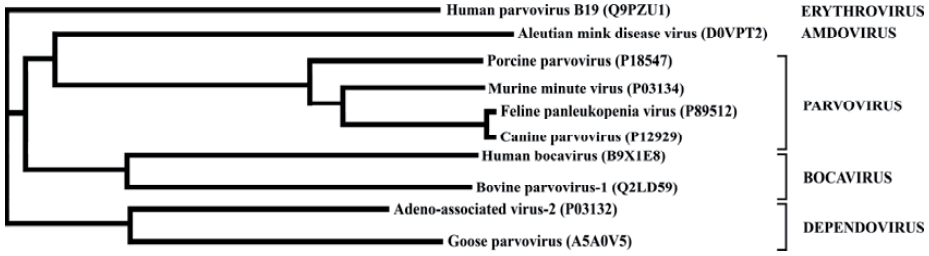

FIGURE 1 Taxonomic tree of *Parvovirinae* subfamily. Tree is based on gene sequences of the non-structural protein and was generated using clustalW (Chenna et al. 2003) at a protein information resources website (Wu et al. 2003) (http://pir.georgetown.edu/). Virus names, UniProtKB accession codes, and viral genera are shown.

2.1.1 Canine parvovirus

Canine parvovirus (CPV) is a dog virus that causes severe symptoms, including gastroenteritis and myocarditis, especially in young dogs (Studdert 1989). The first reported case occurred in 1978 in the USA (Appel et al. 1978). Serological samples, however, indicate that the virus was already present in Europe in 1976 (Schwers et al. 1979) and perhaps as early as 1974 (Koptopoulos et al. 1986). In the following two years after the identification, infection had spread worldwide (Carmichael 2005).

Sequencing of the viral genome (Reed et al. 1988) revealed that CPV is very closely related to feline panleukopenia virus (FPV); only a few mutations were needed to change the specificity from cat to dog (Chang et al. 1992, Parrish et al. 1988). The original CPV strain was named CPV-2 to distinguish it from another more distantly related dog virus: minute virus of canines (CPV-1). Subsequently, new variants have emerged. Appearance of the first variant, CPV-2a, was confirmed in 1984 but had probably been around already from 1979 (Carmichael 2005). Original CPV-2 strain did not infect cat cells, but CPV-2a had regained this ability (Truyen & Parrish 1992). This strain was further mutated to CPV-2b in 1984 (Parrish et al. 1991), and these two variants currently prevail (Hoelzer et al. 2008). The main differences between CPV strains are in the capsid gene (Hoelzer et al. 2008).

Primary CPV infection in dogs occurs by oronasal route, followed by infection of lymphatic tissue of the oropharynx, thymus, spleen, and bone marrow (Carman & Povey 1985). Infection also spreads to gut-associated lymphoid tissue and epithelial cells of small intestine after day six (Carman & Povey 1985). At the cellular level, CPV infection is fairly well characterized. Like its predecessor, FPV, it binds to cell membrane transferrin receptor-1 (TfR), which is necessary for the infection to proceed (Parker et al. 2001) (Fig. 2A). CPV also has some affinity for cell surface sialic acids, but apparently this interaction is not mandatory for infection (Barbis et al. 1992). TfR is a type II membrane protein, and it functions in a cell to bind and internalize iron-bound transferrin (Tf) protein. Normally the receptor is internalized constitutively, with or without Tf, by clathrin-mediated endocytosis, transported in Rab5-Rab7

endosomes, and recycled back to the cell membrane in Rab11-positive vesicles (reviewed in Aisen 2004). Up to two iron-loaded Tf molecules are bound to TfR at neutral pH. They traverse with TfR to endosomes and release iron in lower-pH vesicles. Most of the Tfs recycle back to the cell membrane in 30 minutes (reviewed in Aisen 2004). CPVs, however, remain bound to TfR for up to 2 h after internalization and are released from late endosomes or lysosomes (Parker et al. 2001) (Fig. 2B-D). TfRs are known to be rerouted to similar endosomal pathways when cross-linked with antibodies or in the presence of chemically cross-linked Tf molecules (Marsh et al. 1995). These results suggest that CPV infection might induce cross-linking of TfRs. Endosomal release of CPV is mediated by an N-terminal domain that is unique to VP1. This domain is exposed during the entry process (Suikkanen et al. 2003b) (Fig. 2D, E). The N-terminal domain of VP1 contains a nuclear localization signal and a phospholipase A_2 enzymatic active domain, which is vital for endosomal release by a poorly understood mechanism (Farr et al. 2005, Vihinen-Ranta et al. 2002, Zadori et al. 2001). After the escape from endosomes, viruses are transported to the nucleus using microtubules and dynein (Suikkanen et al. 2003a) (Fig. 2F, G). CPV enters the nucleus in intact form, presumably through nuclear pore complexes (Vihinen-Ranta et al. 2000). It is also speculated that parvoviruses could utilize microtubules, which are in direct interaction with nuclear pore complexes, to enter the nucleus (Vihinen-Ranta et al. 2000). CPV interaction with microtubules *in vitro* supports this idea (Suikkanen et al. 2003a). Some studies suggest that parvoviruses could induce pores to nuclear envelope, but the relevance of these findings in infection still need to be clarified (Cohen et al. 2006).

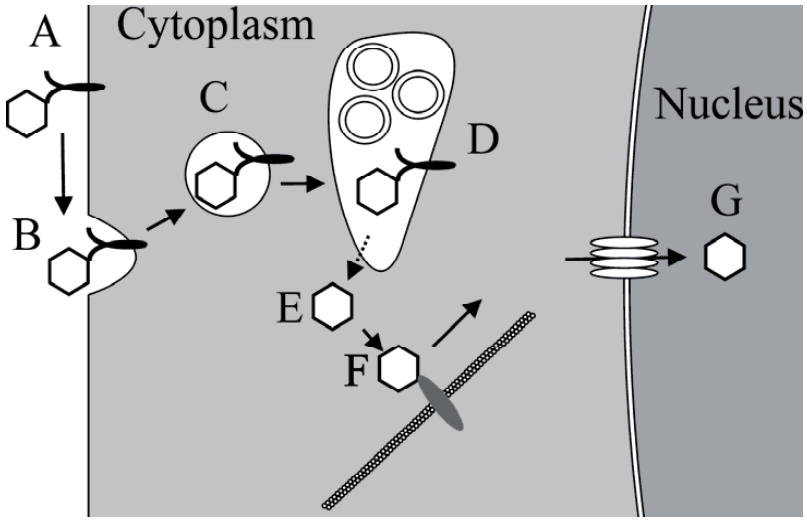

FIGURE 2 CPV entry. CPV binds cell-surface transferrin receptor (A) and is internalized via clathrin-mediated endocytosis (B, C). Capsids escape from late endosomes or lysosomes (D, E) and are transported to the nucleus, presumably utilizing microtubules and dynein motors (F, G). Image according to Vihinen-Ranta & Parrish (2006).

2.2 Parvovirus capsid structure and transcriptional regulation

Autonomous parvovirus virions consist of a viral capsid that is composed of viral proteins VP1 and VP2 and an encapsidated ssDNA genome.

2.2.1 Structure of canine parvovirus capsid

Parvoviruses have three capsid proteins, VP1-3, which are products of the same gene and have identical C-terminal domains. VP1 is the largest capsid protein, and compared to VP2, it has an additional 143-amino-acids-long phospholipase-A2 enzymatic activity-containing domain in its N-terminus (Zadori et al. 2001). VP3 is the smallest capsid protein and is ~19 amino acids shorter than VP2 (Weichert et al. 1998). Changes in the lengths of the N-termini are due to different splicing of the mRNA and proteolytic cleavage of VP2 and VP3, respectively (Clinton & Hayashi 1976, Tattersall et al. 1977). VP1 is a minor capsid protein in all parvoviruses (~10% of capsid proteins in CPV), and ratio of VP2 and VP3 depends on the virus species and the maturation of the virion (Chapman & Agbandje-McKenna 2006).

Autonomous parvovirus capsids are small – only 18-25 nm in diameter. Their protein shell is composed of 60 copies of capsid proteins, which are arranged in T=1 icosahedral symmetry (Fig. 3) (Berns 1990). The first and, thus far, best-studied autonomous parvovirus capsid structure is that of CPV. It was solved in 1991 (Tsao et al. 1991), and since then, several structures of empty and DNA-containing capsids have been solved (Simpson et al. 2000, Xie & Chapman 1996). Only the C-terminal region, common to all VP proteins, is seen in available structures. This domain forms an eight-stranded β-barrel-fold (Fig. 3A; "viral jelly-roll"), similar to many other icosahedral viruses (Rossmann & Johnson 1989, Tsao et al. 1991). Compared to the smooth capsid surface generally found among the members of *Densovirinae*, those of *Parvovirinae* have usually rough capsids. Fivefold symmetry axes of CPV capsids (Fig. 3C; pentagon) have ~14-Å holes and are aligned by 9-Å-deep canyons (Tsao et al. 1991). The threefold axes (Fig. 3C; center of the triangle) have 22-Å-long protrusions, and two fold axes (Fig. 3C; two parallel lines) have 15-Å-deep canyon (Tsao et al. 1991).

Virus-like particles can be formed from VP2 proteins in minute virus of mice (MVM) and CPV, confirming that neither VP1 nor viral DNA is essential for capsid formation (Hernando et al. 2000, Singh et al. 2006). As suggested for MVM, capsid assembly of parvoviruses occurs in two stages. Upon translation, VPs form trimers in the cytoplasm (Fig. 3B). The trimers are subsequently transported to the nucleus, where they are assembled into capsids (Fig. 3C) (Riolobos et al. 2006). The genome is presumably packed from a fivefold pore to the preformed capsids in the nucleus (Bleker et al. 2005, Bleker et al. 2006, Farr & Tattersall 2004). As supported by the 3'-to-5' orientation of the genome packing, the necessity of ATPase activity of the replication protein, and its interaction with capsid in AAV, parvovirus genome packing is suggested to be

driven by NS1 protein docked to the fivefold symmetry axis (Cotmore & Tattersall 2005a, King et al. 2001).

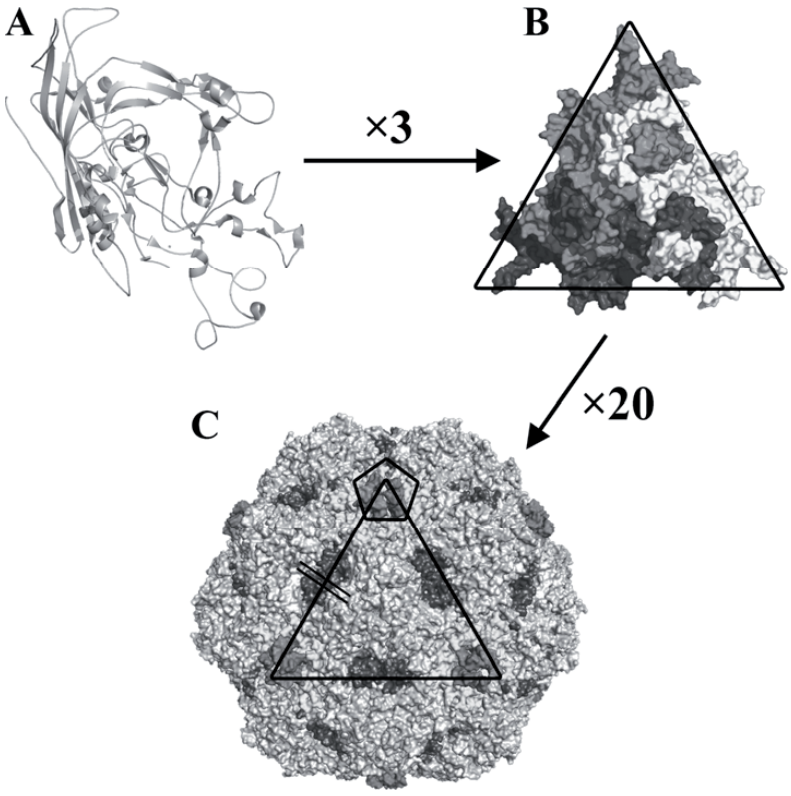

FIGURE 3 CPV capsid structure. Parvoviral capsid proteins have "viral jelly-roll" folds (A). Three capsid proteins form a tightly intertwined complex (B), which are thought to be precursors in the assembly of icosahedral capsid (C). The twofold symmetry axis is highlighted with parallel lines and the fivefold symmetry axis with a pentagon in C. CPV capsid images were produced with PyMol (Protein data bank accession code (PDB ID): 1C8D; Berman et al. 2003, Simpson et al. 2000).

2.2.2 Genome organization and transcription regulation in canine parvovirus

Parvoviruses have linear ssDNA genomes with only two genes, perhaps the simplest possible genomic structure (Fig. 4). Both ends of the canine parvovirus genome contain unique inverted terminal repeats (Reed et al. 1988). Terminal repeats are utilized in various steps of genome replication. The left-hand side of the genome (3'-end in (-)-sense viruses, e.g., CPV) contains gene for nonstructural proteins (NSs), while capsid proteins (VPs) are produced from the right-hand side (Fig. 4) (Reed et al. 1988). Both genes have unique TATA box-containing promoters, but they share a common poly-A sequence (Reed et al. 1988). Transcripts from the NS gene can be detected prior to the VP gene (Clemens & Pintel 1988). In CPV, the NS promoter P4 is in the untranslated

region of the left-hand side. In the closely related parvovirus MVM, this promoter is regulated by general transcriptional regulators through GC box element (Deleu et al. 1999, Pitluk & Ward 1991). P4 is strongly controlled by the cell cycle-dependent transcription factors E2Fs: it is most active in S phase and strongly down regulated in G1 phase (Deleu et al. 1999). To emphasize effective use of the genetic material, the VP promoter P38 is embedded in the coding region of the NS proteins (Reed et al. 1988). This promoter contains a GC box transcriptional regulation element, which is located 16 nucleotides upstream of the TATA box in CPV. In addition, there is a transactivation-responsive (tar) element for NS1-mediated transcriptional regulation (Gu et al. 1992, Rhode & Richard 1987, Storgaard et al. 1993). Originally identified in H1 parvovirus, the minimal tar element was defined as a 19-nucleotide element located 116 nucleotides upstream of the P38 promoter (Rhode & Richard 1987). Depending on parvovirus strain, NS1 enhances transcription from P38 up to 1000-fold, and thus the promoter reaches its full activity only at the later stages of infection (Storgaard et al. 1993). Complete NS1-induced transactivation of P38 requires additional upstream elements in some parvoviruses, but CPV does not appear to belong to them (Storgaard et al. 1993). There is also evidence of an NS1-controlled transcriptional element downstream of the transcription initiation site, but the role of this interaction in CPV has not been studied (Krauskopf et al. 1990).

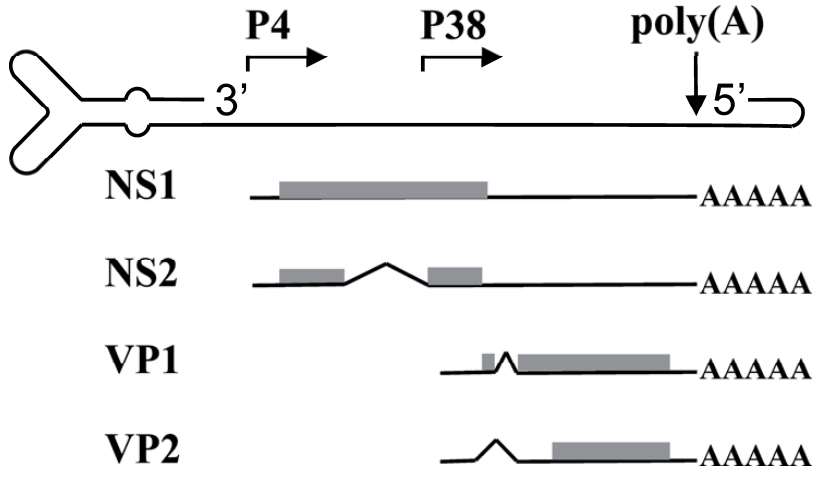

FIGURE 4 CPV genomic organization. The CPV genome is single-stranded DNA with unique inverted terminal hairpins at both ends. Both CPV genes have their own promoters: P4 for non-structural proteins, NS1 and NS2, and P38 for capsid proteins, VP1 and VP2. Both genes utilize the same poly(A) sequence, which is located before the right-hand side terminal hairpin. Four different mRNAs are produced in CPV infection. NS1 mRNA is the longest and spans the whole coding region of the genome (~4.9 kb). NS2 is a splice variant of NS1, and its latter exon is from a different reading frame than NS1 (~3.3 kb). VP1 has a small (~3.0 kb) and VP2 has a larger intron, which removes the first start codon of the VP ORF (~3.0 kb). Image according to Reed et al. (1988).

2.3 Replication of parvoviruses

2.3.1 Genome replication

Parvoviruses are transported through the cell to the nucleus, where their genome replication takes place. The viral genome is released from the virion in the nucleus by a largely unknown mechanism. The 3′-end of the genome is exposed from the capsid, and large amounts of the genome can be artificially ejected from the capsid in 3′-to-5′ direction with ion depletion at physiological temperatures (Cotmore et al. 2010). This finding suggests a possible mechanism of genome release, where cellular cues could trigger the release of the genome and initiate its replication (Cotmore et al. 2010). Replication of the parvovirus genome is a biphasic process, where the initial ssDNA genome is first replicated into concatenated replicative form (RF) double-stranded DNA (dsDNA) and subsequently multiplied to correctly sized single-stranded genomes (Bashir et al. 2000, Cotmore & Tattersall 2005b). The single-stranded genome inherently possesses a problem – it can not be used directly to transcribe viral proteins (Fig. 5A). Only when the host cell progresses to the S phase is the endogenous genome replication machinery activated and able to convert the viral genome into covalently closed RF dsDNA (Fig. 5B)(Cotmore & Tattersall 1987, Wolter et al. 1980). This process is independent of polymerase α primase activity and utilizes a free 3′-hydroxyl group on the left-hand side terminal hairpin as a primer (Christensen et al. 1997a, Cotmore & Tattersall 1987). The initial conversion to RF dsDNA is dependent on cyclin A, which controls G1/S transition, and is most likely carried out by polymerase δ in a process that is dependent on the sliding clamp protein proliferating cell nuclear antigen (PCNA) and the single-strand-binding protein replication protein A (RPA), but independent of viral proteins (Bashir et al. 2000). The RF dsDNA can be used to transcribe viral genes, and the production of NS1 is mandatory for subsequent steps. Transcription from the NS1 promoter P4 is limited to S phase cells, and together with the activation of host replication machinery, these factors are thought to control the initiation of viral replication (Deleu et al. 1999). NS1 recognizes a specific sequence, termed OriR, near the end of the right-hand side of the RF dsDNA genome (Cotmore et al. 2000) (Fig. 5C). This process is dependent on endogenous high mobility group (HMG) proteins and leads to site- and strand-specific nicking of the RF dsDNA (Cotmore & Tattersall 1998). HMGs are a large family of chromatin-modifying proteins that can bind DNA either alone or in complex with other proteins (reviewed in Bianchi & Agresti 2005). They can recognize DNA either sequence-specifically or -non-specifically (Bianchi & Agresti 2005). The binding of an HMG to the DNA leads to modification of the chromatin structure by bending the strands into specific configuration. This altered DNA conformation affects many cellular processes (reviewed in Bustin 1999). In the AAVs of the genus *dependovirus*, the replication protein (Rep), equivalent of NS1, interacts directly with HMG1, leading to its enhanced DNA binding and site-specific nicking (Costello et al.

