The role of the adenovirus DNA binding protein in DNA replication and recombination

De rol van het adenovirus DNA bindend eiwit in DNA replicatie en recombinatie

(met een samenvatting in het Nederlands)

Proefschrift

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Geen enkele fantasie is zo ongelofelijk als de werkelijkheid

M.J. Vreeken-Buijs

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General Introduction

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1. Introduction

Throughout the years adenoviruses have served as a model system to increase the insights in many cellular mechanisms. Early viral regulatory proteins function in transcriptional regulation of host RNA production, induce cell cycle progression and directly participate in viral DNA replication (for review: (171)). Currently many reports can be found about the use of recombinant adenovirus as a vector for gene therapy. This is due to the high efficiency of infection by adenovirus vectors. A well-known example is the use of adenovirus in gene therapy for cystic fibrosis to replace the cystic fibrosis transmembrane conductance regulator gene (for review, (52)).

Adenovirus DNA replication is the main subject of this thesis with particular emphasis on the function of the DNA binding protein, DBP. The following paragraphs will give a general introduction in the adenovirus family and its structural components. Subsequently an introduction in adenovirus DNA replication is given, followed by a more detailed description of the structure and function of the adenovirus DNA binding protein. The last paragraph will discuss the functions of other single-stranded DNA binding proteins in various related replication systems.

1.1 Adenovirus

The adenovirus family consists of many members that can infect a broad variety of hosts, ranging from birds to mammals. The family members are divided into two genera: the aviadenoviridae that infect birds and the mastadenoviridae that infect mammals. Although the adenovirus members within one genus are quite similar, most have a limited host-range. The human adenoviruses form a large group within the mastadenoviridae. Human adenovirus strains are defined by immunological cross-reactivity and are called serotypes. Based on sequence homology the serotypes are grouped into 6 different subclasses, A to F that together encompass over 50 different serotypes. The adenovirus serotype 5 (Ad5) is a member of the group C subclass. The proteins and the genomic DNA from Ad5 were used for the research described in this thesis**.**

1.2 Structure of the adenovirus

The adenovirus particle (virion) is icosahedrally shaped (2-3-5 symmetry) with twelve 360 to 370 Å long fibers protruding from the particle (Fig. 1) (46). When viewed under the electronmicroscope the total size of the virion is approximately 60-90 nm. The shell of the viral particle consists of triangular facets and 12 vertices. All facets are identically composed, and contain 6 different viral proteins (161). Twelve hexon trimers form the facet itself together with a pentameric penton-base located at each vertex. A fiber, which is a trimer of glycoprotein IV, protrudes from the penton-base, and is responsible for heamagglutination. This fiber is composed of a shaft, which has the conformation of a protein triple helix, and a fiber-knob (164). The facet is structurally stabilized by the presence of three additional viral proteins, IIIA, VI and IX.

The core of the Ad5 virus particle contains a 36 kb long double-stranded (ds) DNA genome, covalently attached to a terminal protein at each end. The dsDNA genome is also complexed to two highly basic viral proteins, V and VII, and with the viral μ protein (20). Based on the pattern of micrococcal nuclease

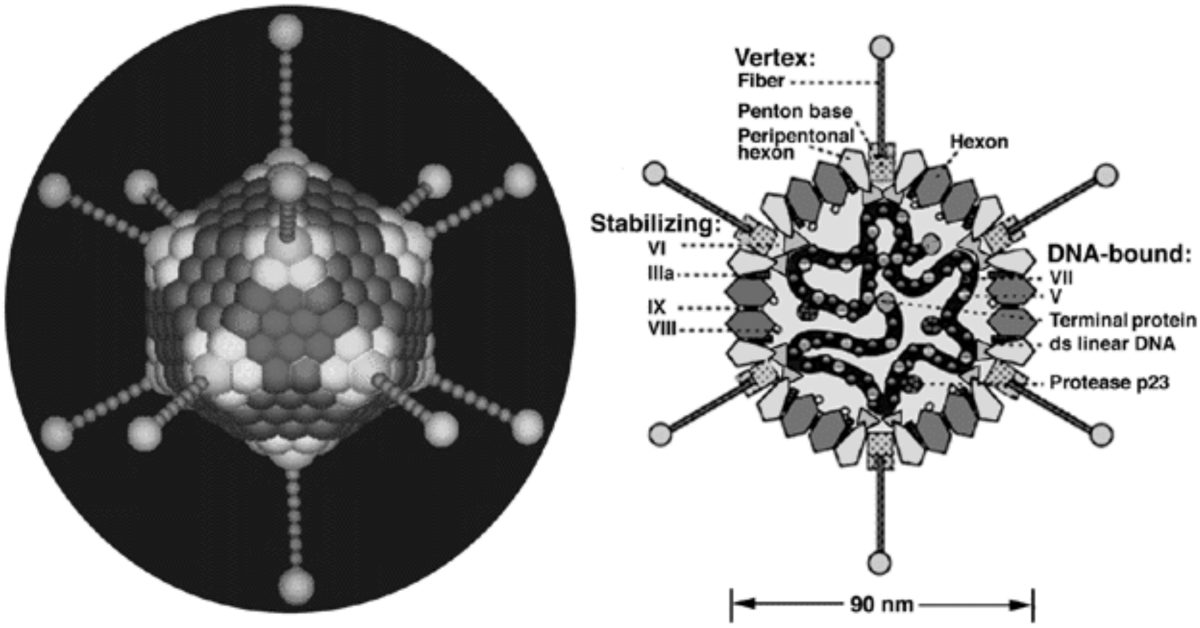

Figure 1: Model of adenovirus type 2 viral particle, which is similar to that of adenovirus type 5. Left: a 3D-model of the viral particle. Right: Schematic representation indicating the proteins involved in the virus structure.
(Source: http://www.unizh.ch/~cellbio/pages/galleryPages/img01.html: **The Greber Lab**)

digestion of adenovirus DNA in pentonless particles it was suggested that the adenovirus DNA is organized into a nucleosome-like structure. Electron microscopy and ion etching led to a model indicating that the internal DNA/protein complex is packaged as 12 large spheres, one below each capsid vertex. However, the exact structure and organization of the DNA/protein complex remains subject of debate (20, 135, 173).

1.3 The viral life cycle

Infection by the human adenovirus is a common cause of respiratory illness. Other inflictions that are associated with adenoviral infection are keratoconjunctivitis, enteric dysentery, pharyngitis, gastroenteritis, pneumonia, hepatitis and acute heamorrhagic cystitis (reviewed in (79)). The adenovirus serotypes 1, 2, and 6 are endemic in many areas in the world. At the age of one almost half of the children in Western Europe and the United States have acquired antibodies to one or more of these viruses. The virus is mainly transmitted horizontally via a fecal-oral route (61). The virus will attach, when in proximity of a susceptible host-cell, via an interaction of the fiber-knob with coxsackievirus and adenovirus (CAR) receptor. Second, an interaction of the penton base capsid protein interacts with alpha(v) integrin receptors (alpha(v)beta1, integrin alpha(v)beta3 and integrin alpha(v)beta5 (9, 17, 106, 150)) to promote internalization. Once the virus is internalized, the shell of the virion is degraded and the viral DNA is transported into the nucleus of the host cell (65). Subsequently, the viral DNA is replicated and virus specific proteins are expressed in the host cell. The structural proteins are first synthesized in a precursor form and are processed into

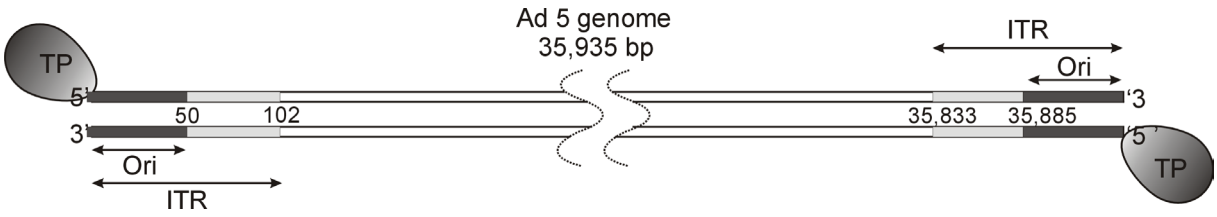

Figure 2: Schematic representation of adenovirus type 5 genome, with its inverted terminal repeats (ITRs) and the origin of replication (ORI) therein. Attached to both molecular 5' ends are the terminal proteins (TP).

mature proteins via proteolytic cleavage by a virally encoded protease. Although virion assembly can also occur with the precursor proteins, only processed proteins will yield infectious particles (174, 190-192). After 24 hours of replication of the virus, up to 10.000 new virus particles per cell have been assembled. Subsequently, the host cell bursts and dies and the newly synthesized infectious virus particles can start a new round of infection.

2. Adenovirus DNA replication

2.1 DNA replication mechanism

To ensure the survival of any living organism, duplication of its genetic material is essential. The DNA replication machinery of a cell is used to copy its genome. This machinery is often complex and under control of many pathways within a cell. The adenovirus DNA replication mechanism has been studied extensively and is one of the first eukaryotic replication systems that could be reconstituted *in vitro* (30, 180).

The adenovirus 5 (Ad5) genome is a 35935 bp long dsDNA strand (34) with a Terminal Protein (TP) attached to each molecular 5' end (Fig. 2). An inverted terminal repeat (ITR) that encompasses 102 bp is also located on each molecular end. The ITRs of different adenovirus serotypes are of variable lengths (78, 154, 170). The first 50 bp of the ITR encompass the origin of replication (ORI), which is a highly conserved region among adenovirus serotypes (156, 163, 172).

The adenovirus genome is replicated from each end of the molecule via a strand displacement mechanism (for review see (29)). Three virally encoded proteins are required for adenovirus DNA replication *in vitro*, DNA polymerase (pol), precursor terminal protein (pTP) and the single stranded DNA binding protein (DBP). In addition, three host proteins are required for optimal replication efficiency, nuclear factor 1 (NFI), Oct-1 (NFIII) and topoisomerase (NFII) (43, 68, 180). Two stages in adenovirus DNA replication can be distinguished. First, initiation of replication takes places followed by elongation of the initiation product in which the DNA is processively replicated. Preceding initiation pol complexes with pTP to form a stable heterodimer. Although the pTP/pol complex can initiate DNA replication in the absence of the host proteins NFI and Oct-1, initiation is significantly enhanced when NFI and Oct-1 are present. Binding of NFI and Oct-1 will recruit pol and pTP, respectively, to the origin. Fig. 3 gives a schematic overview of the mechanism of adenovirus DNA replication (for review, (43)). The multiprotein complex of pTP/pol with NFI and Oct-1 bound to the origin is called the preinitiation complex (PIC) (Fig. 3C). Once the PIC has been

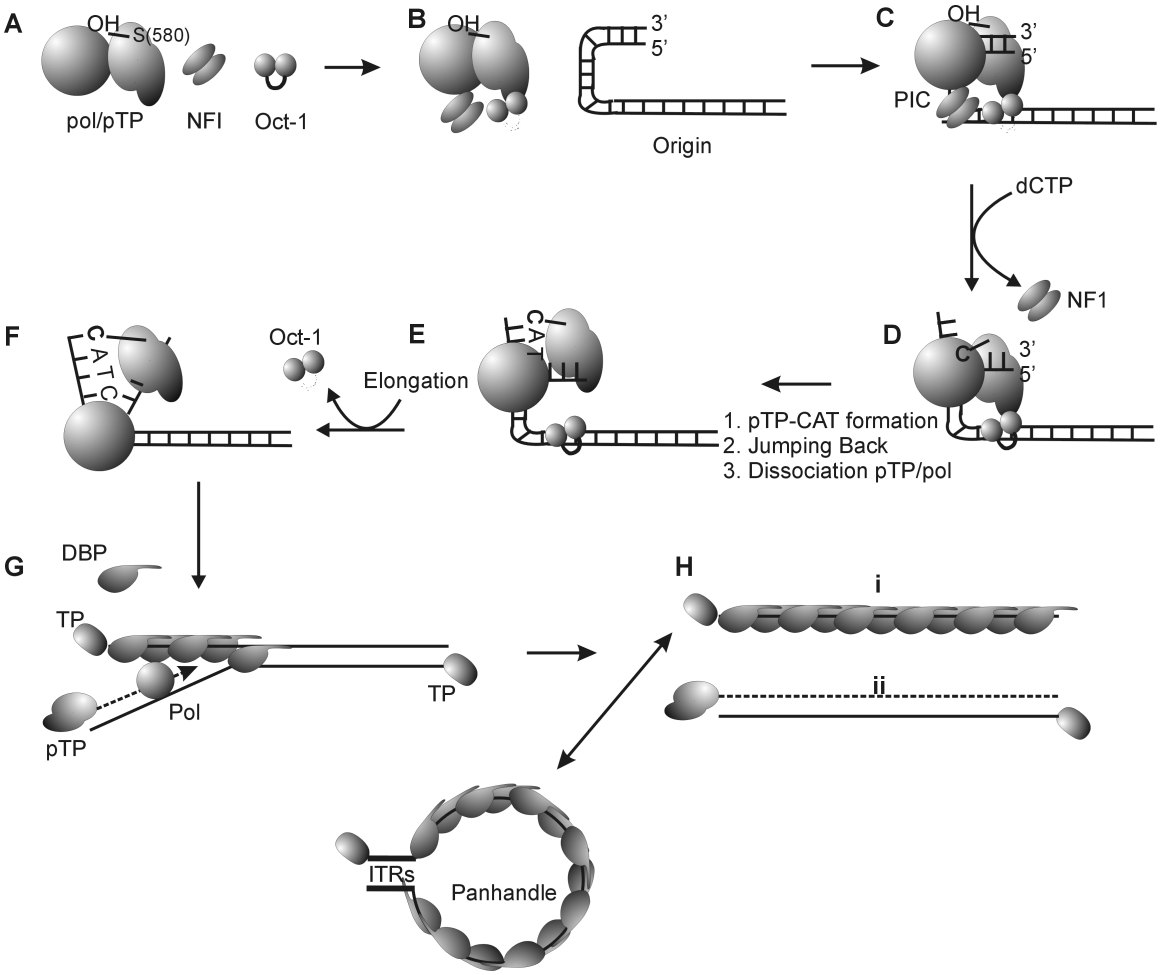

Figure 3: The adenovirus type 5 DNA replication. For clarity DBP and TP are omitted from A-F in this figure. **A**: Protein components of the PIC. **B**: Assembled PIC protein complex and origin DNA, **C**: PIC formation of origin of replication. **D**: The first nucleotide, dCTP is coupled to the pTP opposite the 4th base of the template strand and NFI is released from the origin. **E**: When pTP-CAT is formed opposite to template nucleotides 4-6, the complex jumps back by base pairing to nucleotides 1-3. Oct-1 is released and pTP and pol dissociate. **F**: The pTP-CAT intermediate is elongated. **G**: DBP unwinds the dsDNA ahead of pol. **H:** Two end-products are formed; (**i**) a DBP covered ssDNA (displaced strand) product and (**ii**) a newly synthesized dsDNA replication product. The displaced strand can form a panhandle structure via base pairing of the ITRs. Both products (**i** and **ii**), can be used for a subsequent round of DNA replication.