1997). In autonomous parvoviruses, a model has been suggested, where HMG1/2 activates the nicking activity of NS1 by modifying the structure of the DNA near the NS1 recognition site at the right-hand side origin of replication (OriR) (Cotmore & Tattersall 1998). Initial nicking of the OriR sequence is followed by formation of the first concatenated duplex RF DNA form (Fig. 5C-G). NS1 functions as a helicase to unwind the terminal hairpin structure, and strand synthesis is completed by endogenous replication machinery (Fig. 5D). The self-priming 3'-end of the terminal hairpin (Fig.5 E) is used as a primer, and a new replication complex is formed (Fig. 5F). The whole complementary strand is subsequently synthesized, revealing a new NS1 recognition site at the left-hand side of the genome (OriL) (Fig. 5G). The complex of NS1 and the endogenous replication factor GMEB-1 (glucocorticoid modulatory element binding protein 1) is formed at the OriL, leading to site- and strand-specific nicking of the DNA (Fig. 5G). Subsequent multiplication of the genome occurs in a repetitive, unidirectional strand elongation process, where OriL nicking and DNA synthesis from a residual 3'-hydroxyl group lead to accumulation of positive-sense ssDNA genomes (Fig. 5H).

Parvoviruses differ in their ability to package positive- and negative-sense genomes (Bates et al. 1984, Cotmore & Tattersall 2005b). Virions of most members of the genus *Parvovirus* contain mainly negative-sense genomes. Packaging sense is not controlled by specific packing signals but rather by differences in the genome replication efficacy. Most parvoviruses selectively produce more negative-sense genomes compared to positive ones. This selectivity is a result of differences in the nicking and in the hairpin formation of the OriL and OriR, respectively (Cotmore & Tattersall 2005b). Positive-sense genomes that are the end-products of unidirectional strand elongation processes (Fig. 5) preferentially form hairpins at OriR (Fig. 6A). This end contains a 3'-hydroxyl group and can be used to initiate replication (Fig. 6B-D).

Unlike OriL, the OriR can support NS1-mediated nicking in hairpin conformation (Fig. 6E), which is necessary to complete the synthesis of the genomic end (Fig. 6F-G). Hairpin formation at the OriR leads to synthesis of a new – and concomitant displacement of the old – negative-sense genome (Fig. 6H, I, A). Parvoviruses are packing newly synthesized genomes, which leads to sequestration of the negative-sense ssDNA (Fig. 6J) (Cotmore & Tattersall 2005b). The cyclic complementary strand synthesis leads to alternating forms of right-hand ends of both ssDNA genomes, termed flip and flop, which are inverted complements of each other (Astell et al. 1985, Cotmore & Tattersall 2005b).

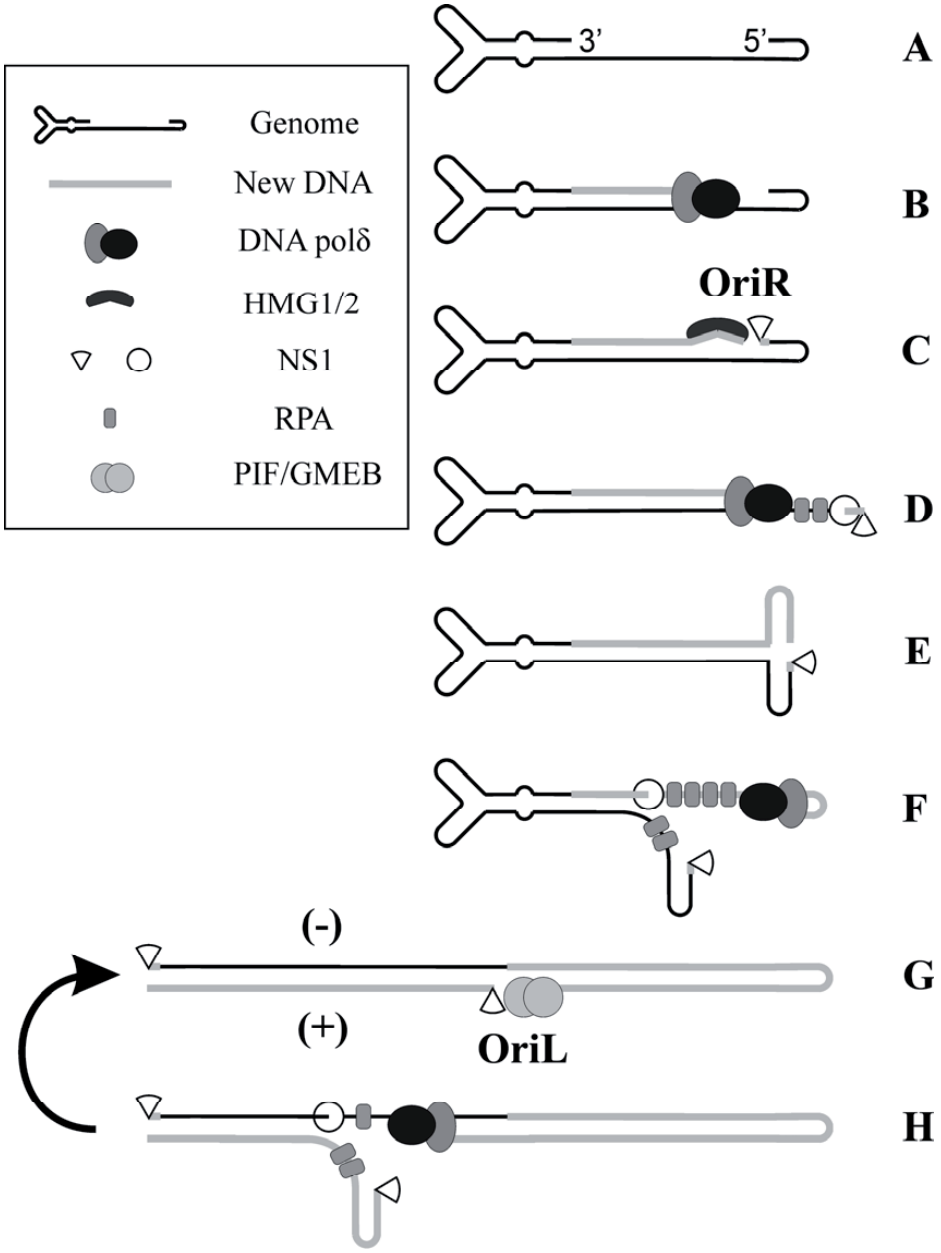

FIGURE 5 Parvovirus genome replication: from incoming ssDNA to replicative form dsDNA. The Parvovirus ssDNA genome is released into the nucleus (A). Activation of host replication leads to synthesis of monomeric RF dsDNA (B). Nicking from the OriR requires NS1 and HMG1/2 proteins (C), and enables the completion of the second-strand synthesis (D). Terminal hairpin (E) is used as a primer for the next round of virus genome replication (F). Duplex replicative form DNA (G) contains a new recognition site for the NS1 and GMEB proteins (OriL). Nicking and strand synthesis lead to accumulation of positive-sense viral genomes (H). Figure according to Cotmore & Tattersall (2005b).

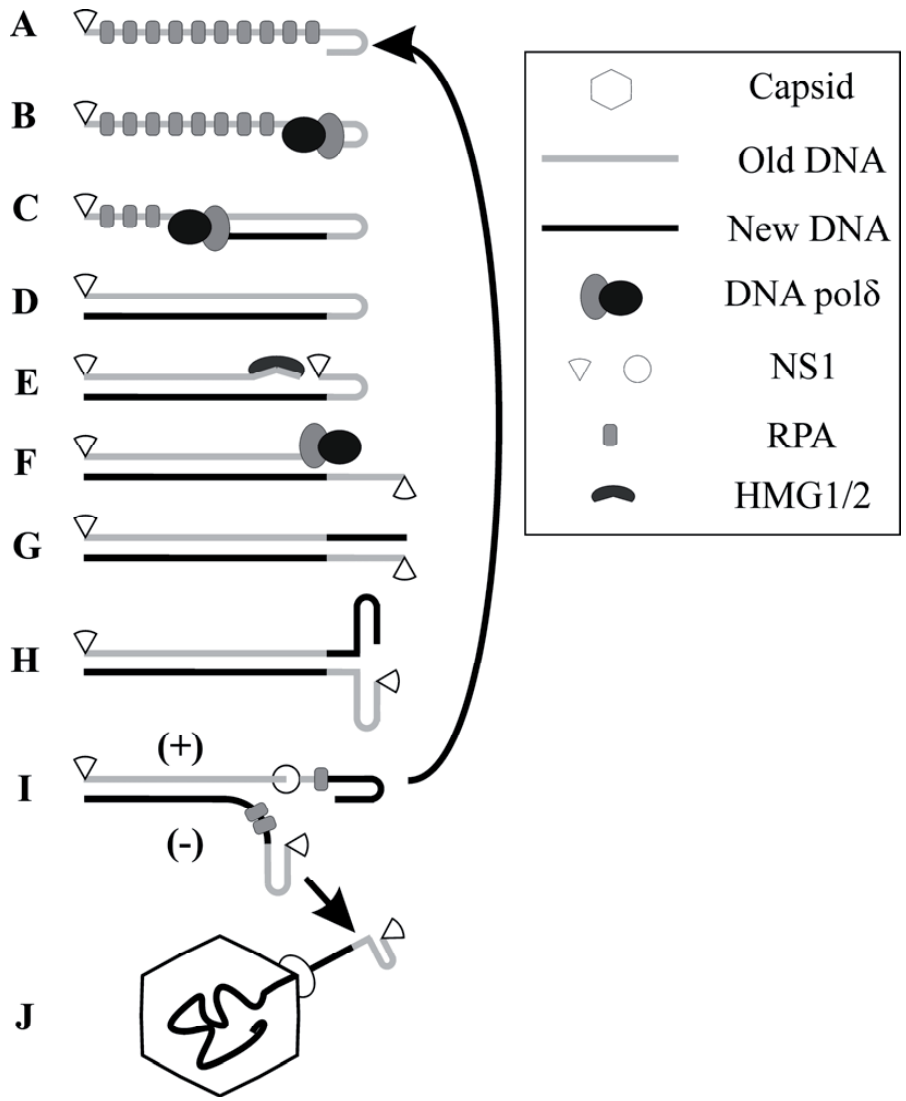

FIGURE 6 Parvovirus genome replication: multiplication of negative-sense genomes. The positive-sense genome from unidirectional strand displacement synthesis preferentially forms hairpins with the OriR (A), which can be used to synthesize a complementary strand (B-D). The OriR nick-site is also active in this hairpin conformation (E), enabling the replication of the genome end (F-G). OriR hairpin formation initiates displacement of the newly synthesized negative-sense genome and a new round of synthesis (I, A). Newly formed genomes are presumably packed to preformed capsids (J). Alteration of the right-hand end sequence between synthesis rounds ("flip-flop") is indicated with black and gray colors.

2.3.2 Autonomous parvovirus replication complex

Autonomous parvovirus infection induces multiple changes in the nuclear architecture. Specific parvovirus-induced structures, termed autonomous

parvovirus replication (APAR) bodies, are formed during the infection. In synchronized cells, APAR bodies first emerge as multiple small foci in the interchromosomal domains at ~12 h after the release from cell cycle arrest (Cziepluch et al. 2000, Ihalainen et al. 2007). In CPV infection, these structures mature to fill the most of the nucleus at ~24 h post-infection (Ihalainen et al. 2009). APAR bodies are defined by the existence of NS1 and ongoing replication of viral genome (Cziepluch et al. 2000). The minor nonstructural protein NS2 concentrates in the APAR bodies but it is also found in the nuclear envelope and cytoplasm (Engelsma et al. 2008, Young et al. 2005). Neither NS1 nor NS2 expression alone is able to form APAR bodies and expression of NS2 is not essential for APAR formation (Young et al. 2005). Besides viral components, many endogenous proteins localize to APAR bodies. They include components of the replication machinery – PCNA, polymerase δ, and RPA – and also proteins with no known function in viral genome replication: polymerase α, cyclin A, small glutamine-rich tetratricopeptide repeat -containing protein, and splicing factor (SR) (Bashir et al. 2001, Cziepluch et al. 2000, Young et al. 2005). Incorporation of the RNA processing survival of motor neuron (SMN) protein is also reported in MVM APAR at later time points (Young et al. 2005).

2.4 Life of DNA-binding proteins

Parvoviral replication proteins or non-structural (NS) proteins carry out multiple functions during the viral life cycle. NS proteins are further divided into two, NS1 and NS2, based on their size. Different parvoviruses have varying number of essential NS proteins. Interestingly, CPV infection is only dependent on the large NS protein, NS1. NS1 and equivalent Rep proteins in AAVs contain a helicase domain with conserved ATP-binding motifs. Besides sequence-independent helicase activity, NS1 proteins also function in dsDNA recognition and nicking and regulate the transcription of promoters. Large AAV Rep proteins also have dsDNA recognition and nicking activities and in addition are able to mediate the integration of viral and host genomes.

This chapter reviews general properties of DNA-binding proteins, concentrating on their binding and dynamics in the nucleus, sequence recognition, and function of helicases.

2.4.1 Mobility of nuclear proteins: diffusion, beyond and below

The proper function of many nuclear proteins – e.g., transcription factors and core-histones – is dependent on their ability to bind DNA either in a specific or non-specific manner. The first requirement for the binding is, however, the encounter of the interacting molecules. Most proteins move in the nucleus by diffusion, which sets the upper-limit for binding reactions. In eukaryotic cells, protein-DNA interactions usually take place in the nucleus which has increased

viscosity compared to water, due to the high concentration of chromatin and many other molecules including proteins, sugars, and RNAs (Fulton 1982). Due to the random nature of the diffusional movement, the average displacement of a molecule is zero, as observation time increases. Hence, the diffusional mobility is described with the mean square displacement (MSD) of the molecule (Wachsmuth et al. 2008). This can be further related to the diffusion coefficient (D):

$$MSD = \left\langle \left[r(t+\tau) - r(t) \right]^2 \right\rangle = 2nD\tau \quad (1)$$

Here, r(t) and r(t+τ) are positions of the molecule before and after time step τ, n is the dimensionality of the system, and D is the diffusion coefficient. On a macroscopic scale, the diffusion coefficient is inversely proportional to the viscosity (η) of the surrounding medium and to the hydrodynamic radius of the molecule (R), according to Stokes-Einstein relation:

$$D = \frac{kT}{6\pi\eta R} \quad (2)$$

Here, D is the diffusion coefficient, k is the Boltzmann constant (~1.38×10^{-23} J/K), T is temperature, η is viscosity, and R is the hydrodynamic radius of the protein (Beaudouin et al. 2006, Sprague et al. 2004). If we assume that the R of the globular protein is related to the cubic root of the molecular mass (Wachsmuth et al. 2008), the diffusion coefficient in three dimensions for a 30-kDa protein in water is ~130 μm^2/s (Fig. 7A). The average distance that the protein travels in a given time is approximately the square root of the diffusion coefficient; a protein with D=130 μm^2/s is moving on average ~11 μm to a random direction in each second. Nuclear viscosity has been estimated to be approximately ~3-4-fold higher than in water (Beaudouin et al. 2006, Lukacs et al. 2000, Seksek et al. 1997). For an inert protein with a molecular mass of 50 kDa, this estimation leads to a nuclear diffusion coefficient of ~30 μm^2/s or movement of ~5.5 μm/s (Beaudouin et al. 2006). The intracellular and nuclear diffusion coefficients are estimated to be between 10-100 μm^2/s for inert particles, depending on their size and shape (Fig. 7B) (Gorisch et al. 2005). The measured diffusion coefficient for an inert 30-kDa protein in the nucleus is ~50 μm^2/s (Dross et al. 2009). This is enough to cover the entire nuclear volume on a time-scale of minutes (Gorski et al. 2006). In addition to viscosity, the mobility in the nucleus is hindered by the chromatin network in a size-dependent manner. Large molecules are either immobile (Gorisch et al. 2005) or confined to diffuse in small volumes (Tseng et al. 2004).

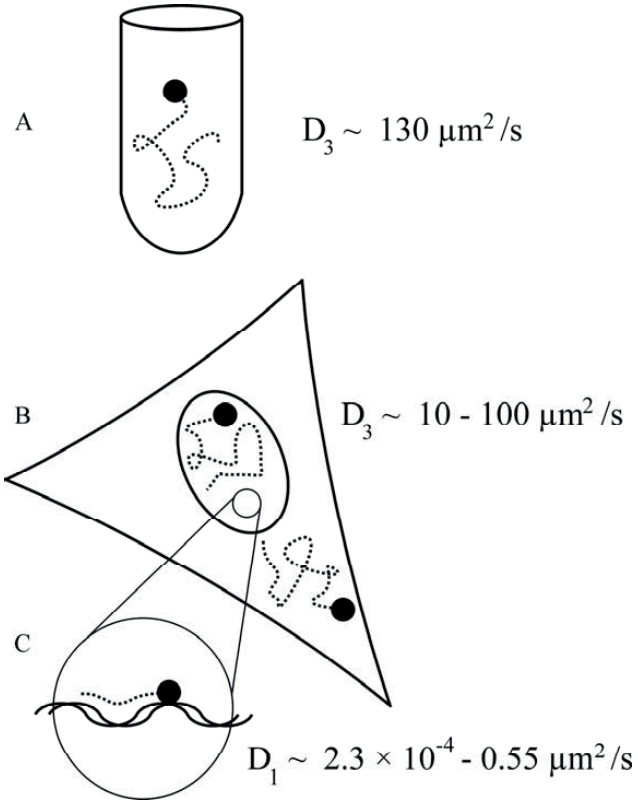

FIGURE 7 The diffusion coefficient is affected by the environment. Diffusion in aqueous buffers is dependent on the temperature and viscosity of the liquid (A). Inside the cell, viscosity increases and macromolecular concentration increases to crowding concentrations. This leads to markedly lower diffusion coefficients (B). Some proteins have natural a affinity for DNA and can engage in 1D diffusion along DNA strands (C).

Protein dynamics in cells is usually studied using the fluorescence recovery after photobleaching (FRAP) technique (Phair & Misteli 2000, Sprague & McNally 2005). In FRAP, the distribution of the fluorescent molecules is perturbed locally by irreversible photobleaching. The recovery of the fluorescence is then monitored and used to estimate the dynamic properties of the molecules. In some cases, the quantitative analysis of the obtained data can be done by using analytical mathematical models (Axelrod et al. 1976, Braga et al. 2004, Sprague et al. 2004) or by using numerical simulations (Ihalainen et al. 2009, Phair & Misteli 2001, Schaff et al. 1997). Both approaches are usually dependent on initial assumptions of the diffusion coefficient of the protein. FRAP studies have shown that the mobility of many nuclear proteins is much slower than equally sized inert particles. In many cases, the decreased mobility is a consequence of binding reactions with immobile targets; e.g., chromatin in the case of DNA-binding proteins (Phair et al. 2004).

2.4.1.1 Anomalous subdiffusion

A closer inspection of the diffusion in the cytoplasm and in the nucleus has revealed abnormal behaviour (Wachsmuth et al. 2000, Weiss et al. 2004). In the case of free diffusion, the growth of an MSD of a particle in time is linear:

$$MSD \propto t^{\alpha}, \alpha = 1 \quad (3)$$

For inert particles in the cells, however, the growth of an MSD in a time is non-linear and slower than in free diffusion ($\alpha<1$, in equation 3)(Wachsmuth et al. 2000, Weiss et al. 2004). This phenomenon, termed anomalous subdiffusion, is speculated to arise from the molecular crowding caused by high molecular concentrations in the cell (Bancaud et al. 2009, Weiss et al. 2004). Molecular crowding also causes increased viscosity in the cell, and it has strong effect on many processes including binding, protein folding, and enzyme kinetics (reviewed in Ellis 2001).

2.4.1.2 Facilitated diffusion

Proteins that bind a DNA sequence specifically – e.g., transcription factors – have to find their target from a large amount of DNA. This process would be very inefficient by pure 3D diffusion due to the huge size of the genome, and it has been long known that proteins are able to accomplish this task much faster (Berg et al. 1981, Riggs et al. 1970). There are several mechanisms by which proteins can facilitate target finding processes compared to 3D diffusion. Presumably due to excessive van der Waals contacts and small diffusion constants of macromolecules, protein-DNA collisions in solution are not elastic (von Hippel & Berg 1989). After an initial encounter, molecules are able to rotate and explore for more favorable interaction surfaces in a series of small-scale collisions (von Hippel & Berg 1989). In the case of DNA-binding proteins, initial transient interactions can lead to changes in the binding mode, which is speculated to help find targets. They include sliding or hopping along DNA fibers, and intersegment transfer, where a protein is able to change between two nearby DNA fibers (Berg et al. 1981) (Fig. 8). Sliding and hopping lead to protein translocation along the DNA on a small scale. Intersegment transfer, however, can transfer a protein to any location in the DNA as long as it is spatially near the original binding site (reviewed in von Hippel & Berg 1989). Importantly, all of these mechanisms operate without full dissociation of the protein from the DNA fiber and, thus, without 3D diffusion and re-binding (Berg et al. 1981). Theoretical analysis of these mechanisms suggests that they can all lead to facilitated target localization when compared to 3D diffusion (reviewed in von Hippel & Berg 1989, von Hippel 2007).

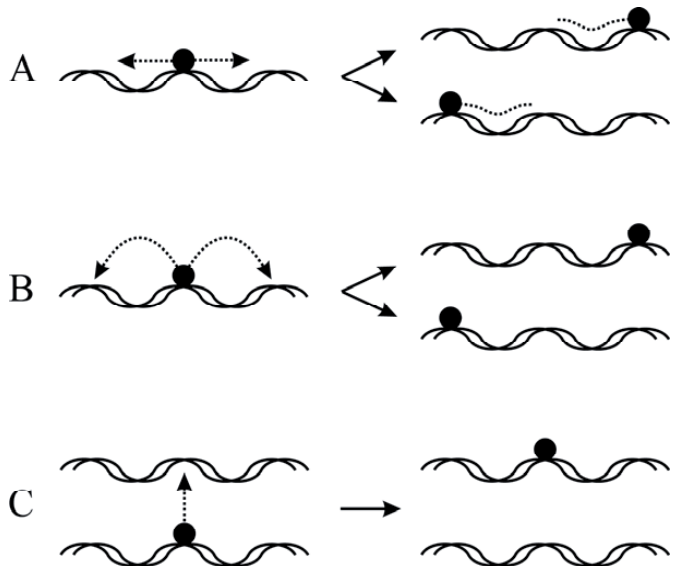

FIGURE 8 Facilitated diffusion in DNA binding. Different modes of facilitated diffusion include sliding or 1D diffusion along the DNA (A), hopping along the DNA (B), and intersegment transfer (C).

Sliding movements has been experimentally demonstrated for many proteins, and is likely to be common to most, if not all, proteins that need to find their specific target from the excess of similar sequences (Barkley 1981, Ehbrecht et al. 1985, Jeltsch et al. 1994). Recent *in vitro* single-molecule measurements of different protein species have revealed a wide distribution in 1D diffusion coefficients (Fig. 7C). Measured values for the transcription factor LacI span the range of 2.3×10^{-4} – 1.3×10^{-1} $\mu m^2/s$, and it is able to travel 120 - 2920 nm (or 390-8600 bp) of DNA in facilitated diffusion mode (Wang et al. 2006). Using mean values of ~2.1×10^{-2} $\mu m/s$ and ~500 nm (~1,500 bp) for the 1D diffusion coefficient and the distance, respectively, the facilitated diffusion is estimated to increase the binding rate of LacI by ~90 times, when comparing with 3D diffusion (Wang et al. 2006). This has very good correlation with the original ~100-times-faster binding rate measured for LacI (Riggs et al. 1970). An even faster 1D diffusion rate constant of 0.55 ± 0.13 $\mu m^2/s$ has been measured for the base-excision repair initiation protein, oxoguanine glycosylase (Blainey et al. 2006). This value, which approaches the theoretical limit, can be at least partly attributed to the small size of the protein (Blainey et al. 2006). Interestingly, proteins that use ATP for DNA binding and active translocation can also utilize ATP-independent 1D diffusion. Eukaryotic Rad51 is a RecA-like recombinase protein, and it assembles as a stable helical filament on ssDNA overhangs to facilitate recombination in homologous recombination of dsDNA breaks. Rad51 can also bind dsDNA, presumably in octameric ring conformation, and engage in 1D diffusion with a diffusion coefficient of 0.001 - 0.21 $\mu m^2/s$ (Graneli et al. 2006).

In the above examples, proteins use facilitated diffusion for locating their target sequences from an excess of bulk DNA. The binding rate in these cases is enhanced by increasing the encounter probability by reducing diffusion dimensions. Facilitated diffusion might also play a part in other important reactions. In some cases (e.g., in promoter binding of RNA polymerase in transcription initiation), the association rate of the protein to the DNA is not the rate-limiting step, and hence the enhancement due to 1D sliding is less important. Instead, 1D diffusion is suggested to control the affinity for specific sites. This could be attained by either allowing or blocking diffusion near the promoter site in strong- and weak-binding promoters, respectively (reviewed in Shimamoto 1999). Interestingly, 1D diffusion also leads to counterintuitive behavior of specific protein-DNA interactions: up to a certain point, an increase in the non-specific DNA concentration enhances specific target sequence binding (von Hippel & Berg 1989).

2.4.2 Protein-DNA binding interaction: a closer look

One way to characterize protein-DNA interactions is through the specificity for the DNA sequence. Sequence-specific proteins include transcription factors and endonucleases that perform their function only when they encounter a specific DNA sequence. Non-specific binding is found in helicases and histone proteins, which function more or less equally irrespective of the DNA sequence. In general terms, these classes differ in their affinity and specificity for their targets. Binding affinity arises from the chemical complementarities of the interacting species, and it defines the dissociation rate for the binding reaction. Specificity is the ability to distinguish between binding partners, and it arises from the differences in the affinity for dissimilar targets; e.g., DNA sequences.

2.4.2.1 At the Protein-DNA interface

A survey of available structures has revealed that protein-DNA binding is governed by the same biochemical interactions as protein-protein complexes (Luscombe et al. 2001). However, the preferred interactions of protein-DNA are electrostatic, while in protein-protein interfaces, they are hydrophobic (reviewed in von Hippel 2007). Electrostatic interactions are very important for non-specific binding that leads to facilitated diffusion, as discussed above. The DNA strand is thought to form an isopotential surface, along which proteins can diffuse in search for their target sequence without dissociating (Winter & von Hippel 1981). Many DNA interacting proteins (e.g., transcription factors) need to switch from non-specific scanning of the DNA to the sequence-specific binding mode. The structural basis of this transition has began to emerge from the work done with LacI (Kalodimos et al. 2004). LacI uses the same interface for DNA binding in both specific and non-specific binding modes, but the role of the amino acids is different. The orientation of the protein relative to the specific complex changes, and specific contacts that are formed by critical amino acids with base pairs of the major groove change to electrostatic interactions with the phosphate backbone (Kalodimos et al. 2004). Compared to

unbound forms, the overall changes in the protein and DNA structure in a non-specific complex are minimal. The overall protein conformation resembles to that of non-bound LacI, and DNA remains in B-form conformation (Kalodimos et al. 2004). In a specific complex, DNA is in a bent conformation, and the protein adopts different, less dynamic conformation (Kalodimos et al. 2004). Similar results are also reported for type II restriction enzymes, suggesting a general mechanism in non-specific interactions (Winkler et al. 1993); minimal conformational changes, compared to unbound forms, enable weak binding that is necessary for 1D diffusion.

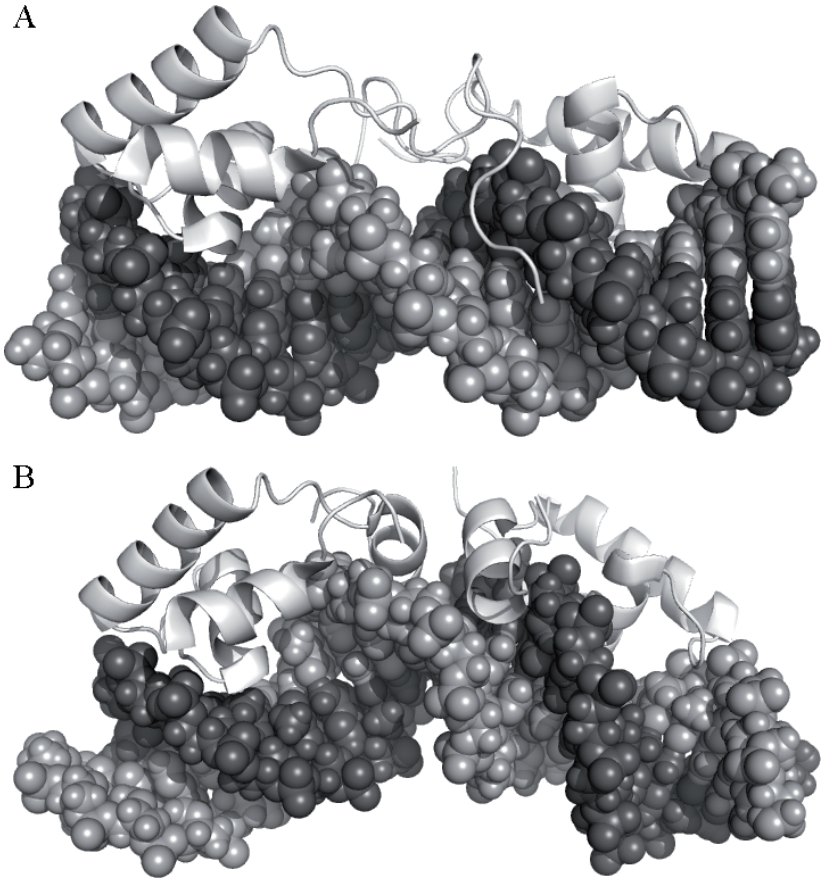

FIGURE 9 Non-specific and specific DNA interactions of LacI. When bound to a non-specific DNA sequence, LacI retains its unbound conformation and DNA remains in B-form (A). Upon sequence recognition, the LacI structure undergoes only small changes, while DNA is clearly bent (B). PDB IDs: 2KEI, operator O1 bound, and 1OSL non-specific. Images according to Kalodimos et al. (2004).

A closer look at specific interactions reveals that most protein-DNA interactions are made with the sugar-phosphate backbone of the DNA and are thus nonspecific and stabilizing in nature (Luscombe et al. 2001). Approximately

two-thirds of observed interactions in known protein-DNA structures are van der Waals contacts, usually with the DNA backbone. They are important for the stabilization of the protein-DNA complex and do not generally confer sequence specificity (Luscombe et al. 2001). The remaining protein-DNA interactions, with equal contributions, are hydrogen bonds and water molecule-mediated contacts (Mandel-Gutfreund et al. 1995). Sequence specificity is generally mediated by hydrogen bonds (Suzuki 1994). Depending on the amino acid, one or more hydrogen bonds can be formed with DNA bases or the phosphate-sugar backbone – especially, bidentate interactions where a single amino acid is forming multiple hydrogen bonds with either one or two DNA bases, are found from sequence-specific interactions (Suzuki 1994). Water molecules form interactions mostly to fill up cavities, but cases of specific water-mediated interactions are also known (Lawson & Carey 1993).

Overall the sequence specific protein-DNA interactions are considered to vary between protein families, and at the present, there is no universal code for describing recognition sequences from nucleic acids (Smith 1998). There have been several attempts to predict sequence-specific protein-DNA interactions. A common theme among them is to rely on statistical analysis of known interactions. Although some clear preferences between particular amino acids and bases have emerged, overall recognition appears to be redundant and flexible – and thus case-specific (reviewed in Sarai & Kono 2005).

2.4.2.2 Effect of the DNA structure in non-specific interaction

The structure of the DNA also affects the protein-DNA interactions. The dsDNA can be found in three helical formations: A, B and Z forms, of which B-DNA is the most common. Depending on the backbone conformation, it adopts the BI or BII form. The selection between these two is dependent on the DNA sequence, and it is controlled mainly at the di-nucleotide level (Heddi et al. 2010b). Positioning of the BI and BII conformations leads to altered DNA flexibility, which directly affects binding of many non-specifically binding proteins; e.g., core-histones (Heddi et al. 2010b, Kaplan et al. 2009) and DNaseI (Heddi et al. 2010a). Interestingly, small differences in DNA flexibility appear to be enough to guide the localization of the nucleosome core particles, which in turn leads to changes in transcription regulation (Heddi et al. 2010b).

2.5 Structure and function of parvoviral replication protein NS1

2.5.1 Sequence-specific recognition in parvoviruses

Sequence-specific dsDNA recognition is important for replication of the viral genome. In parvoviruses, the viral origin of replication (ORI) is recognized by N-terminal domain of the NS1 protein in autonomous parvoviruses and large Rep proteins in AAVs. In addition, the N-terminal domain of both NS1 and Rep contains a catalytic domain for strand- and site-specific nicking of the dsDNA.

Several structures of the N-terminal domain from the Rep protein are available, and they elucidate details of its interactions with DNA. The structures include two complexes with specific DNA sequences: the Rep binding sequence (RBS) and terminal hairpin.

2.5.1.1 Binding at the origin of replication in AAV

The inverted terminal repeat of AAVs contains a Rep binding sequence (RBS), which is a repeated sequence of four nucleotides (GCTC). The Rep-RBS structure has five N-terminal domains of Rep, bound individually to the RBS-containing dsDNA. The interval between domains is four base pairs, and they are revolving around the DNA axis with a ~138º turn (Hickman et al. 2004) (Fig. 10A). Interactions of each subunit are restricted to two major motifs: a loop between β-strands 4 and 5, which binds to the major groove, and the N-terminus of α-helix C, which binds to the minor groove (Fig. 10B). The most notable change in the protein structure, compared to the unbound structure, is the bending of the β3/β4-loop towards the major groove. This allows contacts of the two lysines (K137 and K138) and the backbone carbonyl of glysine (G139) with the DNA (Fig. 10B, C). Most of the interactions of α-helix C are between the phosphate backbone (S101, S109, and Q110) and methionine 102, which forms multiple van der Waals interactions with the bases and the backbone. Sequence specificity is mediated by bidentate interaction of arginine 106 with two bases (Fig. 10B, C). DNA conformation is slightly distorted due to the β3/β4-loop pushing the backbone from the major groove towards the minor groove. Even though Rep subunits are not in contact with each other, their recognition sequences are intertwined (Fig. 10C). (Hickman et al. 2004)

Besides the RBS, the inverted terminal repeat of AAVs also contains a terminal hairpin structure with two distinct stems. One of the stems is enhancing the Rep-mediated nicking of the nearby terminal resolution site, although it is not essential for the nicking (Brister & Muzyczka 2000). The terminal hairpin structure is recognized with a different interface of the N-terminal REP domain, compared to RBS (Hickman et al. 2004). The hairpin has 9-bp stem and loop, consisting of three thymidines (T1-T3), which is partly buried in a positively charged preformed binding pocket (Figure 11A & B). T1 has a flipped-out conformation, and the remaining two thymidines form a continuous stack with the nucleotides at the stem. The binding pocket is outlined by trypthophan (W29) and tyrosine (Y65), which are stacked with T1 and T3, respectively (Fig. 11B). In addition, an extensive network of hydrogen bonds is formed between the thymidine loop and protein (Fig. 11B). Three residues (R58, Q121, and V119) form hydrogen bonds with T1, completing all possible hydrogen-bond interactions. R58 also forms a bond with the phosphate of T2 together with R61.