formed initiation takes place. Strikingly, the initiation starts at position 4 (a G-residue) of the template strand (Fig. 3D). pTP protein offers its serine(580)-OH group as a primer for the covalent coupling of the first nucleotide (dCTP), called protein priming, followed by the dissociation of NFI from the PIC. Subsequently, the pTP-CAT trinucleotide intermediate is formed (90), and the whole complex jumps back to base pair with the first three nucleotides of the template strand (jumping back) (89). After or during the jumping back, pTP dissociates from pol and remains covalently attached to the newly synthesized DNA (Fig. 3E). After initiation the DNA is replicated in a processive manner (Fig 3F). DBP unwinds the dsDNA ahead of the polymerase in an ATP independent manner and enhances the processivity of

Figure 4: The Ad5 origin of replication, with the core origin (9-18 bp) and the auxiliary origin (25-47 bp). Light gray (9-18 bp), the pTP/pol binding site. Black (25-38 bp), the NFI binding site. Dark gray (39-47 bp), Oct-1 binding site.

the DNA polymerase via the removal of secondary structures in the DNA chain (44, 109). For this stage of DNA replication DBP is critical. In addition, NFII is required for elongation of the full-length genome. Once the genome has been replicated a ds- (Fig. 3H**ii**) and a ssDNA (Fig, 3H**i**) end-product have been formed. The ssDNA end-product (displaced strand), can be replicated via a similar procedure initiated from a panhandle structure, where the ITRs base pair to form a double stranded origin (103, 165). In addition, two complementary ssDNA products can reanneal to form a new dsDNA template, which can be used in a subsequent round of DNA replication.

Later during the viral life cycle the pTP proteins that are covalently attached to the molecular ends of the newly synthesized DNA are cleaved by the adenovirus protease to their mature form, TP. The adenovirus DNA, with its associated TP proteins is also referred to as TP-DNA.

2.2 Replication proteins

Adenovirus DNA replication can be reconstituted *in vitro* with only three virus encoded proteins, pTP, pol and DBP, and three cellular proteins encoded by the host, NFI, Oct-1 and topoisomerase. Since the topoisomerase is not absolutely required for *in vitro* DNA replication when templates shorter than ~5000 bp are used it will not be discussed here. The viral proteins pol and pTP and the cellular proteins NFI and Oct-1 will be discussed here in more detail.

The *in vitro* DNA replication has allowed the mapping of important cis-regulatory elements in the origin of DNA replication (Fig 4). The Ad5 origin can be subdivided into two regions. Each of the regions contains a specific binding site for a set of proteins, important for PIC formation and adenovirus replication. The core origin (nt 9-18), which is well conserved in the various adenovirus serotypes, binds the pTP/pol complex. The partially conserved auxiliary origin (nt 25-47) contains the binding sites for NFI and Oct-1 and is involved in enhancing the replication.

2.2.1 (Precursor) Terminal Protein

In vitro replicated Ad5 DNA was shown to be covalently linked to an 80 kDa protein (28). This protein, pTP, was identified as the precursor of the 55 kDa TP (31, 162). During initiation of adenovirus DNA replication the serine(580) residue of pTP protein was shown to be covalently linked to a dCMP residue via a phosphodiester bond forming the pTP-dCMP complex (157). Substitution of serine(580) as well as deletion of the N-terminal 19 amino acids completely eliminated pTP activity in initiation (143), suggesting that this serine(580) residue is essential for

proper Ad5 initiation. pTP forms a tight heterodimer with a second viral protein, pol. (49, 108). This strong complex of pTP and pol can only be separated by urea containing glycerol gradients (108) and is formed immediately when separate fractions of the two proteins are mixed (168). Besides the interaction with pol, pTP has been found to interact with the host transcription factor Oct-1, that is important for the stimulation of adenovirus DNA replication (see below) (42).

Baculovirus expression systems made it possible to express large amounts of pTP protein for detailed studies of its structure and function. (197). These studies revealed that pTP contains a strong nuclear localizationsignal, which was initially mapped between aa 362-373 (198). Linker insertion mutants (57, 151) showed that some domains outside this region might also be important for nuclear localization (56). Linker insertion mutants also identified lethal mutants defective in production of viable virus, either due to defective DNA replication or due to disruption of other functions like encapsidation of the DNA or matrix association (56, 57, 151).

The use of a protein as primer for DNA replication is not only restricted to adenovirus. The genomes of bacteriophages φ29, PRD1 and Cp-1 also contain a terminal protein attached to the molecular ends of their genome. In all these cases the terminal protein is associated to a specific DNA polymerase that catalyzes both initiation and elongation. For instance, φ29 polymerase uses the β-OH group of a threonine residue of its terminal protein as a primer. Remarkably, when the serine(580) of Ad pTP is substituted by a threonine, no initiation was observed, suggesting that the composition of the domains involved in initiation determine what type of amino acid is used for priming (143).

2.2.2 Adenovirus DNA Polymerase

When pTP was purified it appeared to form a strong heterodimeric complex with a 140 kDa protein, which was determined to be a DNA polymerase (pol) distinct from the cellular polymerases, α, β and γ (49, 108). This Adenovirus encoded DNA polymerase shares regions of strong homology especially with φ29, RB69 and PRD1 polymerases, but also with T4 DNA polymerase and with the eukaryotic DNA polymerases of yeast (pol I), human (pol α), herpes simplex virus, vaccinia, and Epstein-Barr virus. (3, 189, 195). Ad pol is an α-like DNA polymerase belonging to the subclass of protein-priming DNA polymerases. Six regions of homology can be detected in the α-like class of DNA polymerases designated I to VI based upon the extent of similarity (189). Regions I-III are conserved in all groups of polymerases and are likely involved in initiation and elongation (I) (82) and in substrate binding (II and III) (115). A seventh conserved motif was also identified in α-like DNA polymerases (11, 80). The crystal structure of the DNA polymerase of RB69 (187) is currently used as a model for α-like DNA polymerases. Recently it was shown that a conserved region, the (**I/Y**)X**GG** motif, is also present in Ad pol. Subsequently, it was demonstrated that this motif plays an important role in the stabilization of the template strand at the polymerase active site, as well as in binding the initiating nucleotide and the transition of initiation to elongation (19).

2.2.3 NFI

Although pTP and pol are able to initiate adenovirus DNA replication, it was found in complementation studies that addition of two host cellular proteins resulted in a significant (200 -fold) increase of DNA replication. A 47 kDa host protein, NFI, was the first to be identified. It is required for efficient initiation of adenovirus DNA replication. NFI is a cellular transcription factor (129) and it is also known as CTF (CCAAT binding transcription factor). NFI binds as a dimer (66) to its recognition site, which, is the palindromic DNA consensus sequence, TGG(C/A)N_5GCCAA, located between nucleotides 25 and 38 in the Ad5 origin (Fig. 4) (104, 130). Structural studies of the NFI protein revealed that the domains for NFI dimerization and the domain that binds to the DNA are contained within the same region of the protein (aa 1-120) (Fig. 5) (63, 120). Binding of NFI to its DNA binding site is stimulated by the viral DBP (167). Although no direct interaction between NFI and DBP was observed, other ssDNA binding proteins cannot complement for DBP (35). It is most likely that DBP stimulates NFI binding via subtle changes in the Ad dsDNA structure (166).

In vitro the level of stimulation of initiation by NFI varies between 2- and 50-fold, depending on the DNA polymerase concentration (124, 127). Deletion studies identified the DNA-binding domain to be solely responsible for this stimulating effect (32, 120). Stimulation by NFI is the result of protein-protein interaction of NFI with pol (32). It is proposed that NFI recruits pol to the origin. It was shown that the DNA-binding domain of NFI between aa 68-150 is sufficient for the pol interaction and stimulation of initiation (4, 16).