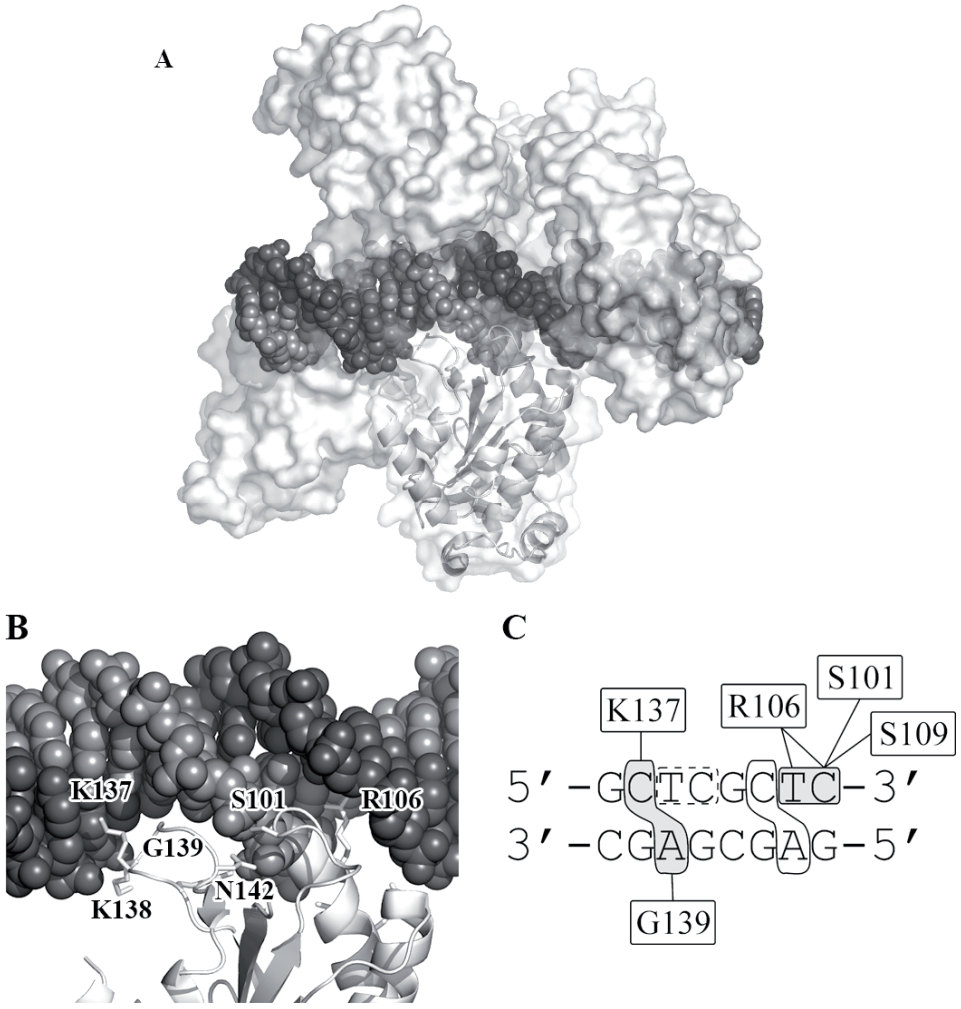

FIGURE 10 Rep-RBS interactions. Five N-terminal domains of AAV Rep protein bound to the dsDNA containing RBS sites (A). Rep proteins are shown as surface presentations and DNA as spheres. A closer look of a single binding site reveals multiple interactions (B). Interacting amino acids are shown in stick model, protein as cartoon, and DNA as spheres. A schematic presentation of the interactions in a single RBS binding site (C). Nucleic acids with gray shading are bound by single Rep domain. Nucleic acids, surrounded with dashed and solid lines, are bound by previous and the next subunits, respectively. PDB ID: 1RZ9. Figure adapted from (Hickman et al. 2004).

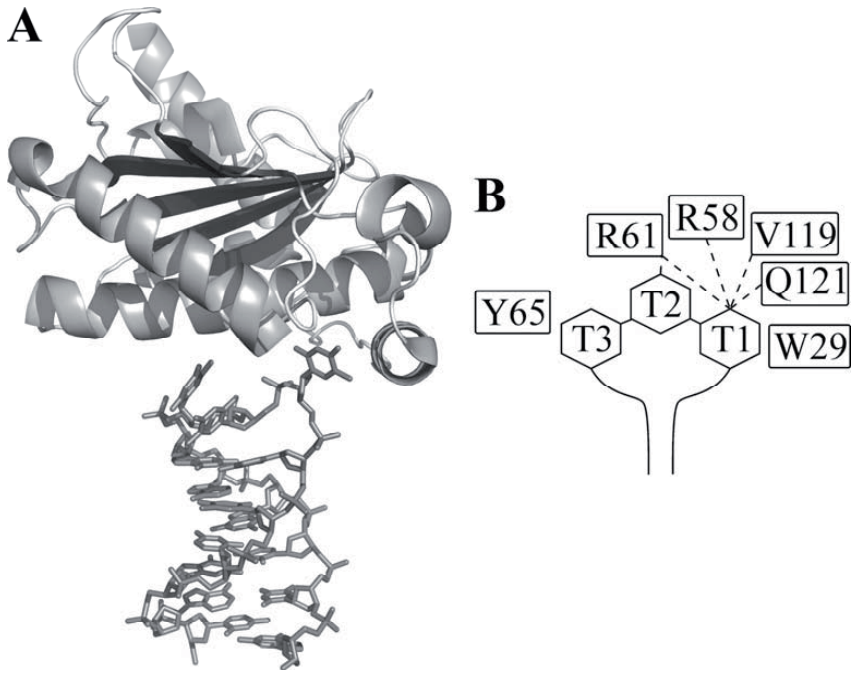

FIGURE 11 AAV hairpin recognition. Structure of the N-terminal domain of AAV5 Rep protein bound to a terminal hairpin stem loop (A). Schematic presentation of the interactions at hairpin loop (B). PDB ID: 1UUT (Hickman et al. 2004).

2.5.1.2 DNA nicking and promoter recognition

Parvovirus genome replication occurs via concatameric intermediates, where multiple viral genomes are covalently linked together (reviewed in Berns 1990). The N-terminal domain of NS1/Rep has the nicking activity that is needed to cut this concatameric DNA to correctly sized fragments. Amino acids that catalyze this reaction were originally identified in a computational comparison of the sequences from proteins of rolling circle replication (RCR) (Koonin & Ilyina 1993). Similar conserved motifs were also found from replication proteins of ssDNA viruses (Koonin & Ilyina 1993). Subsequently, the functional importance of these amino acids was demonstrated in parvoviral NS1 and Rep proteins (Davis et al. 2000, Nüesch et al. 1995). Compared to three motifs found from RCR proteins (Koonin & Ilyina 1993), the catalytic center of parvoviral replication proteins is characterized by two motifs. The first motif contains two histidines that surround a bulky hydrophobic amino acid (HUH-motif, U is the bulky hydrophobic amino acid). The second motif is characterized by a conserved sequence (Y)UXXY$X_{2\text{-}3}$K, which contains a tyrosine that functions as a nucleophile in a cleavage reaction and remains covalently linked with the 5′ end of the DNA (Cotmore & Tattersall 1988, Im & Muzyczka 1990). The structure of the Rep N-terminal domain revealed five antiparallel β-strands, flanked by three α-helices on both sides (Hickman et al. 2002). This fold is classified as an RNA recognition motif-containing protein (Burd & Dreyfuss 1994). The two most closely related structures are ORI recognition domains

from the viral proteins LTag of SV40 (Luo et al. 1996) and E1 of bovine papillomavirus (Enemark et al. 2000). A distant evolutionary relationship between these proteins is expected, because homology is not detectable at the sequence level, and neither LTag nor E1 contains RCR motifs (Hickman et al. 2002).

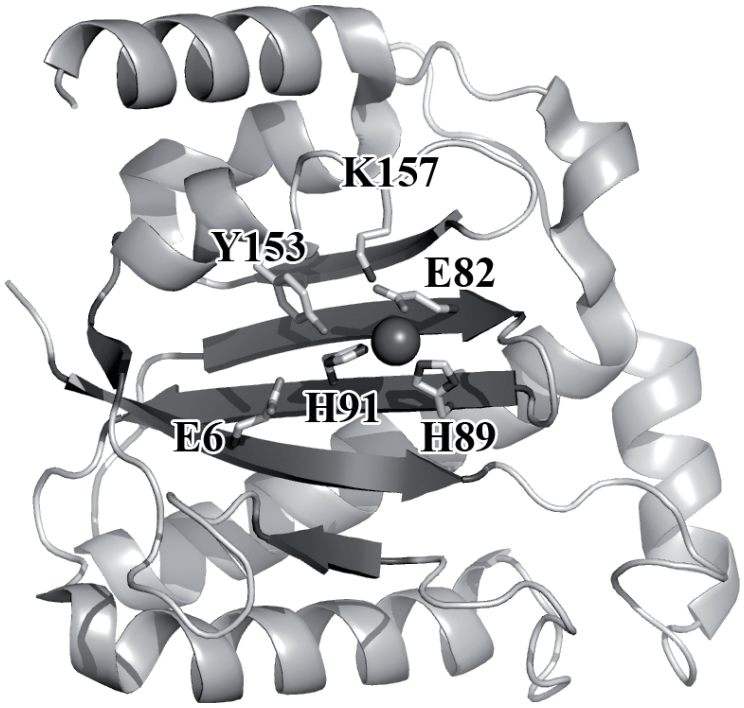

FIGURE 12 Catalytic domain from AAV5 Rep protein. Cartoon presentation of the N-terminal domain of the AAV5 Rep protein (PDB ID: 1M55; Hickman et al. 2002). Amino acids at the catalytic center that participate in nicking activity are presented in stick models. Zn^{2+}-ion is shown as a sphere.

The linking tyrosine in AAV5 Rep (Y153) is located in the α-helix (αD), and together with the hairpin loop between the β2/β3-sheets, it is lining a positively charged canyon composed of four β-strands (Fig. 12) (Hickman et al. 2002). The αD also contains another tyrosine (Y149) that is pointing towards the pocket. Some RCR proteins use two tyrosines in the cleavage reaction, but parvoviruses apparently use only one: Y153 in AAV5 Rep and its homologs in other viruses (Davis et al. 2000, Hanai & Wang 1993, Nüesch et al. 1995). At the bottom of the canyon, the HUH motif histidines (H89 and H91) and a glutamate (E82) are coordinating a zinc ion (Figure 12). Proper orientation of the histidines is controlled by two glutamates (E6 and E82), which are essential for nicking activity (Hickman et al. 2002, Urabe et al. 1999). In addition, a lysine (K157), which is part of the linking tyrosine motif sequence, is pointing towards the active site. This amino acid is also needed for nicking in AAV2, and it is speculated to assist in substrate orientation or act as a general acid in re-

protonation of the 3'-hydroxyl group of the nicked DNA (Hickman et al. 2002, Urabe et al. 1999). In general, the cleavage reactions that utilize the tyrosine OH group as a nucleophile do not need a metal ion for activation. Thus, the role of the ion that is coordinated by the HUH motif could be structural (Hickman et al. 2002). Moreover, it has been speculated that the scissile bond of the DNA template is oriented by a metal ion and K157 (Hickman et al. 2002).
Promoter regulatory function is also associated with parvoviral replication proteins. In autonomous parvoviruses, the capsid promoter P38 is strongly activated by NS1 (Gu et al. 1992, Rhode & Richard 1987, Storgaard et al. 1993). Transactivation activity is located at the C-terminus, while the DNA-binding domain is the same as in ORI recognition (Mouw & Pintel 1998). The promoter recognition sequence contains the same repeat as the ORI sequence (Cotmore et al. 2007).

2.5.2 Ring-shaped helicases

Parvovirus replication proteins have helicase and ATPase activity, both of which are located in the central domain in autonomous parvoviruses and are present in all Rep proteins in AAVs. The helicase activity is essential for their functions in genome replication.

2.5.2.1 Classification of the helicases

Helicases are proteins that are able to unwind double-stranded nucleic acids into single-stranded components. This activity is energy-dependent, and it is typically powered by consumption of ATP, and thus the helicases are also ATPases. The original classification, which was based on sequence comparison, divided helicases into three superfamilies (SFs) (Gorbalenya & Koonin 1993). Accumulation of sequence and structural data has expanded the number of SFs to five or six, depending on the classification of the AAA+ fold-containing proteins (reviewed in Singleton et al. 2007). The current classification also includes proteins termed translocases, which use energy to traverse along nucleic acids without unwinding activity. The ATP-binding pocket of all known helicases and translocases contain at least one copy of the two evolutionarily related folds: RecA or AAA+ (reviewed in Berger 2008). These folds are highly similar, and AAA+ is sometimes considered a subgroup of the RecA fold (reviewed in Wang 2004). In both folds, the C-terminal domain is built on five parallel β-strands that are connected by short loops or α-helices (Fig. 13). The N-terminal domain shows more variation between the superfamilies. In SF3 helicases, the N-terminal domain is α-helical and mediates oligomerization of the protein.

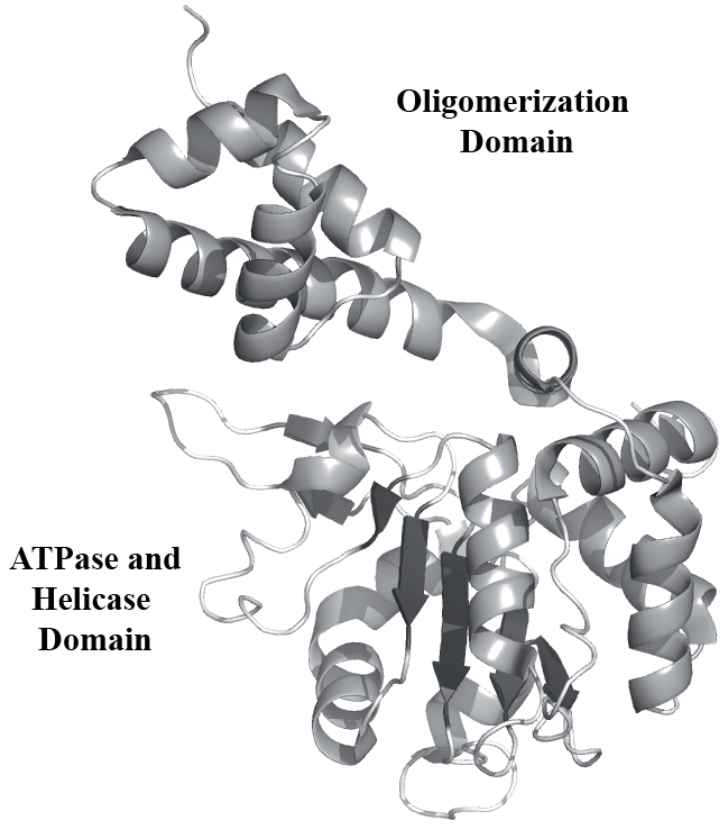

FIGURE 13 AAA+ fold. Cartoon presentation of the AAA+ fold protein: E1 helicase from bovine papilloma virus (PDB ID: 2GXA) (Enemark & Joshua-Tor 2006). The structure consists of two domains: the N-terminal oligomerization domain and C-terminal ATPase/helicase domain.

Helicases and translocases can be further specified in many ways, reflecting their function: by their target (RNA, DNA, or both), binding to either single- or double-stranded nucleic acids, or based on their directionality (5′-to-3′ or 3′-to-5′). Some SFs contain representatives with different binding specificities and directionalities, and thus this classification does not always correspond with the function of the protein (reviewed in Berger 2008).

2.5.2.2 Structure of ring shaped helicases

Although the basic function of all helicases (i.e., to unwind nucleic acids) is the same, they vary in their structure and mode of function, including oligomeric state. Members of SF1 and SF2 are monomeric and contain two ATP-binding domains (Fig. 14A). Helicase activity is achieved by ATP hydrolysis-driven ratchet-like movement of the two domains along the nucleic acid strand (Gu & Rice 2010). A quite different structure is found in SF3-6 helicases, which are functional as multimeric rings of either a RecA or AAA+ fold domain (Fig. 14B; reviewed in Singleton et al. 2007). Regardless of the fold, each subunit has an N-terminal multimerization domain and a C-terminal helicase domain, containing

a single ATP-binding pocket (Fig. 14C). Parvoviral NS1 and Rep proteins belong to the SF3 helicases and have single an AAA+ fold helicase domain with an ATP-binding pocket (James et al. 2003). The monomeric structure of the AAV2 Rep helicase domain (James et al. 2003) and hexameric structures of SV40 LTAg (Li et al. 2003) and bovine papillomavirus (BPV) E1, including a structure with ssDNA (Enemark & Joshua-Tor 2006), have been solved.

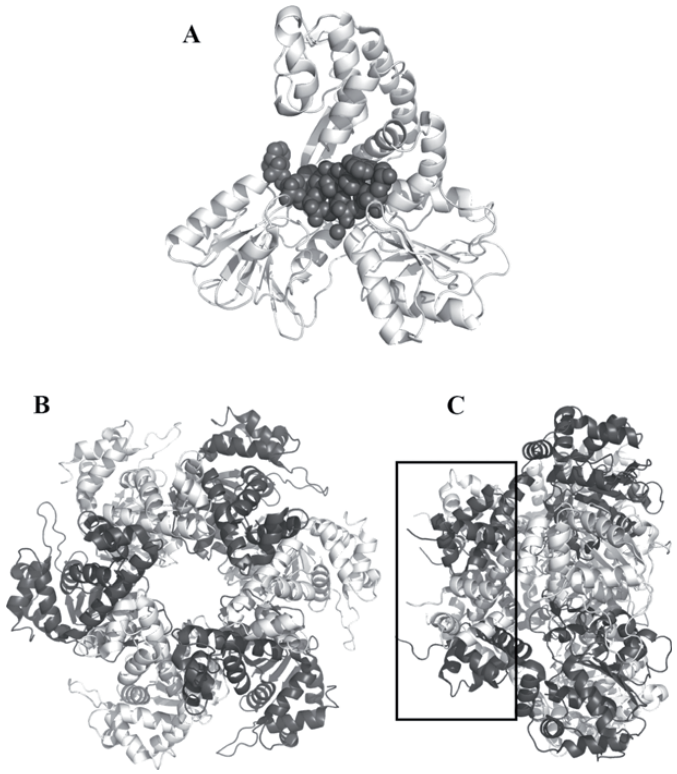

FIGURE 14 Helicase structures. Helicases of SF2 are functional as monomers that contain two RecA-like domains (A). Unwinding activity is achieved by "ratchet" like movement of the domains relative to the nucleic acid strand. Hepatitis C virus NS3 protein is shown as a cartoon, and ssDNA as a spherical presentation (PDB ID: 3KQH) (Gu & Rice 2010). Helicases of SF3 function as multimeric rings where DNA is translocated through the central cavity (B). Localization of the N-terminal multimerization domain is highlighted with a black box in the side-view image of the SF3 helicase ring (C). Structure of E1 protein from BPV is shown as a cartoon presentation (PDB ID: 2GXA) (Enemark & Joshua-Tor 2006).