2.2.4 Oct-1

The second cellular protein found to stimulate the initiation of adenovirus DNA replication was a 90-95 kDa protein,

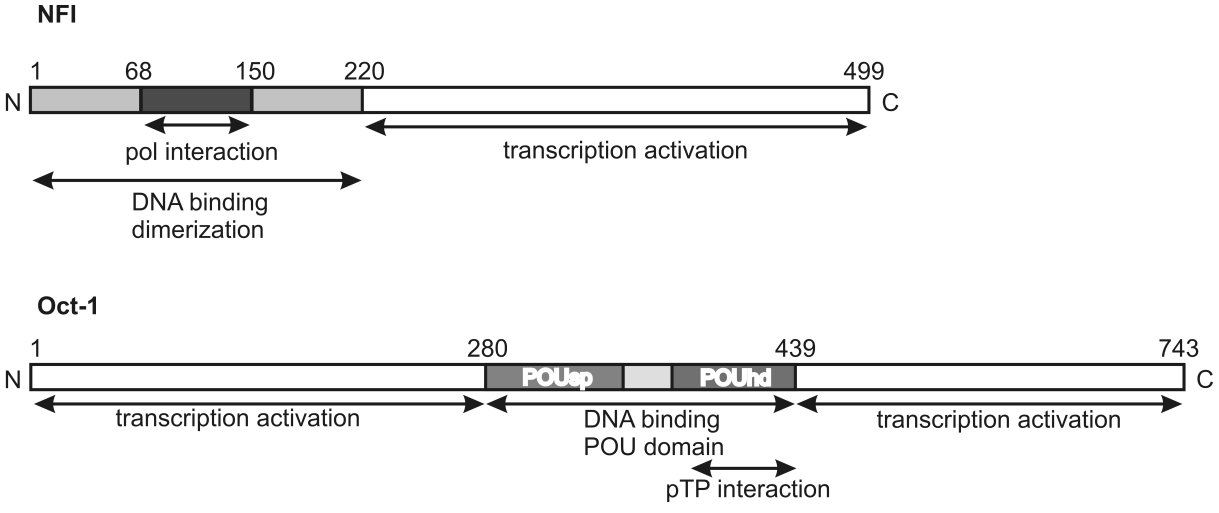

Figure 5: Primary structure of transcription factors NFI and Oct-1. NFI contains an N-terminal DNA-binding and dimerization domain and C-terminal transcription activating regions. The NFI-pol interaction was mapped between aa 68 and 150. Oct-1 contains transcription-activating regions at both the N- and C-termini and a DNA-binding POU domain in the center of the protein. The POU domain consists of a conserved POU-specific domain (POU_{SP}) and a POU homeodomain (POU_{HD}) spaced by a non-conserved linker region (light gray). Residues involved in the interaction between Oct-1 and pTP resides in the POU homeodomain. (Source: (42, 43)).

identified as Oct-1 (141, 145, 146). Like NFI, only the DNA binding domain of Oct-1, the POU domain, is sufficient for stimulation of adenovirus DNA replication (Fig. 5). The POU domain, named after its homology with the binding domains of **P**it-1, **O**ct-2 and **U**nc-86 (74, 75, 169), binds to the Ad DNA between nucleotides 39-47 (Fig. 4). The Oct-1 binding site in the Ad5 origin is a degenerate octamer motif, which is bound by Oct-1 with a lower affinity than for example the Oct-1 binding site present in the Ad4 origin, which contains a perfect octamer binding site (183, 184).

The protein structure of the Oct-1 POU domain consists of two highly conserved regions connected to each other by a non-conserved flexible linker. Although each domain can bind DNA, both domains are required for efficient DNA binding. The N-terminal domain, the POU specific domain (POU_{sp}) recognizes gAATAT(G/T)CA, whereas the POU homeodomain (POU_{HD}) binds to the DNA sequence RTAATNA, as was determined by DNA-binding site selection. The binding consensus of the complete POU domain a(a/t)TATGC(A/T)AAT(t/a)t contains both partially overlapping subdomain binding sites (183).

In vitro Oct-1 can stimulate Ad DNA replication 6 to 8 fold depending on the pTP/pol concentration. This could be due to protein-protein interaction with the pTP/pol complex as well as due to DNA-mediated interactions of Oct-1 with the ORI. In accordance with DNA-mediated interactions, Oct-1 was shown to induce bending of DNA in the presence of an Oct-1 binding site (185). Alternatively, a direct interaction with pTP and Oct-1 was shown using GST-POU domain fusion proteins (38, 182), suggesting a role in recruitment of the pTP/pol complex together with NFI (Fig. 3B,C). Recently it was shown that the Oct-1-pTP interaction domain resides in the POU_{HD} and that pTP and DNA cannot be bound simultaneously at the same time (42). In addition, origin templates with insertions and deletions to alter the spacing between the NFI and Oct-1 binding sites resulted in mutants defective in NFI or Oct-1 stimulation (193). This suggests the necessity for a network of precise protein-protein and protein-DNA interactions in the Ad PIC. For a more complete and extensive review on NFI and Oct-1 see (43).

3. Adenovirus DNA binding protein, DBP

The 59,049 Da Ad5 DBP was the first adenovirus encoded non-virion protein to be discovered (181). This is in part due to the high abundance of the protein in adenovirus infected cells, where about $2*10^7$ molecules of DBP per cell are produced early in infection. Compared to pol and pTP it is expressed about 100-fold more (25). DBP is encoded in the viral E2A transcription unit, a transcription unit that is transcribed early in adenovirus infection. The next sections will discuss the functions and structure of DBP in more detail.

3.1 DBP functions

DBP has been found to play a role in many steps during the adenovirus life cycle. This section will briefly discuss the functions of DBP in transcription, host-range specificity, translation control, virus assembly and recombination. Second, DBP has been found to play a role in the adenovirus associated virus (AAV) DNA replication. Section 3.2 will discuss the function of DBP in the adenovirus DNA replication into more detail, since it is the main subject of this thesis.

3.1.1 The role of DBP in Adenovirus transcription

DBP has been implicated in transcriptional control in a number of studies. First it was shown that DBP enhances the transcription from the E1A and E2 viral transcription units as well as from the AAV P5 promoter. While at the same time the transcription from the E4 region is inhibited. Mutations affecting the phosphorylation of DBP showed a decrease in the enhancement of its own synthesis, suggesting that phophorylation plays a role in the regulation of viral protein synthesis. (37, 67, 122, 149). The inhibition of the E4 transcription unit was further investigated by Northern blotting where probes against the 3' and 5' end of the transcription unit showed a diminished amount of all transcript products, suggesting that initiation of transcription rather than premature termination is affected by DBP (133). Second, DBP has been found to stimulate the binding of transcription factors to their binding sites on DNA, like upstream stimulatory factor (USF) and NFI (35, 199). DBP increases the binding affinity of NFI for its specific site due to structural alteration favoring NFI binding rather than an NFI-DBP protein interaction (167).

3.1.2 The role of DBP in Host-range determination

DBP is able to determine the host-range specificity at the level of mRNA. No infectious progeny is produced when monkey cells are infected by human adenovirus serotypes. The host-range restriction can be relieved when mutations in the N-terminal domain of DBP are introduced, suggesting the involvement of DBP in host-range specificity. The exact mechanism however, has not been determined. There is some indication that DBP influences splicing of a specific subset of late viral mRNAs, notably mRNA coding for the fiber protein (2).

3.1.3 The role of DBP in Translation control

In addition to the role of DBP in transcription regulation, DBP also influences the level of protein expression, thereby acting post-transcriptionally. When a temperature sensitive mutant of DBP was used (H5ts125), the level of early viral mRNAs during infection is raised at permissive temperatures when compared to the levels of early viral mRNAs found during wild-type infection. However, also the stability of the mRNAs is increased in this mutant, suggesting the requirement of a functional DBP protein for the rapid turnover of the early viral mRNAs (5, 27).

3.1.4 The role of DBP in Virus assembly

A class of thermosensitive DBP mutants was identified that were temperature-independent revertants of the H5ts107 mutant (which is identical to H5ts125) mutant but contain a second mutation at aa 352. These mutants are able to grow wild-type like on HeLa cells however they are still temperature-sensitive for growth on 293 cells, a human cell line transformed by Ad5 DNA that constitutively expresses E1A proteins. The mutants are unable to produce infectious particles in 293 cells, although viral DNA replication is only slightly repressed and late protein synthesis, including fiber synthesis, is normal (136). This suggests that the mutant DBP protein is functional but due to the second mutation it is unable to function properly in the virus assembly pathway. At what stage of the assembly DBP exerts this function and what the mechanism of this action is, remains to be elucidated.

3.1.5 The role of DBP in Recombination

Homologous recombination is an important fundamental biological process. In addition to its role in meiosis, it serves to repair DNA damage or to reinitiate DNA replication stalled at replication forks. Upon adenovirus infection high levels of adenovirus recombination is observed. It was demonstrated that, although the host cell has its own efficient recombination machinery, adenovirus recombination is interrelated with the adenovirus DNA replication machinery (53, 61). A role for DBP in recombination has been implicated for long (61). DBP mutants that are defective in DNA binding or helix destabilization, are also deficient in adenovirus DNA replication and hence are difficult to study for their recombination phenotype. Nevertheless it can be envisioned that DBP plays a role in this process. One mechanism of homologous recombination involves the formation of a triple DNA helix from a single DNA strand and a homologous DNA duplex, as has been described for *E. coli* RecA, the principal *E. coli* recombination protein. RecA catalyzes strand transfer of either a single strand and a homologous DNA duplex or two homologous duplexes (Reviewed in (40, 148)). Analogous functions have been described for the Herpes Simplex Virus I single stranded DNA binding protein ICP8 that will be discussed in more detail in a later section. In chapter 4, the role of DBP in the strand exchange step of homologous recombination is discussed in more detail.

3.1.6 The role of DBP in AAV DNA replication

Adeno associated virus (AAV) DNA replication is greatly stimulated upon coinfection with adenovirus of herpes virus. DBP seems to play a role in this stimulation, although the data on DBP in the AAV DNA replication have been ambiguous (26). A possible role of DBP during AAV replication could be similar to that of DBP during adenovirus DNA replication, as will be discussed in 3.2 in more detail. Here DBP enhances the processivity of the AAV replication complex. Indeed immunodepletion of the infected cell extract with an antibody to DBP substantially reduces the ability of these extracts to support AAV DNA replication. In addition several point mutants of DBP with mutations in its DNA binding domain failed to support the synthesis of AAV DNA (132, 147). A second possible mechanism is the recruitment of the AAV helicase Rep78 by DBP. Evidence has been found for a direct interaction between DBP and Rep78. This interaction will be discussed in Chapter 5 of this thesis.

3.2 The role of DBP in adenovirus DNA replication

Ad DNA replication can be subdivided into two separate phases, initiation and elongation. DBP performs different actions in each phase. During initiation, DBP can stimulate the pTP-C formation in two different ways. First, DBP stimulates the initiation indirectly via NFI binding. Addition of DBP to dsDNA enhances the binding of NFI to its dsDNA binding site. For this mechanism a direct interaction between NFI and DBP is not required, but more likely a conformational change in the dsDNA structure is responsible for this enhancement (35, 167). Second, DBP can lower the K_m for the incorporation of the first nucleotide (125), thereby stimulating the initiation up to 15-fold depending on the pol concentration. It is thought that this is due to a direct

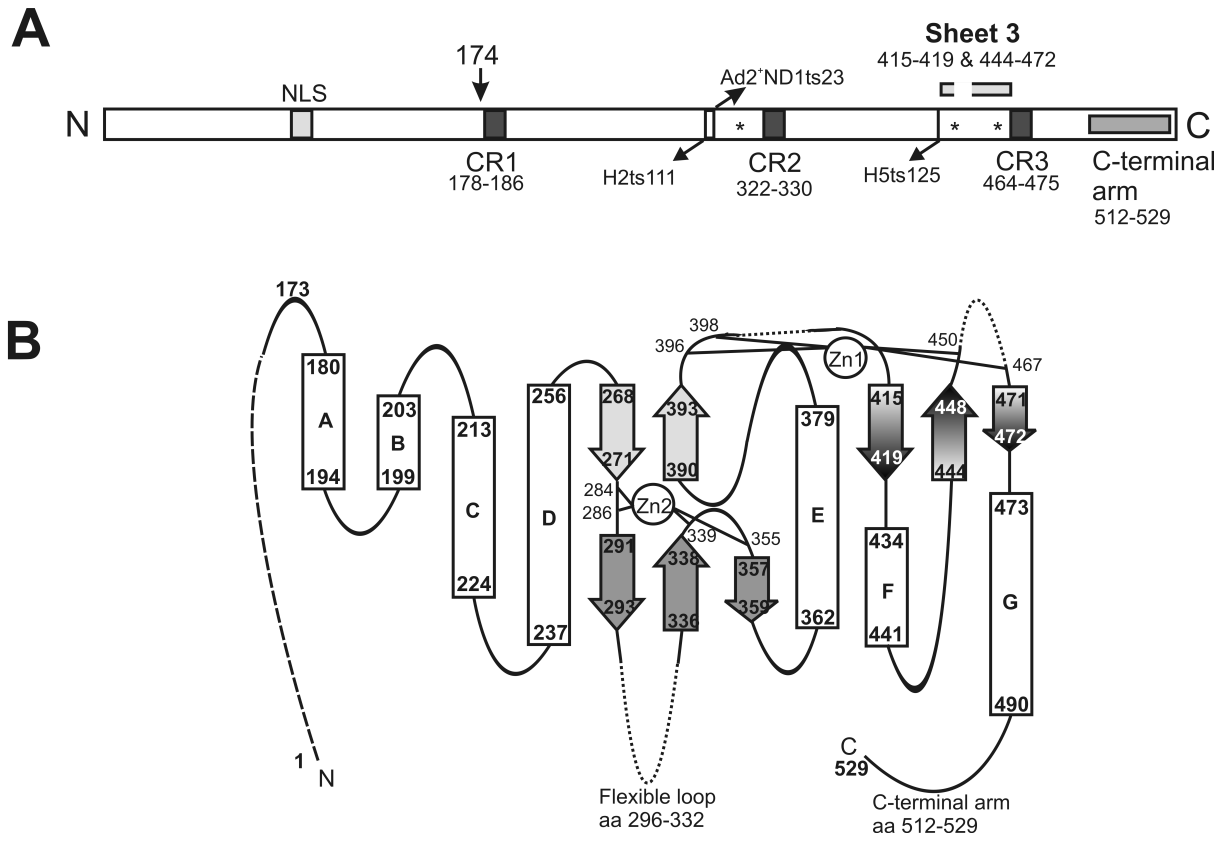

Figure 6: **A**: Schematic representation of Ad5 DBP (aa 1-529), indicating the position of several characteristic domains and amino acids. Indicated with a thin line are the temperature sensitive mutants. An * indicates an amino acid that can be crosslinked directly to DNA. Black boxes indicate conserved regions 1-3 (CR1-3). Adenovirus DBP can be cleaved at position 174 by mild chymotrypsin cleavage into two domains. The N-terminal part (aa 1-173) harbors a nuclear localization signal (NLS). The C-terminal part (aa 174-529) harbors the DNA binding domain (Sheet 3) and the flexible C-terminal arm. **B:** Topological diagram of the DBP structure. aa 1-173 (dashed line) can be cleaved by mild chymotrypsin treatment, no topological data are known. aa 174-512, C-terminal fragment of DBP, containing 7 α-helices and 3 β-sheet-regions. Helices are numbered A-G, sheet 1 light gray, sheet 2, dark gray and sheet 3 (DNA binding sheet) fountain fill. Zinc atoms and residues coordinating them are shown. The topology shows the importance of the Zinc atoms for structural stability. Regions of the polypeptide chain that we are currently not able to model are shown by dashed lines. See text for details. Adapted from Tucker et al 1994.

interaction of DBP with pol. Indeed evidence exists of a DBP/pol interaction, such as the increased stability of polymerase in the presence of DBP and the altered sensitivity of the polymerase for certain inhibitors (51, 109, 126). However, attempts to show a direct interaction by DBP and pol have not been successful up to now. Alternatively DBP could act indirectly by inducing a better fit for dCTP in the active site of pol, either by introducing structural changes in the template or via origin unwinding. Chapter 3 will discuss additional template requirements for an optimal stimulation of initiation via DBP. It should be noted that for the initiation DBP is not absolutely required.