SF3 helicases share a similar overall structure with many ring-shaped ATPases including the SF4 helicase of phage T7 (Sawaya et al. 1999), protein translocase subunit of chaperones (Neuwald et al. 1999), and ATPsynthase F1 (Abrahams et al. 1994). All multimeric x-ray structures of SF3 helicases show hexameric rings, but octameric rings have also been reported (Mansilla-Soto et al. 2009). In the LTAg and E1 structures, multimerization is achieved through interactions of the N-terminal α-helical domains of neighboring subunits (Fig. 14C). As

demonstrated by the DNA-containing structures, DNA is transported through the ~13-Å central pore (Enemark & Joshua-Tor 2006).

2.5.2.3 ATP binding in ring-shaped helicases

In SF3 helicases, the originally relatively open ATP-binding pocket is formed by three conserved ATP binding motifs: Walker A and B, and motif C, and it is complemented by *trans* interactions with neighboring helical domain (Fig. 15A). The role of the amino acids of the Walker A and B motifs is to coordinate phosphates of the ATP. All phosphates of bound ATP in the LTAg structure are extensively bound (Fig. 15B, C): α-phosphate is bound to threonine (T434) from A motif and *in trans* by lysine (tK418), β- and γ-phosphates by lysine (K432) of the A motif, and magnesium, which is coordinated by the A motif threonine (T433). The γ-phosphate also binds aspartate (D474) from the Walker B motif, asparagine (N529), and an *in trans* arginine finger residue (tR540). In addition, there are multiple water molecule-mediated interactions between ATP and the protein. A similar bonding network is observed in BPV E1 protein (Enemark & Joshua-Tor 2006) and most likely present in parvoviral replication proteins, although multimeric x-ray structures of these have not yet been solved (James et al. 2003).

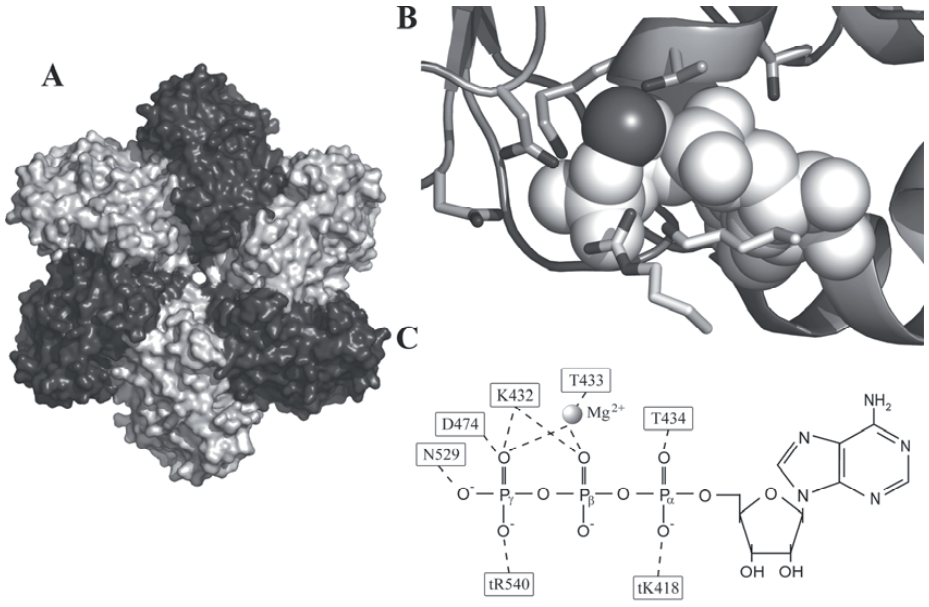

FIGURE 15 ATP binding in hexameric helicases. ATP-binding pocket resides between subunits (A). Surface presentation of the hexameric LTag from SV40 is shown, with colors alternating between adjacent subunits. Close-up of one ATP-binding pocket (B). Amino acids that participate in ATP binding are shown as stick models: A and B motif amino acids from the *cis*-binding subunit, and arginine and lysine from the *trans*-binding subunit. ATP molecule is show as light gray spheres and magnesium-ion as dark gray sphere. Schematic presentation of ATP-binding interactions shown in (B), including amino acid numbering (C). (PDB ID: 1SVM) (Gai et al. 2004).

ATP binding changes the overall structure of the protein complex in many ways. In LTAg, ATP binding causes tighter interactions between subunits and leads to an increase of the buried surface area from 2474 Å^2 to 4344 Å^2 (Gai et al. 2004). Also, the diameter of the central cavity is decreased from ~15 Å to ~7 Å, which is largely due to the movement of the N-terminal multimerization domain relative to the ATPase domain (Gai et al. 2004).

2.5.2.4 DNA binding interactions in ring-shaped helicases

SF3 helicases transport DNA through the central cavity that is formed by multiple protein subunits. This protein family is exclusively found in viruses and includes viruses with both dsDNA and ssDNA genomes. Regardless of the genome type, SF3 helicases apparently translocate only ssDNA (Enemark & Joshua-Tor 2006, Gai et al. 2004). In the central pore, the β-hairpin loops of B' motifs protrude into the central cavity and are mainly responsible for protein-DNA interactions (Fig. 14). Mutations here abolish the helicase activity, as demonstrated for LTag (Shen et al. 2005) and AAV2 Rep (Walker et al. 1997). The β-hairpin reacts to the ATP binding by moving ~17 Å along the cavity axis in LTAg (Gai et al. 2004).

The structure of the BPV E1 helicase with ssDNA has elucidated details of the DNA-binding interactions in SF3 helicases (Enemark & Joshua-Tor 2006). When bound to DNA, the β-hairpins are arranged in a progressive manner, with a rise along the axis of the central cavity that corresponds to a single nucleotide (Fig. 16C, D). The difference of the first and last β-hairpin in the E1 structure is in good correlation with an ATP induced ~17-Å movement along the cavity axis observed in LTAg structures (Gai et al. 2004). Each hairpin makes the same amino acid contacts with the sugar-phosphate backbone of the encircled ssDNA substrate. The phosphates of two consecutive bases are hydrogen-bound with the main chain amide of H507 and ammonium group of K506 (Fig. 16C, D). A sugar ring between these phosphates makes van der Waals contacts with H507, F464, and the aliphatic chain of K506. This overlapping pattern is repeated from the first subunit to the fifth, leading to stepwise escorting of the DNA strand through the central cavity (Enemark & Joshua-Tor 2006) (Fig. 16C).

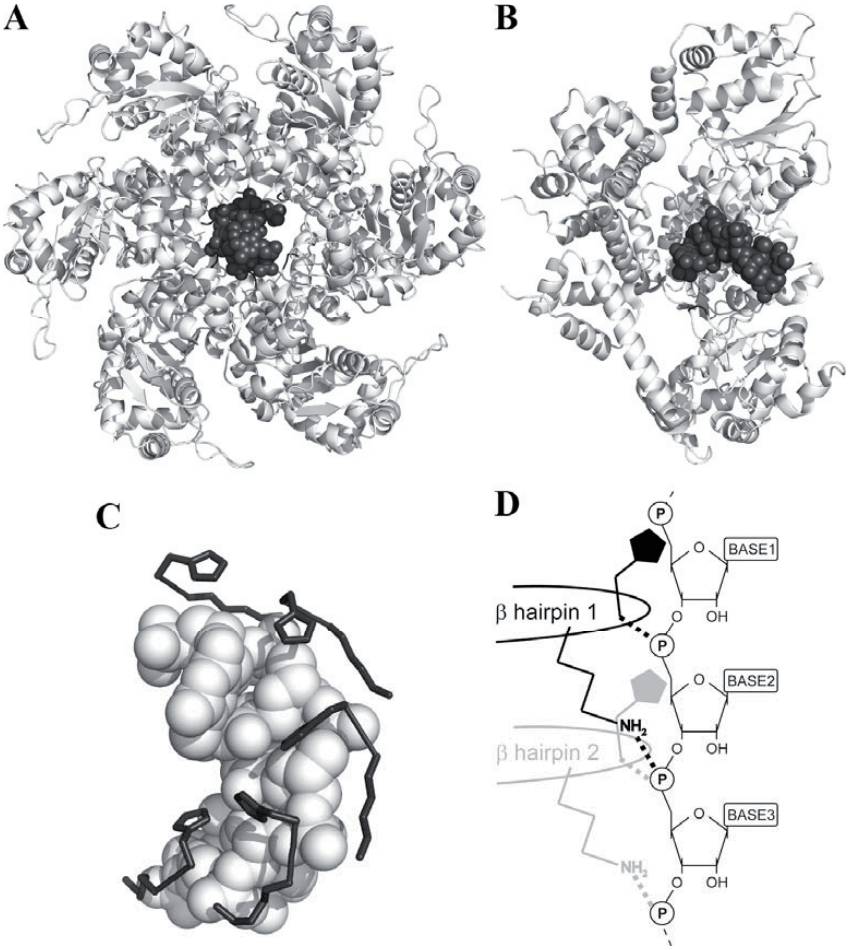

FIGURE 16 Single-stranded DNA binding in ring shaped helicases. A cartoon presentation of the hexameric E1 protein from BPV (A). Single-stranded DNA (dark gray spheres) resides at the central cavity. Cross-section of the hexameric ring showing only three subunits and the ssDNA (B). Five of the six E1 subunits are contacting the ssDNA with histidine (H507) and lysine (K506) residues of their β-hairpins forming a right handed "staircase" (C). Schematic presentation of the interactions showing intertwined interactions and one nucleotide rise per subunit (D). Structure of E1 protein from BPV was used (PDB ID: 2GXA; image adapted from Enemark & Joshua-Tor 2006).

2.5.2.5 Function of ring-shaped helicases

Monomeric helicases and translocases are thought to move along their target in an "inchworm" manner, where the alternating binding and release of the two domains leads to the movement (Gu & Rice 2010, Singleton et al. 2007). Ring-shaped helicases have one ATPase domain in each subunit, and movement is thus attained by motion of individual subunits relative to the others or to translocated DNA.

Two functional mechanisms, sequential and concerted, have been proposed for the SF3 helicases (Enemark & Joshua-Tor 2006, Gai et al. 2004). In the sequential mechanism, subunits function successively in DNA binding, β-hairpin movement, and release, leading to rotational translocation of the DNA. In concerted models, subunits function all at once and pump the DNA through the central cavity. Based on ssDNA-containing E1 structures, a sequential mechanism, termed coordinated escort was proposed. In this model, each subunit undergoes a repetitive movement along the central axis, which is driven by the binding and hydrolysis of ATP and release of ADP. Escorted ssDNA remains bound to the β-hairpin during the movement and is released together with the ADP. After the release, the subunit is free to start a new cycle with ATP binding (Enemark & Joshua-Tor 2006). This model explains the 3′-to-5′ directionality observed for the E1 helicase and links different conformations of the subunits in the structure to their ATP hydrolysis status. One nucleotide would be bound by a single subunit and translocated through the central cavity, powered by hydrolysis of a single ATP molecule (Enemark & Joshua-Tor 2006). A more recent structure of hexameric apo E1 supports the idea of the sequential movement of the subunits (Sanders et al. 2007). However, as the different β-hairpin conformations were observed in the apo E1 structure, the necessity of the ATP hydrolysis in each of the subunit was challenged (Sanders et al. 2007).

In LTag structures, all subunits adopted the same conformation, which was dependent on ATP binding status (Gai et al. 2004). This observation led to a model where ssDNA translocation is achieved by concerted iris like opening and closing of the central pore (Gai et al. 2004). Perhaps the most rigorous attempt to unravel the mechanism of hexameric ATPases does not come from helicase but from the hexameric protein translocase ClpX that belongs to the AAA+ protein family (Martin et al. 2005). The activity of covalently linked hexameric polymers containing functional and non-functional ClpX domains was studied. The results suggested that a single subunit initiates protein translocation in this protein, ruling out the concerted mechanism. Furthermore, even though activity of the ClpX was reduced, it was clearly functional with multiple non-functional subunits, ruling out the strict sequential mechanism. A probabilistic sequential model was proposed, where ATP hydrolysis of a single subunit can be followed in not only consecutive but also more distant subunits (Martin et al. 2005). This mechanism is clearly more robust than the strictly sequential model and could, although not yet tested, also explain the function of ring-shaped helicases.

3 AIMS OF THE STUDY

Parvovirus replication protein NS1 has multiple essential functions in viral infection. The functions and factors that determine its DNA binding have not been characterized for NS1 in living cells before. The specific objectives of this thesis were:

I To characterize the changes in intranuclear organization and dynamics of endogenous and viral proteins in CPV infection

II To determine the role of ATP binding and hydrolysis in the dynamics of CPV NS1

III To localize the DNA-binding regions and to define their role in the intranuclear dynamics of CPV NS1

4 SUMMARY OF THE MATERIALS AND METHODS

This chapter summarizes the materials and methods that were used in the studies. Histone H3 and H4 localization studies are not included in the publications. A detailed description of other materials and methods can be found from the original publications.

TABLE 1 Antibodies used in publications.

Antibody (type)	Antigen	Source	Publication
A3B10 (Mab)	Intact CPV Capsid	Gift from Parrish CR, USA	I, II, III
BrdU (Mab)	5-bromo-2'-deoxyuridine	Santa Cruz biotech, USA	I
Cornell #2 (Ab)	CPV Capsid protein	Gift from Parrish CR, USA	I, II
Goat anti-mouse and anti-rabbit Alexa-488/555/633 conjugated IgG	Mouse or rabbit IgG	Invitrogen, USA	I, II, III
Goat anti-mouse and anti-rabbit horseradish peroxidase conjugated IgG	Mouse or rabbit IgG	Bio-Rad Laboratories, UK	II, III
NS1 (Mab)	NS1 protein	Gift from Astell C, Canada	I, II, III
PCNA (Ab)	PCNA protein	Abcam, UK	I

TABLE 2 Fluorescent fusion constructs used in publications.

Construct	Source	Publication
EGFP	Clontech, USA	I
EYFP	Clontech, USA	I, II, III
H2B-ECFP	Weidemann et al. 2003	I, II, III
H2B-EYFP	Weidemann et al. 2003	I, II
H3-EGFP	Gift from prof. Langowski J.	-
H4-EGFP	Gift from prof. Langowski J.	-
PCNA-EYFP	Essers et al. 2005	I
pBI265 (renamed pIC)	Parrish 1991	II
PAGFP-VP2	-	I

TABLE 3 NS1 plasmids used in publications.

NS1 and NS1 mutants	Context (usage)	Publication
NS1	deYFP (microscopy: localization and FRAP)	I
NS1, K406M, E444Q, E445Q, R508A, R510A	EYFP (P38 transactivation)	II
NS1, K406M, E444Q, E445Q, R508A, R510A, E121A, AUA, K2A, Y212F, K470/2A, dC67	deYFP (microscopy: localization and FRAP)	II
K406M, E444Q, E445Q, R508A, R510A	pIC (virus production, P38 transactivation)	II
NS1, K406M, E121A, AUA, K2A, Y212F, K470A, K472A, K470/2A, dC67, AUA-K2A, AUA-K470/2A, AUA-dC67, AUA-K470/2A-dC67, K2A-K470/2A, K2A-dC67, K2A-K470/2A-dC67, K470/2A-dC67	deYFP (microscopy: localization and FRAP)	III

TABLE 4 Reagents used in publications.

Reagent	Source	Publication
40 kDa and 500 kDa FITC-dextran	Invitrogen, USA	I
146 kDa TRITC-dextran	Invitrogen, USA	I
ADP	Sigma-Aldrich, USA	III
AMP-PNP	Sigma-Aldrich, USA	II
ATP	Sigma-Aldrich, USA	II, III
ATP-γS	Sigma-Aldrich, USA	II
BrdU (5-bromo-2′-deoxyuridine)	Sigma-Aldrich, USA	I
DAPI	Invitrogen, USA	I, II
Digitonin	Sigma-Aldrich, USA	II, III

TABLE 5 Experimental procedures used in publications.

Experimental procedure	Instrument or program	Publication
Cloning	-	I, II, III
Confocal imaging	Zeiss LSM510, Olympus FV1000	I, II, III
Comparative modeling	Bodil, Modeller	II, III
FRAP	Zeiss LSM510	I, II, III
FRAP and photoactivation simulations	Virtual Cell	I, II, III
Microinjection	Eppendorf Transjector	I
Photoactivation	Olympus FV1000	I
Transfection	-	I, II, III
Widefield microscopy	Zeiss CellObserver	I

4.1 Localization of H3-EGFP and H4-EGFP in CPV infection

NLFK cells were cultivated on coverslips for 24 h. Cells were transfected with H3-EGFP or H4-EGFP constructs (plasmids were a generous gift from Professor J. Langowski) using the TransIT-LT2 transfection reagent (Mirus Bio LLC, WI, USA) and infected with CPV. At 24 h post-transfection and –infection, cells were fixed using 4 % paraformaldehyde (20 min., RT) followed by antibody labelling with mouse anti-NS1 antibody and goat anti-mouse Alexa-555 conjugated secondary antibody (Bio-Rad). Samples were mounted with Mowiol-Dabco (Sigma-Aldrich, USA), and imaged using an Olympus FV1000 confocal microscope with the appropriate settings.

5 REVIEW OF THE RESULTS

5.1 Fluorescent fusions as protein markers (I, II, III)

Most of the results in this thesis were obtained using fluorescent fusion proteins. The correct function of the protein fusions is therefore utmost important. The fluorescent fusion construct of CPV NS1 (NS1-deYFP) appeared to function similarly to wt NS1 protein in all our experiments. Western blot analysis showed that only one product, which can be detected with both anti-NS1 and anti-GFP antibodies is present in transfected cells. Similar to wt NS1, the apparent size of the NS1-deYFP fusion was ~20 kDa larger than the theoretical size (I). When expressed in mammalian cell lines, it localizes to cell nucleus and, in the case of infection, to the replication body (I, II, III). The same localization is observed for wt NS1 in infected cells with antibody labeling. NS1-deYFP transfectants are infected with high efficiency, indicating that expression of the fusion protein is not hindering the initiation or progression of the infection (II, III). NS1 proteins function as transcriptional regulators. The most prominent regulatory effect of CPV NS1 is its ability to enhance transcription from the capsid promoter P38. This property was also retained in the fluorescent fusion of the NS1 (II). Fluorescent NS1 fusion appeared to retain the cytotoxicity reported for parvoviral NS1 proteins, as suggested by the inability to produce stable cell line from transfectants. In all, NS1 protein with the C-terminal fluorescent fusion functions as wt NS1 in all aspects that we have studied.