In contrast to the initiation of Ad DNA replication, addition of DBP during the elongation is absolutely required, and DBP cannot be replaced by any other SSB. Again this is suggestive for a specific DBP/pol interaction during elongation. DBP functions as a helix destabilizer. DBP can bind both to

ssDNA and dsDNA with high affinity (55, 153). In addition DBP binds ssDNA in a cooperative fashion with a cooperativity constant ω of ~20 to 30, occupying 10-15 nt per DBP molecule depending on the base-composition, thereby imposing a regular and extended structure of the DNA (97, 179). These nucleic acid binding properties enable DBP to unwind the dsDNA template ahead of the replication fork and to remove secondary structures, like hairpins in the template. Unwinding by DBP is ATP-independent and without polarity. The cooperativity is the driving force for DNA unwinding and it is suggested that the growing DBP chain on the displaced strand invades the ssDNA-dsDNA junction (200). In addition, DBP protects the displaced ssDNA strand against nucleolytic breakdown.

3.3 DBP structure

Ad5 DBP is a 529 aa long protein which can be cleaved into two domains by mild chymotrypsin treatment (Fig. 6A) (176). The N-terminal domain (aa 1-173) is not well conserved among different serotypes. It harbors a nuclear localization signal and most of the phosphorylation sites. Furthermore it is involved in host-range determination (1) as well as in virus maturation (136).

The C-terminal domain (aa 174-529) contains the DNA binding domain of DBP. It is composed of seven α-helices, three β-sheets and two Zinc atoms, that together form a compact and globular protein with a remarkable protruding C-terminal arm (aa 490-529) of ~40Å. The C-terminal domain is well conserved among different serotypes. It contains three well-conserved regions, CR1 to CR3 located at aa 178-186, 322-330 and 464-475, respectively (91).

Many mutants of the C-terminal fragment have been created to elucidate the functions of the various DBP domains. A number of thermosensitive DBP mutants were produced with mutations at positions 413 (H5ts125 and H5ts107), 280 (H2ts111) and 282 ($Ad2^{+}$ND1ts23), all located outside the conserved regions. Those mutants had a reduced binding affinity for DNA at the restrictive temperature, which resulted in replication deficient phenotypes (96, 132, 144, 177, 181) (Fig. 6AB, shows the positions of these mutants).

The first zinc atom, coordinated by four cysteines at positions 396, 398, 450, and 467 (Fig. 6B), is loosely bound and easily removed from the crystal by soaking the crystals in EDTA. No mutations have been described involving the cysteine residues coordinating this Zinc atom. These findings combined with the observation that DBP is insensitive to Zinc chelators suggest that this Zinc atom is not involved in DNA binding. However, when the second Zinc atom coordinated by three cysteine residues at position 284, 339, and 335 and one histidine residue at position 286 is targeted, ssDNA binding mutants were generated. Mutant H2ts111 and $Ad2^{+}$ND1ts23, both have mutations near one of the residues (C284) coordinating the second Zinc atom (Fig. 6), and show conformational changes as detected by an altered proteolytic breakdown pattern, suggesting that the second Zinc atom is mainly required for protein stability.

Mutations in the CR1-3 regions often lead to reduced DNA binding phenotypes (131, 132). Cross-linking studies have identified residues 299, 418 and 455 to be in close contact with DNA (36). These mutants together with the availability of a crystal structure of DBP (178) have revealed detailed information about the DNA binding domain of DBP

(Fig. 7A). Although only low-resolution crystal structures of DBP in complex with ssDNA $(dT)_{16}$ have been obtained extra information on DNA binding residues and regions has become available. Extra electron density above sheet 3, (Fig. 6 aa 415-472 and Fig 7A) was measured (85). This together with the calculated electrostatic potential of DBP shows that sheet 3 and the connecting loops form the most positive charged region on the surface of the protein, indicating that it is important in ssDNA binding. In addition to sheet 3, several other aa and regions involved in DNA binding were identified. The conserved aromatic residues F234, F418, F469, and Y455, may interact with the DNA. Alternatively the positive charged aa R410, K414 and K470 can contact the phosphate backbone. Indeed mutation of K470 lowers the affinity for ssDNA (131) and aa F418 and Y455 can be crosslinked to DNA (36). In addition, Y455 is located in a loop region, which is flexible in the DBP crystal, but becomes structured upon DNA binding (85). Additional DNA contacts are made by residues in a large loop called the flexible loop (aa 296-332). This flexible loop contains an aa sequence (NRWGR) that is well conserved in ssDNA binding loops of different ssDNA binding proteins (12, 18). Mutation of these residues or deletion of the entire loop reduces the affinity for ssDNA (45, 131). Taken together, the DNA binding interface is probably formed by sheet 3, the flexible loop and the loop between aa