5.2 Changes in the nuclear architecture of infected cells (I)

CPV infection begins from small NS1-containing intranuclear foci that grow to fill the nucleus. These replication bodies are sites for viral genome replication, and necessary components (e.g., PCNA and NS1) are highly concentrated and localized there at all time points of the infection (I). Concomitant with the

enlargement of the replication bodies, the endogenous chromatin is marginalized towards the nuclear envelope (I). This can be seen by the labeling of endogenous DNA with BrdU – or by fluorescent histone markers in the cells. At approximately 16 h post-infection, the replication body is fully grown, and the histone proteins are highly concentrated to the nuclear periphery, along with the host DNA. Interestingly, there was a clear concentration of histones H3 and H4 but not H2B in the replication body in the beginning of the infection (Fig. 17). Compared to H2B, H3 and H4 were also more present in fully grown replication bodies (Fig. 18). Using DAPI staining, the total amount of the DNA was estimated to increase ~2.5-fold at 24 h p.t., which is explained by the synthesis of viral genomes (I). Concomitantly, the volume of the nucleus was also increased approximately 2- and 3-fold at 8 h p.t. and 24 h p.t., respectively (I). Viral capsids showed slightly different localization in the replication bodies, compared to histones or NS1. In the majority of the infected cells, capsids were concentrated in the vicinity of the nuclear envelope, as shown by antibody labeling (I).

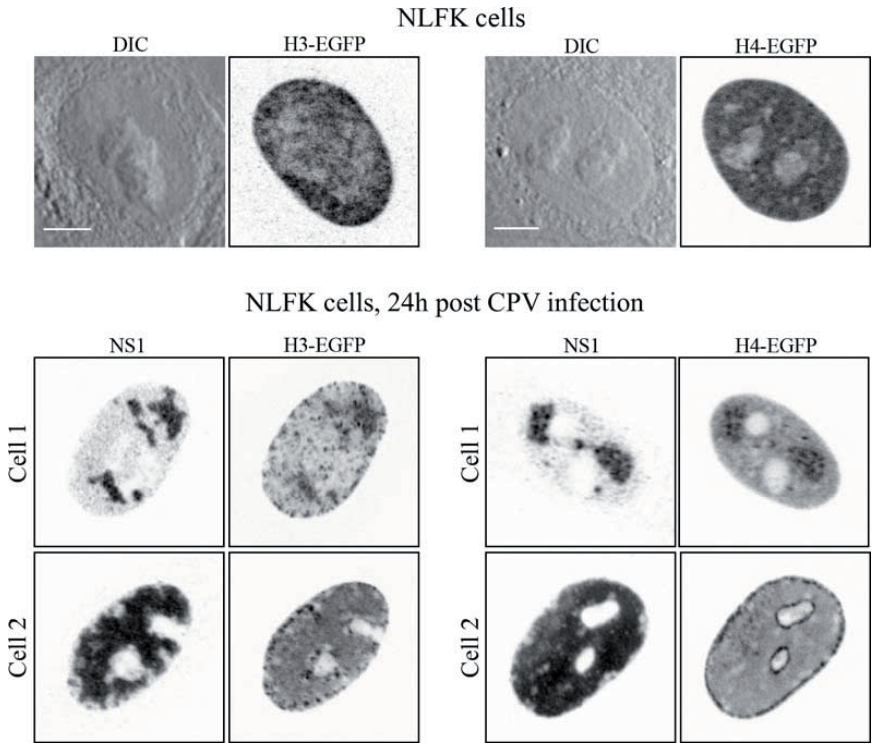

FIGURE 17 H3 and H4 histones concentrate in early replication bodies. H3-EGFP and H4-EGFP are homogeneously distributed in the nuclei of transfected NLFK cells (above). In infection, they concentrate in emerging replication bodies (below). Two cells at 24 h post-infection, but at different replication body formation stages, are shown for H3-EGFP and H4-EGFP transfectants. Scale bar, 5µm.

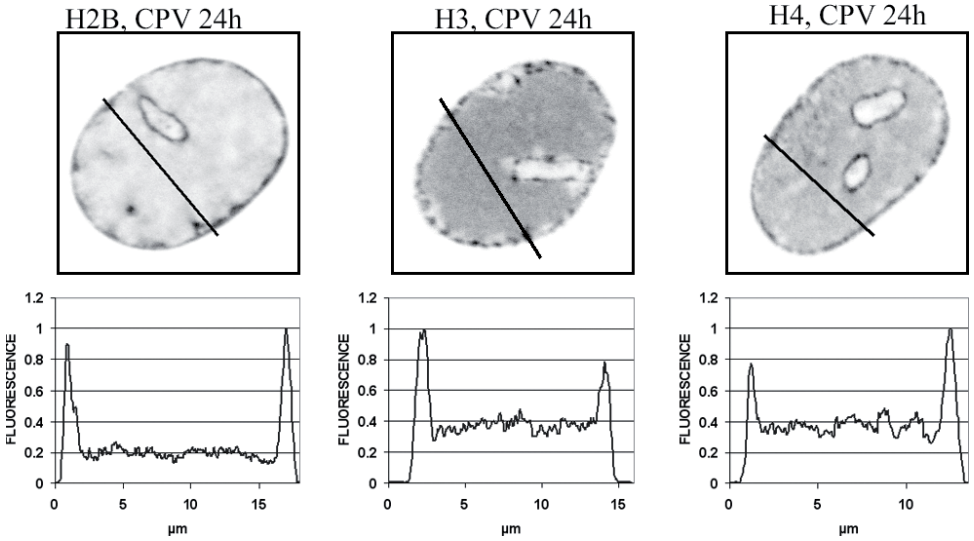

FIGURE 18 Localization of different histone species in infection. Histone H2B-ECFP shows less replication body localization, compared to H3-EGFP and H3-EGFP. Infected transfectants at 24 h post-infection are shown. Line profile is normalized between the smallest and highest values (area indicated with a black bar in the fluorescent image).

The structural properties of the replication bodies were analyzed by microinjecting various-sized dextrans into the nuclei of infected cells (I). The data suggested that replication bodies were more homogeneous, compared to the nuclei of noninfected cells. In addition, they were freely accessible to 40-kDa (radius of gyration, r_g ~ 7 nm) and 146-kDa (r_g ~ 13 nm) dextrans and somewhat inaccessible to the larger 500-kDa (r_g ~ 24 nm) dextran (I).

5.3 Intranuclear mobility of proteins is affected by infection (I)

To study the changes in the diffusion properties of the replication bodies, we used an enhanced yellow fluorescent protein (EYFP), which is inert and small (~27 kDa). EYFP was transfected into the cells, and the effect of the infection on its mobility was monitored using the FRAP method and computer simulations in the Virtual Cell simulation environment (I). A dark rim around the nucleus, presumably caused by the dense packing of the endogenous chromatin, was used to determine infected cells. Best fit to the experimental data was obtained with Virtual Cell simulations using two EYFP populations. The existence of two components in the free diffusion of EYFP was also supported by fluorescent fluctuation microscopy studies of noninfected cells (I). In noninfected cells, EYFP was concluded to have diffusion coefficients of 50±5 µm^2/s (96 % of the EYFP) and 1±0.1 µm^2/s (4 % of the EYFP). In infected cells, only the faster population with the diffusion coefficient of 50±5 µm^2/s was observed.

PCNA is an essential protein for parvovirus infection. Its role is presumably to enhance processivity of polymerase δ. We used fluorescent PCNA-fusion transfectants to assess PCNA dynamics in infection. In noninfected cells, PCNA-EYFP showed fast recovery and a diffusion coefficient of 9±2 $\mu m^2/s$. When comparing to the diffusion of free EYFP in similar imaging conditions, this was expected diffusion coefficient for trimeric PCNA-EYFP. In infected cells, the recovery of the PCNA-EYFP was slower. The FRAP model that takes into account the diffusion and binding of the protein was used to describe the data. In the infected cells, PCNA-EYFP was concluded to have one binding site, to which it was bound for 83 s (k_{off}=0.012±0.002 s^{-1}) and diffusing freely for 111 s (k_{on}=0.009±0.002 s^{-1}).

Mobility of the viral capsids was also studied using fluorescent fusion proteins. The major capsid protein VP2 was fused to photoactivatable green fluorescent protein (VP2-PAGFP), and its mobility was monitored in transfected cells. Studies were conducted by activating VP2-PAGFP proteins from a circular area inside the nucleus and by monitoring the spreading of the fluorescence. Obtained results were further reproduced in the Virtual Cell modeling environment. In noninfected cells, fluorescence filled the entire nucleus in a 60-s observation period. Similar mobility was obtained in the simulations using two populations with diffusion coefficients of 5.0 $\mu m^2/s$ (81 % of the VP2-PAGFP) and 0.02 $\mu m^2/s$ (19 % of the VP2-PAGFP). In contrast, radically reduced mobility of VP2-PAGFP was seen in the photoactivation experiments in infection. The best simulation was again obtained with the two-component system and with diffusion coefficients of 5.0 $\mu m^2/s$ (26 % of the VP2-PAGFP), and 0.001 $\mu m^2/s$ (74 % of the VP2-PAGFP).

Finally, the dynamics of the major nonstructural protein NS1 was also studied using the fluorescent EYFP fusion protein. Conserved mutations were made to the capsid promoter region which is located in the NS1 gene and non-conserved to the start codon of the EYFP to prevent production of free EYFP protein. This construct (NS1-deYFP) was transfected into cells, followed by infection, and its dynamics was studied using the FRAP method and Virtual Cell simulations. Existing models for quantitative description of the FRAP results did not fit the experimental data. These models are limited to cases where there is only one immobile binding partner. Virtual Cell models were used to test two hypotheses: binding to the mobile binding site and binding to two independent binding sites. Both models fit well with the recovery of NS1-deYFP (I, Fig. 6). In the former model, the mobile binding partner had a diffusion coefficient of 0.01 $\mu m^2/s$, and NS1 was bound for 250 s (k_{off}=0.012±0.002 s^{-1}) and diffusing freely for 42 s (k^*_{on}=0.012±0.002 s^{-1}). In equilibrium, 86 % of the NS1 was bound, while 14 % was diffusing freely. In the latter model, NS1 was bound to the first higher-affinity binding site for 83 s (k_{off}=0.012±0.002 s^{-1}), to the second binding site for 10 s (k_{off}=0.10±0.002 s^{-1}) and diffusing freely for 8.1 s (for the first site k^*_{on}=0.024±0.004 s^{-1}, and for the second k^*_{on}=0.10±0.02 s^{-1}). In equilibrium, 50 % of the NS1 was bound to the higher-affinity site, 25 % to the lower-affinity site, and 25 % was diffusing freely. In both Virtual Cell models, the binding time of NS1 was in the order of

tens of seconds. Intriguingly, in both models, the binding of the NS1 contained a component where its binding time was the same as for PCNA.

5.4 Comparative model of CPV NS1 (II, III)

To further understand the dynamics of the CPV NS1 protein, we chose to characterize the contribution of different properties to its binding. Parvoviral NS1 functions are mediated through interactions with DNA or other proteins. Common to many, if not all, NS1 functions is their requirement for ATP binding. We wanted to investigate how ATP binding affects NS1 dynamics (II). To be able to reliably identify amino acids that are part of the ATP-binding pocket in CPV NS1, we would need a structure of the protein. There are no structures available for autonomous parvovirus NS1 proteins. However, the structure of a closely related adeno-associated virus 2 Rep40 protein has been solved. This protein is a helicase and the helicase domain has a high sequence identity (~38 %) with that of the CPV NS1. Hence, it was used to build a comparative model of NS1 (II, Fig. 1). Based on the model, the helicase domain of the NS1 is formed by five parallel β-strands that are connected through α-helices of varying length. Based on the overall fold, NS1 belongs to the superfamily III helicases (SF3) together with many viral helicases. This superfamily is characterized by four conserved sequence motifs (A, B, B', and C), which are also found from the CPV NS1 sequence. An ATP molecule is bound by conserved amino acids in the A, B, and C motifs, while the B´ motif is interacting with translocated DNA.

SF3 helicases are thought to function as ring-shaped homo-oligomers, containing six to eight subunits. In this arrangement, the ATP-binding pocket is situated at the inter-subunit interface: the first subunit provides conserved A and B motifs, while the other provides positively charged sensor element called arginine finger. The DNA strand is thought to be translocated through the central cavity. We constructed a hexameric NS1 model to further investigate amino acids at this interface. To this end, we fitted six monomeric NS1 models with the hexameric structure of the simian virus 40 LTag protein (II, Fig. S1; III, Fig. 1). According to the sequence alignment with AAV2 Rep40 protein, the arginine 510 was the candidate arginine finger in CPV NS1. This residue was close to the proposed ATP-binding pocket in the hexameric model. In addition, there were several positively charged amino acids in its vicinity, which might be important in ATP binding or hydrolysis.

The DNA-interacting B' motif of the helicase domain forms a short α-helix at the subunit interface and continues as a β-hairpin that is flanked with two anti-parallel β-sheets (Enemark & Joshua-Tor 2006). This hairpin structure harbors two conserved lysine residues, first one of which is important for the helicase activity of Rep protein (James et al. 2003). In hexameric NS1 model, both lysines are pointing towards the central cavity (III, Fig. 1).

5.5 ATP binding controls NS1 function … (II)

Based on the NS1 structural model, we made charge-neutralizing mutations to the conserved A and B motifs, proposed arginine finger, and another arginine in its vicinity at the subunit interface. The motif A lysine (K406) was mutated to methionine, motif B glutamates (E444 and E445) were mutated to glutamines, and arginines (R508 and R510) were mutated to alanines. All mutated NS1s were cloned as enhanced yellow fluorescent protein fusion constructs, with similar mutations as described for NS1-deYFP (II, Fig. 2). The same mutations were also made to infectious clones to monitor their effect on viral viability. The properties of the NS1 mutants are summarized in a table 6.

TABLE 6 Properties of NS1 mutants. Infection is infectivity of mutants NS1 expressing cells, virus production is the ability of mutated infections clone to produce infectious viruses, P38 is the P38-promoter transcription activation level, and enhanced binding in buffers with different substituent (ATP, ADP, AMP-PNP, or ATP-γS) compared to buffer. – is no activity, + is very low activity, ++ is reduced activity compared to non-mutated NS1, +++ is activity comparable to non-mutated NS1, and N.D. is not determined.

Mutant	Infection	Virus Production	P38	Enhanced Binding in ivFRAP			
				ATP	ADP	AMP-PNP	ATP-γS
K406M	+	-	-	-	N.D.	-	N.D.
E444Q	+	-	++	+	N.D.	-	N.D.
E445Q	+	-	++	+++	N.D.	-	+++
R508A	++	-	+++	++	N.D.	+	N.D.
R510A	++	-	++	++	N.D.	-	N.D.
E121A	++	N.D.	N.D.	+++	+++	N.D.	N.D.
AUA	++	N.D.	N.D.	+++	++	N.D.	N.D.
K2A	++	N.D.	N.D.	+++	+++	N.D.	N.D.
Y212F	+	N.D.	N.D.	+++	+++	N.D.	N.D.
K470/2A	+	N.D.	N.D.	++	++	N.D.	N.D.
dC67	++	N.D.	N.D.	+++	+++	N.D.	N.D.

First, the ATP-binding properties of the mutants were monitored by *in vitro* FRAP (ivFRAP) method. In this method, the plasma membrane of the transfectants is first permeabilized with digitonin to wash out all small molecules, including ATP. FRAP experiments are then conducted in a buffer which can be supplemented with the appropriate small molecules. All mutants, except K406M, showed slower recovery in ivFRAP experiments in the presence of ATP, compared to just the buffer (II, Fig. 5). This indicates that ATP binding or hydrolysis occurs in these mutants and enhances DNA-binding of the protein. Furthermore, the non-hydrolysable ATP analog AMP-PNP was used to characterize the role of ATP hydrolysis in NS1 binding. The ivFRAP recovery in

all, except the E445Q mutant, was slower compared to recovery in buffer but faster compared to buffer with ATP. In E445Q, the recovery was indistinguishable from the recovery without ATP. We used another non-hydrolysable ATP analog, ATP-γS, to solve this discrepancy. In this case, the ivFRAP recoveries of NS1 and E445Q were comparable to the corresponding recoveries in the presence of ATP.

To further characterize the ATP-binding mutations with regard to function of NS1, we tested their effect on viral replication and capsid promoter transactivation (II, Fig. 3). All mutations rendered the infectious clone unable to produce infectious viruses and showed dominant-negative effect on infection. In P38-promoter transactivation assays, only the R508A mutant retained non-mutated NS1-like activity. The lowest activity was seen for the A motif mutant K406M, while intermediate activity was observed for other mutants (E444Q, R508A, and R510A). In conclusion, ATP-binding mutants had strong effects on NS1 function.

5.6 … and dynamics (II)

As ATP binding is important for many parvoviral NS1 functions, we wanted to monitor how different ATP-binding mutants affect the dynamics of NS1. To monitor the dynamics of NS1 mutants, we used the FRAP technique in non-infected tranfectants (II, Fig. 6). In addition, Virtual Cell simulations were used to find binding parameters that would reproduce the data. Mutants showed large changes in their recovery. When compared to half recovery time ($t_{½}$) of non-mutated NS1 ($t_{½}$=17.9±1.2 s), the fastest mutant was K406M ($t_{½}$=3.0±0.2 s), and the slowest was E445Q ($t_{½}$=40.2±6.9 s). Only the recovery of the R508A mutant was similar to non-mutated NS1. Slower recovery was seen for the E445Q and faster for all other mutants, compared to non-mutated NS1.

To analyze the recovery further, FRAP recoveries were reproduced as Virtual Cell simulations. Recovery of the K406M mutant was reproduced using a model with a single binding site. It was bound for 4.76 s (k_{off}=0.18 s^{-1}) and diffused freely for 5.56 s (k^*_{on}=0.21 s^{-1}). All other mutants required a second binding reaction to obtain a reasonable fit. Based on the ivFRAP results, we assumed that K406M binding reflects ATP-independent binding of NS1 and added another independent binding reaction for the remaining proteins. This approach successfully reproduced the recoveries of NS1 and the other remaining mutants, excluding E445Q. NS1 was bound to this second site for 71.43 s ($k_{2,\ off}$=0.014 s^{-1}), and diffusing freely for 5. 06 s ($k^*_{1,\ on}$=0.18 s^{-1} and $k^*_{2,\ on}$=0.0175 s^{-1}). Compared to NS1, the R508A mutant had slightly faster dissociation. It was bound for 55.56 s ($k_{2,\ off}$=0.018 s^{-1}) and diffused freely for 5.06 s ($k^*_{1,\ on}$=0.18 s^{-1} and $k^*_{2,\ on}$=0.0175 s^{-1}). Even faster turnover was seen for E444Q (bound for 22.2 s; $k_{2,\ off}$=0.045 s^{-1}) and R510A (bound for 17.18 s; $k_{2,\ off}$=0.0582 s^{-1}). Free diffusion times for E444Q and R510A were 4.76 s and 4.78 s, respectively. Different binding characteristics for both reactions were needed to

describe the recovery of E445Q, as it did not contain the fast population comparable to K406M. It was bound to the first binding site for 50 s ($k_{1, off}$=0.024 s^{-1}) and to the second for 357 s ($k_{2, off}$=0.0028 s^{-1}) and diffused freely for 43.48 s ($k^*_{1, on}$=0.02 s^{-1} and $k^*_{2, on}$=0.003 s^{-1}). In the FRAP experiments, the E445Q mutant did not recover fully during the 600-s observation period. From the simulations, the full recovery time point was approximated to be 1120 s. To conclude, the DNA-binding kinetics of NS1 is highly dependent on its ability to bind ATP.

5.7 DNA-binding interactions of ORI-binding domain and helicase domain have major effects on NS1 dynamics (III)

The function of parvoviral NS1 is closely related to its ability to bind DNA. Many stages of viral genome replication rely on NS1. We used biochemical data obtained from other parvoviruses and structural data from AAV Rep proteins to pinpoint the amino acids that are important for DNA binding in CPV NS1. The N-terminal domain of parvoviral NS1 proteins harbors the sequence-specific ORI recognition domain and the catalytic cleavage domain (Mouw & Pintel 1998, Nüesch et al. 1995). The sequence similarity between AAV5 Rep and CPV NS1 in the N-terminal region is only ~17 %, which effectively inhibits reliable comparative model building. However, many amino acids that are critical for the function of Rep protein are also conserved in CPV NS1. These include the metal ion coordination site (containing the conserved HUH motif) and a linking tyrosine of the nicking region, and two lysines at one of the DNA-interacting loops. The helicase domain of NS1 is well conserved between AAVs and parvoviruses and also in other SF3 helicases, including BPV E1. Thus, the structural model of NS1 was reliable in estimating the DNA-interacting regions of the SF3 helicase domain.

Based on the reduced infectivity of the transfectants, all DNA-binding mutations targeted functions that are important for NS1 (III). As all mutants clearly bound better in the presence of ATP, the mutations were not targeting the ATP-binding ability of the NS1. Importantly, enhanced binding upon introduction of ATP was also seen for the helicase mutant, which resides in the same domain as the ATP-binding pocket. The recovery in the presence of ATP was, however, somewhat faster in this mutant compared to others.

In infected cells, the recovery of DNA-binding mutants was divided roughly into two groups. The binding characteristics of the nicking mutant Y212F and C-terminal deletion dC67 were highly similar to those of NS1. This group was characterized by slightly smaller on-rates in the slower turn-over reaction, compared to NS1. Markedly faster dynamics was observed for metal ion coordination mutants, E121A and AUA, the ORI-binding mutant K2A, and the helicase mutant K470/2A. These mutants had increased on- and off-rates in faster turnover reactions, and over two-fold larger off-rates in slower turnover reactions, compared to NS1.

Without infection, DNA-binding mutants grouped roughly into three categories in FRAP experiments. Y212F was again most similar to non-mutated NS1 but significantly faster during the first 57 s of the recovery. In this case, the dC67 mutation showed noticeably slower binding. The recovery of dC67 was statistically slower than NS1 during the whole imaging time. We speculated that the reason could be a change in the electrostatic interactions, because the C-terminal deletion changes the theoretical isoelectric point of the protein from 6 to 7.3. In vitro FRAP experiments conducted in varying salt concentrations supported this idea: in high salt, the binding of NS1 and dC67 proteins was more similar than under low-salt conditions. The group of fastest recoveries was formed again by the metal ion coordination mutants (E121A, AUA), ORI-binding mutant K2A, and helicase mutant (K470/2A). Without infection, this group was also more heterogeneous. The E121A recovered significantly faster during the first 15 s of the recovery, compared to AUA. This could arise from a larger freely diffusing population in E121A, as suggested by the simulations. Interestingly, the recoveries of the K2A and K470/2A mutants were indistinguishable. Simulations suggested that both mutants had slight increases in the turnover in slower binding reactions and increased off-rates in the slower binding reactions, compared to non-mutated NS1. Together, these changes led to an increase of the freely diffusing population to ~60 % (~30 % in NS1) and a decrease of the strongly bound population to ~7 % (~40 % in NS1). Finally, the recovery of the mutant that was unable to bind ATP, K406M, was faster than any of the DNA-binding mutants, regardless of infection status.

The results above indicated that the N-terminal domain and helicase domain have similar roles in determining the intranuclear dynamics of NS1. To gather more information on the possible roles of these domains, we combined the AUA, K2A, K470/2A, and dC67 mutations in combinations of two and three, and studied their dynamics in non-infected cells with the FRAP method. When combined with the metal ion coordination mutant AUA, the dC67 deletion resulted in stronger binding, while the K470/2A and K2A mutations resulted in weaker binding. Also, when the AUA, K470/2A, and dC67 mutations were combined, the recovery was identical to AUA-K470/2A. The ORI-binding mutant K2A had faster recovery when compared to AUA. When these mutations were combined, the recovery was even faster, indicating further reduced DNA-binding. Similar recovery was also observed for the triple mutant K2A-K470/2A-dC67. Interestingly, when combined with K2A, K470/2A did not change, and dC67 increased its binding. The changes in the K470/2A mutant were less pronounced upon addition of mutations. Unlike in AUA or K2A, the dynamics of K470/2A was increased slightly when it was combined with the dC67 mutation. A similar effect was also seen with AUA and AUA-dC67, and an even faster recovery was seen in K2A-dC67. Interestingly, the double mutant K2A-K470/2A had an identical recovery with K2A and with K470/2A.

In conclusion, the recoveries of the predicted ORI binding (K2A) and helicase (K470/2A) mutants was indistinguishable in the FRAP experiments regardless of the infection status. This observation suggests that both mutations

have the same effect on the DNA-binding properties of NS1. The double mutant where both ORI binding and helicase domains were mutated was also identical with both single mutants in noninfected cells. In addition, the C-terminal deletion (dC67) was recovering slower than NS1 in noninfected cells, and when combined with ORI binding (K2A) or metal ion coordination mutant (AUA), it slowed down their recovery.

6 DISCUSSION

6.1 Changes in nuclear architecture

CPV infection causes major changes in the nuclear architecture. The most prominent change is the marginalization of the host chromatin towards the nuclear envelope. BrdU labeling confirmed that practically all endogenous DNA is densely packed and close to nuclear envelope and nucleoli. This rearrangements leads to three different compartments in the nucleus: closest to the nuclear envelope is the endogenous chromatin, innermost is the APAR, and sandwiched between these two are small nuclear organelles; e.g., PML bodies. Similarly to host chromatin, the histone proteins were concentrated towards the nuclear envelope. However, their exclusion from the replication area was only partial, and some histones were found homogeneously distributed in the replication body area. This was less pronounced for H2B compared to H3 or H4, suggesting that localization is dependent on the histone species. Differences in the dynamics of the histones is an unlikely explanation for their different localization, as H2B is more dynamic than H3 or H4 (Kimura & Cook 2001). Interestingly, H3 and H4 histones are found in CCAAT box-containing promoters under active conditions, while H2A and H2B are substituted in this nucleosome-like complex by the histone-mimicking transcription factor NF-Y (Gatta & Mantovani 2008). Both viral promoters could potentially bind this kind of complex. NF-Y has been reported to control the NS1 promoter P4 in MVM (Gu et al. 1995), and the CCAAT box enhances capsid promoter P38 transcription *in vivo* in MVM (Gavin & Ward 1990). It therefore seems plausible that distinct localization of the H3-H4 histones to the replication bodies is related to transcriptional control in infection. These results suggest that parvoviruses use host histones to build up viral chromatin, as reported for herpes simplex virus-1 (HSV-1; reviewed in Kutluay & Triezenberg 2009). HSV-1 infection can be either lytic or latent. The viral chromatin formation is associated with the latter and is hypothesized to function as a switch between the two (reviewed in Kutluay & Triezenberg 2009). Unlike HSV-1, parvoviral

genomes are small and single-stranded, and latent infection has not been reported for CPV. The possible role of viral chromatin could be related to transcription, as readily suggested by the importance of NF-Y (Gu et al. 1995). In related AAV2, which is capable of latent infection, the role of histones could be more diverse.

The size of the infected nuclei was ~2.9-times larger than in non-infected cells. The total amount of DNA in the cells was also increased over 2-fold compared to noninfected cells. Increases in the amount of DNA are caused by viral replication. In BrdU experiments at 24 h p.i., newly synthesized ss- and dsDNAs were found only in the replication body area. These results suggest that synthesis of novel viral genomes and their double-stranded precursors are responsible for the increase in nuclear volume.

6.2 Changes in the nuclear dynamics

The intranuclear dynamics of the inert fluorescent protein EYFP increased in infected cells, compared to non-infected. Interestingly, the simulations suggest that observed increase was not due to a change in the diffusion coefficient of EYFP, but rather, it was caused by the disappearance of a slowly moving population that is observed in non-infected cells. Recent results have elucidated the structure of chromatin in a detailed manner. Chromatin appears to be packed in the nucleus as a fractal globule and divided into spatially segregated, dense heterochromatin and less dense euchromatin (Lieberman-Aiden et al. 2009). Both chromatin types restrict the diffusion of the particles, leading to anomalous sub-diffusion but to a slightly different extent (Bancaud et al. 2009). The results obtained in infected cells indicate that the nuclear architecture, and not viscosity of the nucleoplasm, has changed to more homogeneous. This could simply be due to a less complex nature of the replication body that is filled with viral genomes, compared to a nucleus filled with chromatin. More homogeneous distribution of various-sized dextrans in replication body areas, compared to non-infected nuclei, supports this idea.

The parvoviral genome is presumably replicated by polymerase δ in a process that requires the sliding clamp protein PCNA. The changes in the localization and in the dynamics of PCNA correlate well with its proposed role in replication. In the nuclei of G phase cells, PCNA is heterogeneously distributed, and its dynamics resembles diffusion. In S phase cells, PCNA concentrates to replication foci, with an estimated binding half-time of ~25 s (Solomon et al. 2004) or longer (Essers et al. 2005). In CPV infection, PCNA is localized to the replication body area and is bound, on average, for 83 s. Importantly, its dynamics are well reproduced with the single binding site model. The current model for parvovirus genome replication suggests that unit-length (~5.3 kb) genomes are most frequent replication products (Cotmore & Tattersall 2005b). If we assume that PCNA stays bound in the replication complex for the 5.3 kb genome replication period, the synthesis rate of the CPV

genome would be ~64 bases/s. Similar estimates of 33 bp/s and 58-78 bp/s are reported for genome replication in HeLa cells and for the Epstein-Barr virus, respectively (Jackson & Pombo 1998, Norio & Schildkraut 2004).

To investigate capsid mobility, we used virus-like particles (VLPs), which were formed by expressing photoactivatable VP2 fusion proteins (PAGFP-VP2) in target cells. It has been reported that VP2 proteins can form VLPs without other viral components (Hurtado et al. 1996), and that the N-terminal GFP fusion does not hinder the formation of VLPs (Gilbert et al. 2004). Nuclear localization and capsid antibody labelling suggested that when expressed in cells, PAGFP-VP2s formed VLPs in the nuclei. Capsid mobility was drastically affected by the infection. In non-infected cells, the majority of fluorescent VLPs diffused with a diffusion coefficient of ~5 $\mu m^2/s$. This is close to the theoretical value of 3 $\mu m^2/s$, derived for VLP-sized molecules, suggesting that this population corresponds to assembled VLPs. In infection, a small population had identical ~5 $\mu m^2/s$ diffusion coefficient, while the majority was moving much slower. The diffusion coefficient for this population was approximated to be 0.001$\mu m^2/s$, which corresponds to very slow movement of ~30 nm/s. The data suggest that virus-sized particles can diffuse in infected cell nuclei, but the majority appears to be bound somewhere. This slowly moving population could represent packing intermediates with partially exposed genomes, as has been demonstrated for LuIII (Muller & Siegl 1983).

6.3 Many faces of CPV NS1

6.3.1 NS1 dynamics

NS1 is the first viral protein that is produced and its functions are mandatory for the advancement of viral infection. Starting from the small interchromosomal foci in the nuclei of infected cells, NS1-positive structures enlarge and fill the nuclei almost completely. A fluorescent fusion protein was used to analyze NS1 dynamics in the nuclei of infected and non-infected cells. Its dynamics appear to be fairly complex, irrespective of the infection status; it is not reproduced with the diffusion or single binding site models. Instead, a more elaborate model with at least two binding sites is needed. The binding target that affects its dynamics in detectable amounts is most likely DNA. The observed slow recovery in FRAP experiments of infected cells could be explained with two models. Either NS1 could be bound to a slowly diffusing molecule (e.g., viral genome) or it has multiple binding modes with distinct kinetics. The former hypothesis is unlikely, because the viral genome-sized DNA strands would be essentially immobile in the nuclei (Lukacs et al. 2000). Moreover, it does not explain the complex dynamics observed in noninfected cells. Using the model with two independent binding sites, the binding times for NS1 in infection were somewhat dependent on the used FRAP protocol. With a 2-µm-diameter circular bleach area, the obtained binding times were 10 s

for fast turnover and 83 s for slow turnover reactions, while protein was diffusing freely for 8.1 s between bindings (I). Using a 2-µm-wide rectangular bleach area, the binding times were 20 s and 142.9 s, and protein was diffusing for 17.2 s between bindings (III). The ~2-fold differences in the estimated binding times are likely arising from the differences in the FRAP parameters. The larger bleaching area leads to more extensive bleaching of the diffusing proteins due to their faster movement. In the NS1 binding model, both binding reactions are exchanging proteins with the diffusing fraction, and hence the reduction of fluorescent proteins therein would readily explain the apparent slower binding times. On the other hand, the smaller bleach is more prone to a noise due to the smaller data acquisition area, which could lead to underestimation of the contribution from the smaller populations. Nevertheless, the results are reasonably similar in both cases: binding time in the slower turnover reaction is ~7-times longer than in the faster turnover reaction, which in turn is similar to the free diffusion time. Importantly, the results show that the comparison of the results obtained with different FRAP protocols should be made with caution.

In non-infected cells, devoid of viral ssDNA, the NS1 shows qualitatively similar dynamics compared to infected cells: a model with two binding sites is needed to reproduce its behaviour. The binding times in non-infected cells with a 2-µm-wide rectangular FRAP area were 6.25 s and 45.5 s, while free diffusion time was 5 s between bindings (III). This strongly suggests that binding to non-specific dsDNA gives rise to the complex dynamics of NS1.

6.3.2 ATP (in)dependent binding of NS1

To function correctly, NS1 is dependent on ATP. Its ATP-binding pocket is located in the conserved SF3 helicase domain, residing in the middle of the polypeptide chain. According to our results, CPV NS1 is a typical SF3 member in its ATP-binding properties. The base for the ATP-binding pocket is formed by the conserved, A, B, and C motifs, while the B' motif is responsible for interactions with transported DNA. SF3 helicases are generally thought to function as hexameric rings, and the ATP-binding pocket is complemented by the residues of the neighboring subunit. Many SF3 proteins are reported to use an arginine finger in ATP sensing, and CPV NS1 appears to be one of these.

The DNA-binding of CPV NS1 is enhanced in the presence of ATP. The mutation in the conserved lysine of the A motif, K406M, abolishes this function, indicating that this mutant is no longer able to bind ATP. The overall binding properties of the K406M mutant also change dramatically. In non-infected cells, the FRAP recovery of K406M mutant can be simulated with the single binding site model. Based on this observation, we hypothesized that NS1 binding can be explained with ATP-dependent and -independent binding reactions – a model that was later proven to be too simple. Following the hypothesis, we modeled recoveries of other ATP-binding mutants using parameters of the K406M mutant for the faster turnover binding reaction and adjusting the second binding reaction to match the recovery. For the B motif mutant E444Q and two

potential arginine finger mutants, R508A and R510A, this approach produced excellent fits to the experimental data. However, for the mutation E445Q, which targets the second conserved glutamate in the B motif, it did not. The dynamics of E445Q were drastically different from that of the others in two ways. First, its recovery was not dependent on diffusion. This already confirms that its binding kinetics is very different from that of the others. Second, the recovery of E445Q could not be reproduced with the fast turn-over reaction comparable to K406M, again different from the others. In MVM NS1, the mutant corresponding to E445Q in CPV, is binding ATP while its ATPase activity is severely impaired (Jindal et al. 1994). To test the role of ATP hydrolysis in the dynamics of CPV NS1, we used the non-hydrolyzable ATP analogs AMP-PNP and ATP-γS in an *in vitro* FRAP assay. The binding of NS1 was clearly enhanced by both analogs compared to binding in buffer, although slightly less with AMP-PNP. Interestingly, the binding of the E445Q mutant did not change in the AMP-PNP-containing experiments, compared to mere buffer, while its binding was clearly enhanced in ATP-γS. Although both AMP-PNP and ATP-γS are generally referred to as non-hydrolyzable ATP analogues, some ATPases can hydrolyze them, even with an efficiency comparable to ATP (Peck & Herschlag 2003, Taylor 1981). If we assume that ATP hydrolysis is also needed to promote binding, the discrepancy between NS1 and E445Q could be explained by differences in their ability to hydrolyze ATP analogs. This hypothesis is further supported by our recent results. Using the same *in vitro* FRAP setup, we showed that NS1 binding is also enhanced in the presence of ADP, a hydrolyzed ATP.