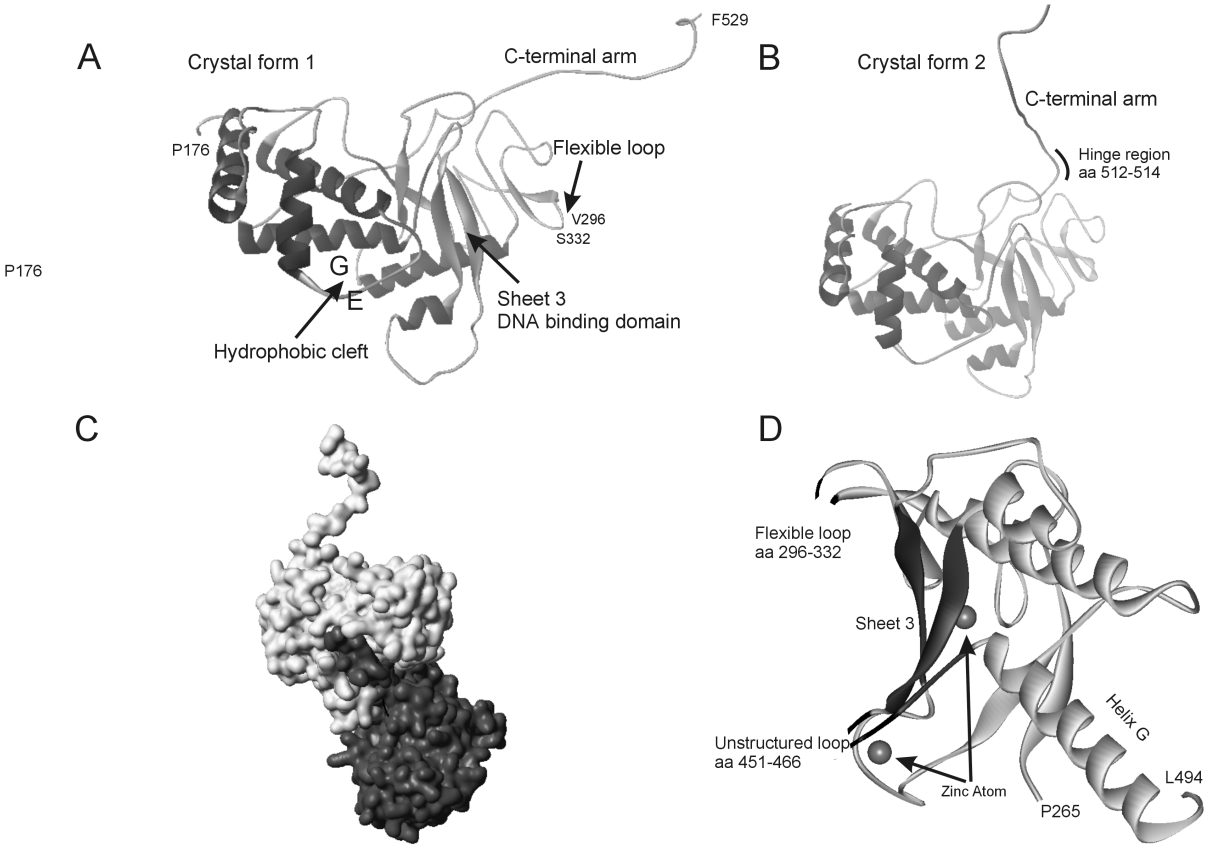

Figure 7: Crystal structures of DBP. **A**: Crystal form 1 (178). Indicated are the hydrophobic cleft and c-terminal arm required for multimerization and sheet 3 and the flexible loop required for efficient DNA binding. **B**: Crystal form 2 (84). The position of the C-terminal arm has been rotated around aa 512-514. **C**: Crystal structure of a DBP dimer. **D**: Magnification of DNA binding domain, composed of Sheet 3, Flexible loop and unstructured loop (black), see text for details.

451 and 466 (Fig. 6 and 7A,D).

Another remarkable feature of DBP is its C-terminal arm (aa 512-529) (Fig 7A,B), which is required for the cooperative multimerization of DBP on ssDNA (44). This DBP multimerization plays an important role in cooperative binding to ss- and dsDNA and unwinding by DBP during adenovirus DNA replication. Indeed, deletion of the C-terminal arm resulted in DBP phenotypes negative in unwinding and in cooperative DNA binding (44, 178). Multimerization of DBP involves the binding of the C-terminal arm in a hydrophobic cleft, located between helices E and G of a second DBP monomer (Fig. 7A,C).

When a second crystal structure of DBP was solved an interesting difference with respect to the position of the C-terminal arm of the first crystal structure was observed. The orientation of the C-terminal arm is different when both crystal structures are superimposed (Fig. 7A,B) (84). Apparently, amino acids 512 and 514 form a “hinge” region, around which the C-terminal arm can rotate, resulting in a flexible C-terminal arm. Chapter 2, will discuss the function of this flexibility in more detail.

4. SSB proteins

Adenovirus is not the only organism that encodes for a sequence-independent single stranded DNA binding protein (SSB). Research throughout the years has identified many different SSBs from several origins, ranging from virus to higher eukaryotes. In this section I will discuss the properties and functions of a selection of well studied SSBs and compare their similarities and differences with respect to DBP. The selected SSBs are: Gp32, the bacteriophage T4 SSB, which was the first SSB discovered and serves as the prototype for SSBs; *E. coli* SSB, the prokaryotic prototype SSB; ICP8, the herpes simplex virus SSB which is like DBP one of the best studied eukaryotic viral SSB; φ29 p5 SSB, the phage 29 SSB, a virus that like adenovirus employs protein primed DNA synthesis; and human RPA, the human counterpart of *E. coli* SSB.

4.1 Structural similarities shared by SSBs

In search for a common motif little homology was found on DNA sequence- and amino acid- level between SSB proteins of different origins. However, a remarkable structural feature, called the OB-fold has been found to be common in a large group of proteins. The SSBs that will be discussed in this section, such as bacteriophage T7, gp2.5, *E. coli*. SSB, human RPA and T4 gp32, share this common motif. At this moment the crystal structures of ICP8 and φ29 SSB have not been determined. In addition to these SSBs the structures of the SSBs from the bacteriophage T7 gp2.5 and

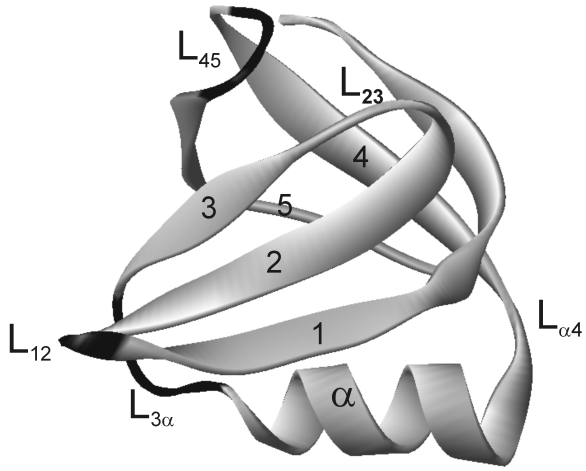

Figure 8: An example of the OB-fold, based on its smallest representative, the B subunit of verotoxin-1. Five β-strands (1-5) form a closed β-sheet, capped by an α-helix. Three variable loops (black) contribute residues in the oligomer binding site.

filamentous bacteriophage M13 gene V (54) protein are also compared in this section.

An OB-fold consists of five β-strands that form a closed β-sheet that is capped by an α-helix. The conformation was first described for the staphylococcal nuclease anticodon binding-domain of asp-tRNA synthetase and for the B-subunits of heat labile enterotoxin and verotoxin-1 (Fig 8). Three variable loops (e.g. L_{12}, $L_{3\alpha}$ and L_{45}) contribute residues in the oligomer binding site and are responsible for the DNA-protein or oligosaccharide-protein interactions (128). OB-folds can also be involved in protein-protein interaction as was demonstrated for the telomeric end binding protein (TEBP) of *oxytrichia nova.* TEBP, contains four OB-folds of which 3 are involved in DNA binding and one is involved in protein-protein interaction (oligopeptide interaction) (77), suggesting a versatile function for this structure.

The crystal structures of the OB-fold containing DNA binding domains of T7 gp2.5, *E. coli* SSB, hRPA and M13 Gene V protein, are shown in Fig. 9A. It should be noted that hRPA contains four OB-folds in its structure, each contributing in a different manner to its ssDNA binding property. In Fig. 9A only one OB-fold from hRPA70 is shown. Although the sequence homology and peptide binding homology of the three SSB proteins is negligible it is remarkable that when superimposing the DNA binding domains of gp2.5, *E. coli* SSB and human RPA results in a large overlap (Fig. 9B). The major difference between these three OB-folds is the structure of the variable loops. It is proposed that these variations reflect the different DNA binding properties (76). The crystal structure of the T4 gp32 does not entirely predict an OB-fold, when compared to the verotoxin model, because an extra structural element, a zinc binding domain, is inserted between strands 1 and 2 (155). However all structural characteristics, like five β-sheets and an α-helix are present, therefore this structure is called a putative OB-fold (Fig. 9C). Like gp32, the OB-fold of the M13 gene V protein is called a putative OB-fold. In this case, the OB-fold contains all OB-fold elements, like the L_{12}, L_{45} loops and the concave DNA binding surface, but the structure is more loosely defined and therefore cannot be superimposed on T7 gp2.5, *E. coli* SSB or hRPA (Fig 9D).

Even though crystal structures of DBP have been solved an OB-fold as such has not been found. Interestingly several similarities between the OB-fold and the DNA binding domain of Ad DBP are present (Fig. 9E). First, the OB-fold uses a β-sheet as a DNA
binding surface. This is comparable to sheet 3 of Ad DBP DNA binding domain. Second, the ssDNA binding domain of Ad DBP is surrounded by different loops that become more structured upon DNA binding. The flexible loop can be compared to loop L_{45} that contributes most of the residues for DNA binding. The unstructured loop (aa 451-466) can be compared to L_{12}, also important in DNA binding. Third, the overall structure of the DNA binding domains is a concave structure, forming a DNA binding cleft (Fig. 9E).