Based on these results, tantalizing similarities are emerging between the function of CPV NS1 and bovine papillomavirus (BPV) E1 initiator protein. The E1 protein is the BPV analogue of NS1: it has an N-terminal ORI-binding domain, followed by helicase domains. Incidentally, the N-terminal domains of E1 and AAV Rep protein (and presumably other members of *Parvoviridae*) share the same fold but hardly any sequence identity (Hickman et al. 2002), suggesting a distant common origin for the two virus families. In addition, both E1 and Rep/NS1 proteins are SF3 helicases, and the N-terminal part of the helicase domain is needed for multimerization (Enemark & Joshua-Tor 2006, James et al. 2003). E1 has a minimum of three different DNA binding modes. First, it can bind dsDNA non-specifically (Liu et al. 2010). Second, E1 forms a head-to-head double trimer on the dsDNA ORI sequence. Interestingly, this binding mode is dependent on nucleotide binding and can be induced by ADP (Schuck & Stenlund 2005). Finally, E1 is able to melt dsDNA at the ORI and assemble a head-to-head double hexameric helicase complex to emerging ssDNA strands. The trimer-to-hexamer transition and the helicase activity are dependent on ATP hydrolysis (Schuck & Stenlund 2005). Our results point out to a similar ATP-dependent function for parvovirus NS1 proteins. The non-specific dsDNA binding mode is mimicked by the K406M mutant, which is unable to bind ATP, and hence unable to multimerize. The multimeric form, equivalent to the E1 trimer, is enriched in E445Q, a mutant with severely

reduced ATP hydrolysis. The hexameric helicase activity would be retained in non-mutated NS1.

To conclude, we hypothesize that NS1 dynamics is determined by at least three different components: ATP-independent non-specific dsDNA binding, ATP-dependent sequence-specific dsDNA binding, and ATP-hydrolysis dependent non-specific ssDNA helicase activity.

6.3.3 Effect of DNA binding on NS1 dynamics

In addition to ATP binding, the function of NS1 is dependent on its different DNA-binding domains. First, we confirmed that the binding of all mutants was enhanced by ATP. This observation established that none of the mutated sites is solely responsible for ATP-dependent binding of NS1. Importantly, this was also seen for the helicase mutant, in which the mutations reside in the same domain with the ATP-binding pocket. Most of the mutations in the N-terminal ORI-binding domain and the helicase domain had major effects on NS1 dynamics in living cells. The mutation Y212F that targeted the covalent linking tyrosine in the N-terminal domain showed slightly but significantly reduced binding in infected cells, compared to non-mutated NS1. The increased dynamics seen for the Y212F mutant could arise from the difference in the covalently bound fraction of the protein. In the FRAP experiments, this covalently bound fraction would appear as immobile. Intriguingly, the simulations suggested that the immobile fraction of Y212F was 2 %, compared to 4 % in others. However, as determined experimentally in MVM, only as little as 0.1 % of the NS1 proteins are covalently linked to the viral genome, and this population is most likely too small to be observed in the FRAP experiments (Cotmore & Tattersall 1988). It therefore seems plausible that the Y212F mutation has some effect on the DNA-binding properties of the N-terminal domain. This is perhaps not surprising, because an equivalent amino acid in AAV5 Rep structure is in close proximity to the metal ion coordination center, a position where dsDNA is thought to bind in a catalytic reaction (Hickman et al. 2002).

In infected cells, both the C-terminal deletion (dC67) and the nicking mutant (Y212F) showed slightly faster dynamics, compared to non-mutated NS1. To our surprise, the dynamics of dC67 without infection was much slower than that of the NS1. According to the simulations, binding kinetics of the dC67 were almost the same in infected and non-infected cells, excluding the immobile fraction observed in case of infection. The C-terminal deletion changes the theoretical isoelectric point of the NS1 from 6 to 7.3. This could lead to changes in the electrostatic interaction of the proteins. *In vitro* FRAP analysis, done in different salt concentrations, supports this hypothesis. Analogous results were recently shown for nucleic acid remodeling chaperone (NC) of human T-cell leukemia virus (Qualley et al. 2010). The NC protein has cationic N-terminal and anionic C-terminal domains. Deletion of the C-terminal domain enhanced its chaperone activity, presumably by removing intramolecular interactions of

the termini. The dC67 deletion in CPV NS1 could enhance multimerization and DNA binding by simple removal of charge-repulsing interactions.

According to the alignment with AAV5 Rep protein, the metal ion coordination center in CPV NS1 is composed of two histidines, H129 and H131, and a glutamate, E121. These sites were targeted with two mutations, AUA (H129A, H131A) and E121A. Both mutations showed significantly faster dynamics compared that of the NS1, regardless of the infection status. Recoveries of the mutants were identical in infected and fairly similar in non-infected cells. Simulations revealed that both mutants had two binding sites with reduced binding, compared to NS1. These results suggest that both AUA and E121A are targeting the same function in NS1, although differences were seen in their recovery in non-infected cells. In MVM, the mutation of amino acid that is equivalent to H131 in CPV abolishes nicking activity of the NS1, renders the virus unable to replicate, and reduces ORI recognition (Nüesch et al. 1995). In line with this, the AUA and E121A transfectants had reduced infectivity, compared to cells expressing non-mutated NS1.

The putative ORI-binding mutant K2A (K196A, K197A) and helicase domain mutant K470/2A (K470A, K472A) had identical recoveries in both infected and in non-infected cells. Their binding was characterized by two binding sites with reduced binding, compared to NS1. The N-terminal domain of NS1 contains the ORI recognition domain (Mouw & Pintel 1998). Based on the large reduction in the binding of the K2A mutant, the identified amino acids are part of the ORI recognition motif. Proteins that bind DNA in sequence specific manner (e.g., transcription factors) are thought to find their targets by scanning the DNA in a non-specific sliding mode (Barkley 1981, Ehbrecht et al. 1985, Jeltsch et al. 1994). The best-studied example of this binding mode is the LacI repressor of *Escherichia coli*. Structures of LacI in solution, bound to a non-specific DNA sequence and bound to its recognition sequence, have revealed a possible general mechanism for the change from non-specific DNA binding to the sequence recognition. When comparing the solution and non-specifically bound structures, only minute changes are apparent in the LacI structure. When bound to the recognition sequence, the structure of LacI undergoes some changes, while the target DNA is clearly bent. Importantly, the DNA-interacting interface of the protein is almost the same in non-specific and specific bindings. This has led to a general model for target site searches by DNA-binding proteins. A protein stays bound to DNA via non-specific interactions and can undergo 1D diffusional "searching" movement. When a specific sequence is encountered, a tighter interaction is formed, which might include changes in the protein structure. We propose that this scheme explains the changes in the dynamics of the ORI recognition mutant K2A.

Interestingly, a change in the binding dynamics, that was identical to K2A, was observed for the helicase domain mutant K470/2A. To analyze the role of the B' motif β-hairipin, we first compared mutants where one or both of the conserved lysines were mutated to alanines. FRAP studies in non-infected cells showed reduced binding for all mutants. The fastest dynamics were observed for the K472A and K470/2A mutants. This domain has not been reported to

have a role in non-specific dsDNA binding in parvoviral NS1 proteins. The change in the binding dynamics in non-infected cells (i.e., devoid of viral genome or ssDNA) strongly indicates that the helicase domain is participating in the non-specific binding of dsDNA. Similar results have been recently reported for BPV E1 protein (Liu et al. 2010). When bound to ssDNA in the helicase mode, the β-hairpin of the B′ motif mediates interactions with the phosphate atoms of the translocated DNA (Enemark & Joshua-Tor 2006). Importantly, the same amino acids in β-hairpin are also important for non-specific dsDNA binding (Liu et al. 2010).

Like in the ATP-binding study, these observations suggest remarkable similarities in the function of parvoviral NS1/Rep and BPV E1 proteins. This level of conservation in the mechanism is somewhat unexpected, considering the differences in their genomes – circular dsDNA in papillomaviruses and linear ssDNA in parvoviruses. However, both viruses use their own helicase, E1 and NS1/Rep, in genome replication. Moreover, the structural conservation in their N-terminal and helicase domain folds, suggests a common evolutionary origin for these virus families. The results obtained here for CPV NS1 imply that the DNA-binding strategy of NS1/Rep and E1 is also very similar, further supporting their common ancestry.

Based on these results, some adjustments can be suggested to the NS1 binding model. Instead of two independent binding sites, it is more likely that NS1 binding consists of an initial non-specific binding to the dsDNA, which leads to tighter binding if the recognition sequence is found or to unbinding. The non-specific binding is mediated by the two lysines of the N-terminal ORI recognition domain and the β-hairpin of B′ motif of the helicase domain. This non-specific dsDNA-binding mode is likely to be enhanced by ATP-induced multimerization of NS1. In addition, both the ORI binding and helicase domains have previously recognized functional roles in sequence specific dsDNA recognition and ssDNA helicase activity, respectively. Moreover, in BPV, the sequence recognition of E1 is enhanced by E2 protein, and the role of E2 is to mask the non-specific binding of the E1 helicase domain (Abbate et al. 2004). By analogy, the nicking and preceding tight binding to dsDNA in NS1 is dependent on the auxiliary host cell factors HMG1/2 and GMEB/PIF (Christensen et al. 1997b, Christensen et al. 1999, Cotmore et al. 2000).

Our studies on the dynamics of CPV NS1 have revealed some of the major factors that determine its DNA binding in living cells. However, more studies are still needed to clarify the details that control NS1 binding properties in infection. The role of auxiliary factors in NS1 binding is demonstrated in *in vitro* studies, but their role in living cells is not studied. It is possible that NS1 interactions with the HMG1/2 or GMEB/PIF could lead to observed tighter binding mode. In addition, different functions of the parvoviral NS1 protein are regulated by phosphorylation. Changes in the NS1 phosphorylation pattern could directly function in regulation of different binding modes or binding preferences. I hope that our results will stimulate further investigation in this field.

7 CONCLUSIONS

The main conclusions in this thesis are:

1. CPV infection leads to substantial reorganization of the nuclear architecture. The host chromatin is marginalized towards the nuclear envelope and nucleoli, and the center of the nucleus is filled with viral genome and the proteins needed in its replication. These changes increase intranuclear mobility of inert proteins. This is presumably an effect of a more uniform environment in the replication body, compared to the nucleus of a non-infected cell.

2. NS1 binding is highly dependent on ATP. Its dynamics can be described using a model with two independent binding reactions with an immobile binding partner. The two binding reactions have highly different binding times. The faster and slower binding sites are related to ATP-independent and -dependent double-stranded DNA-binding modes of NS1, respectively.

3. The binding of NS1 is mainly determined by conserved regions in the N-terminal ORI-binding domain and in the helicase domain. Charge-neutralizing mutations in the proposed ORI recognition region of the N-terminal domain and the β-hairpin of helicase domain cause identical reductions in NS1 binding. These results indicate that the helicase domain is participating in non-specific binding of single-stranded and double-stranded DNA.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Parvovirusten replikaationproteiini NS1:n dynamiikka

Solu on elämän perusyksikkö. Uudet solut syntyvät jakautumisen kautta jo olemassa olevista soluista ja tämä on ainut tapa tuottaa uutta elämää. Jokaisen solujakautumisen kautta myös solun genomi, joka sisältää sen toiminnalle ja kehittymiselle välttämättömät ohjeet monistetaan ja siirretään muodostuviin soluihin. Monistusprosessissa satunnaisesti tapahtuvat virheet johtavat pohjimmiltaan elämän monimuotoisuuteen ja evoluutioon. Yksi monimutkaisimmista ja läheisesti solujen ja eliöiden elämään vaikuttavista elottomista tekijöistä ovat virukset. Virukset muistuttavat monilta osin eläviä soluja. Ne lisääntyvät tuottaen itsensä kaltaisia jälkeläisiä ja kykenevät reagoimaan ympäristön muutoksiin, joskin rajallisesti. Virukset ovat kuitenkin täysin riippuvaisia soluista elinkierrossaan ja ovat niiden ulkopuolella kuin horroksessa, odottaen ulkopuolelta tulevaa ärsykettä herätäkseen. Virusten ja solujen rinnakkaiselo on alkanut todennäköisesti hyvin varhain, ehkä jo ennen eri solutyyppien ilmestymistä. Tästä kertoo eräiden bakteeri-, arkki- ja tumallisia soluja infektoivien virusten samankaltaisuus. Virukset koetaan arkielämässä usein haitallisina sairauksien aiheuttajina, mutta niiden merkitys elämän monimuotoisuudelle on kiistaton. Ihmisen genomisekvenssistä jopa 8 prosenttia on peräisin retroviruksista, kun esimeriksi proteiineja koodaavia eksonialueita on vain noin 1,5 prosenttia.

Virusten fyysinen sekä genominen koko vaihtelevat suuresti. Suurimpien virusten proteiinikuori on jopa 400 nm halkaisijaltaan ja niiden genomi koostuu sadoista geeneistä. Parvovirukset edustavat toista ääripäätä. Niiden proteiinikuori on halkaisijaltaan alle 30 nm ja genomissa on vain kaksi geeniä. Yhteistä kaikille viruksille on kuitenkin niiden tarve käyttää solujen koneistoja monistumisessaan. Niinpä viruksia onkin menestyksekkäästi hyödynnetty solujen toimintamekanismien tutkimisessa. Esimerkiksi bakteereja infektoivien faagien avulla osoitettiin, että viruksen perimäaines koostuu DNA:sta, eikä aminohapoista.

Viruksia on myös ryhdytty valjastamaan lääketieteen käyttöön. Yksi kiinnostavimmista uusista hoitomuodoista lääketieteessä on geeniterapia, jossa yleisimmin pyritään tuottamaan hoitavaa proteiinia kohdesolussa. Jotta tähän päästäisiin, on hoitavaa proteiinia tuottava geeni saatava solun tumaan. Osa viruksista kuljettaa oman perimäaineksensa tumaan saakka. Tällaisia viruksia muokkaamalla on pyritty saamaan aikaa kuljettimia, jotka ohjaisivat solut tuottamaan haluttua proteiinia. Pitkäaikaisen proteiinin tuottamisen aikaansaamiseksi on toivottua saada haluttu geeni liittymään osaksi kohdesolun perimää. Tähän liittyy kuitenkin huomattavia ongelmia. Jos geeni liittyy väärään kohtaan, saattaa se saada aikaan solun toiminnassa hallitsemattomia muutoksia. Pahimmillaan tämä voi johtaa esimerkiksi syövän kehittymiseen halutun hoitovaikutuksen sijasta. Parvoviruksiin kuuluva dependovirus AAV2 on erittäin lupaava geenisiirtovektori. Sen luontaiseen elinkiertoon kuuluu viruksen ge-

nomin liittäminen osaksi kromosomia 19. Tämän prosessin ei ole havaittu häiritsevän solun toimintaa, eikä AAV-infektioihin ole liitetty sairauksia.

Tässä väitöskirjatyössä on selvitetty koiran parvoviruksen replikaatioproteiinin NS1:n toimintaa elävissä soluissa. Lisäksi havainnoimme parvovirusinfektion aiheuttamia muutoksia tuman rakenteessa sekä infektion vaikutusta solun proteiinien toiminnalle. Erilaisilla valomikroskooppisilla menetelmillä on ollut keskeinen osa tutkimuksessa. Konfokaalimikroskoopin avulla pystytään havaitsemaan fluoresoivan molekyylin kolmiulotteinen sijainti solun sisällä. Liittämällä kohdeproteiini osaksi luontaisesti fluoresoivaa proteiinia voidaan sen toimintaa tutkia myös elävissä soluissa. Fluoresoivien fuusioproteiinien avulla voidaan myös karakterisoida proteiinien sitoutumisominaisuuksia seuraten fluoresenssin palautumista osittaisen valovalkaisun jälkeen. Tässä koeasetelmassa proteiinin vahva sitoutuminen johtaa hitaaseen palautumiseen. Merkittävänä etuna tässä menetelmässä verrattuna perinteisempiin biokemiallisiin menetelmiin on proteiinin luontaisen toimintaympäristön säilyminen. Tämä on erityisen tärkeää tutkittaessa proteiineja, kuten parvovirusten replikaatioproteiini NS1:stä, jotka sitoutuvat moniin kohteisiin.

Parvovirukset monistuvat jakautuvan solun tumaan muodostuvissa replikaatio-rakenteissa käyttäen hyväksi solun DNA:n monistuskoneistoa. Tutkimuksissamme havaitsimme, että koiran parvovirusinfektio johtaa laajaan uudelleenorganisoitumiseen tuman sisällä. Solun kromosomaalinen DNA ja suurin osa siihen sitoutuneista histoni-proteiineista pakkautuvat tumakalvon läheisyyteen. Tämän aiheuttaa oletettavasti viruksen genomin monistuminen sekä virusproteiinien suuri määrä tumassa. Infektio vaikutti myös NS1-proteiinin dynamiikkaan tumassa. Analysoimalla NS1:stä tehdyn fluoresoivan fuusioproteiinin palautumista havaitsimme, että NS1:n liikettä voidaan kuvata mallilla, jossa se sitoutuu kahteen hyvin erilaiseen sitoutumispaikkaan. Parvovirusten NS1-proteiinien tiedetään tarvitsevan ATP-molekyylejä toimiakseen oikein. Käyttäen hyväksi rakenteellista mallia koiran parvoviruksen NS1-proteiinista pystyimme tunnistamaan aminohapot, jotka ovat tärkeitä proteiinin kyvylle sitoa ATP-molekyylejä. Muuntamalla kriittiset aminohapot toisiksi, ja tutkimalla saatujen mutanttien ominaisuuksia, pystyimme osoittamaan NS1:n sitoutumisen olevan vahvasti riippuvaista sen kyvystä sitoa ja pilkkoa ATP-molekyylejä. Lisäksi havaitsimme, että dynamiikka ATP:a sitomattomassa NS1-mutantissa oli selitettävissä yksinkertaisemmalla mallilla, jossa sitoutumispaikkoja on vain yksi. NS1-proteiini sitoutuu tumassa sekä yksi- että kaksijuosteiseen DNA:han. Vertailemalla koiran parvoviruksen ja sen lähisukulaisten sekvenssejä pystyimme tunnistamaan NS1:stä useita potentiaalisia DNA:n sitoutumista välittäviä aminohappoja. Näihin kohdennettu mutageneesi heikensi NS1:n sitoutumista tumassa, vahvistaen tunnistettujen aminohappojen roolin DNA:han sitoutumisessa. Tarkempi tarkastelu osoitti, että useat aminohapot proteiinin N-terminaalisessa osassa osallistuvat sen sekvenssistä riippumattomaan sitoutumiseen kaksijuosteiseen DNA:han. NS1:n keskellä sijaitsevan osan on aikaisemmin osoitettu toimivan DNA-juosteita toisistaan irrottavana helikaasina. Tuloksemme kuitenkin osoittavat, että myös se osallistuu sekvenssistä riippumattomaan sitoutumiseen kaksijuosteiseen DNA:han. Samankaltainen havainto

on vastikään raportoitu papilloomaviruksiin kuuluvan BPV:n replikaatioproteiini E1:lle. Väitöskirjan tulokset osoittavatkin hämmästyttävää samankaltaisuutta näiden kahden proteiinin toiminnan välillä.

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