4.2 Functional similarities of SSBs

Not only on a structural level do similarities among SSB proteins exist. On a functional level SSB proteins share a range of properties, of which a few will

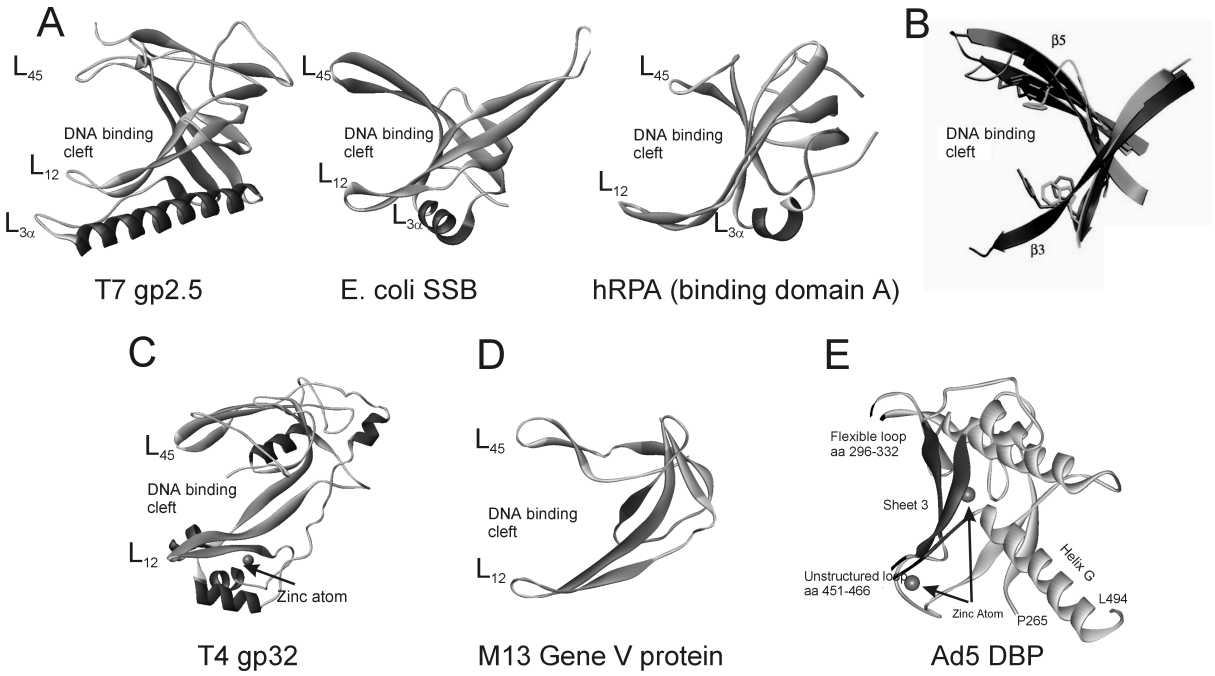

Figure 9: **A**: Crystal structures of the OB-folds of phage T7 gp2.5, *E. coli* SSB and human RPA (Binding domain A) (PDB codes; 1JE5, 1QVC, 1JMC respectively. **B**: Superposition of OB-folds of T7 gp2.5, *E. coli* SSB and hRPA, showing high structural homology (Source: (76)). **C**: Crystal structure of the putative OB-fold found in phage T4 gp32. (PDB: 1GPC) **D**: Crystal structure of the putative OB-fold found in filamentous bacteriophage M13 gene V protein (PDB: 1VQB) **E**: DNA binding domain of adenovirus type 5 DBP (PDB: 1ADT).

be discussed here. First, SSB proteins bind preferentially to ssDNA. This happens in a cooperative manner although the cooperativity constant varies. The cooperativity is defined as a measure of protein-protein association upon binding DNA, i.e. the probability of a protein monomer binding next to an already DNA bound protein relative to binding anywhere else on the DNA. Second, the binding to ssDNA is sequence independent, alhough differences in the amount of nucleotides bound by the SSBs (binding size) exist. Third, binding to ssDNA results in helix destabilization and removal of secondary structures. In some cases the SSB can unwind DNA actively without the requirement of ATP, e.g. DBP, ICP8 and RPA. An overview of the cooperativity constants and binding sizes of the SSBs shown in this chapter is shown in Table I.

4.3 Functional differences between SSBs

From Table 1 it can be seen that although SSBs have many functions in common, their intrinsic properties are different. These differences in binding-size, cooperativity and ssDNA affinity might be reflected in their specific functions that are not shared among each other, hence making them in many functionally related mechanisms like DNA replication, non-exchangeable. This section will discuss the specific functions of these SSBs during DNA binding, replication and recombination.

4.3.1 Herpes Simplex Virus, Infected Cell Protein 8, ICP8

The infected cell protein 8 (ICP8), a 128 kDa SSB protein of the *Herpes simplex*

TABLE I

ssDNA binding and cooperativity of SSBs

Protein	molecular weight kDa	ssDNA binding Affinity Kω (0,2 M NaCl, 25°C)	Cooperativity constant ω	Binding size (nt)
Ad DBP	59	$1.7*10^5$	20-30[d]	10-15
HSV ICP8	128	$1,35*10^7$	10-20[b] and 40[c]	12-40
T4 gp32	33	$2*10^8$	1000[b]	7-8
φ29 SSB	13	$1*10^5$	50-70[a]	3-4
E. coli SSB_{35}*	18.8×	$2,6*10^6$	$1*10^5$ [b]	35
E. coli SSB_{65}			420±100[b]	65
human RPA	70	$1,1*10^9$	10-20[b]	30

DNA binding measured on Poly(dA) except for ICP8 (32mer ssDNA)

[a] From (159)

[b] From (64)

[c] Measured on $Poly(dT)_{50}$. From: (114)

[d] From (97)

* The *E. coli* SSB_{35} binding mode is the major binding mode for helix-destabilization.

× This is the molecular weight of the *E. coli* SSB monomer.

virus, binds with high cooperativity to ssDNA. Like DBP, ICP8 is able to unwind dsDNA, up to 2500 nt (137). Presently the exact mechanism of how ICP8 binds cooperatively to HSV ssDNA is not known. Experimental evidence suggests a polar nearest neighbor interaction model (like DBP) in which the C-terminal portion of the ICP8 protein plays an important role in cooperative chain formation (114).

The replication of Herpes Simplex virus starts with the local melting of an A/T-rich region contained in the HSV-1 origin. These A/T-rich regions in the DNA are at 37°C sufficiently single-stranded to permit ICP8 binding, which in turn permits binding of two origin binding/DNA helicase proteins (UL9) on each side of the ICP8 proteins (105). Binding of the UL9 proteins is restricted to their binding sites adjacent to the A/T-stretch on the HSV-1 ORI. After ICP8 forms a heterodimer with UL9, the two proteins together can unwind the HSV-1 ORI(14, 69, 102). The initial step of HSV-1 ORI unwinding by ICP8 is an ATP-independent step. Subsequently, ATP is required for unwinding of the DNA and it permits the ICP8-UL9 protein complex to unwind the rest of the HSV-1 origin (70). After opening of the HSV-1 ORI the DNA is first replicated via a *theta* mode of replication, which is transient and is rapidly followed by rolling circle replication (Fig. 10) (For review see, (105)). During the *theta* mode of replication ICP8 protects the ssDNA from renaturing. When rolling circle replication occurs ICP8 stimulates the DNA polymerase-clamp (UL42) complex (73) and it assists the helicase-primase by preventing the reannealing of complementary single strands produced by its helicase action (50).

During elongation ICP8 can only assists other replication proteins such as UL9 and the pol α/primase complex in their function by preventing renaturation of the ssDNA.

A role for ICP8 in recombination

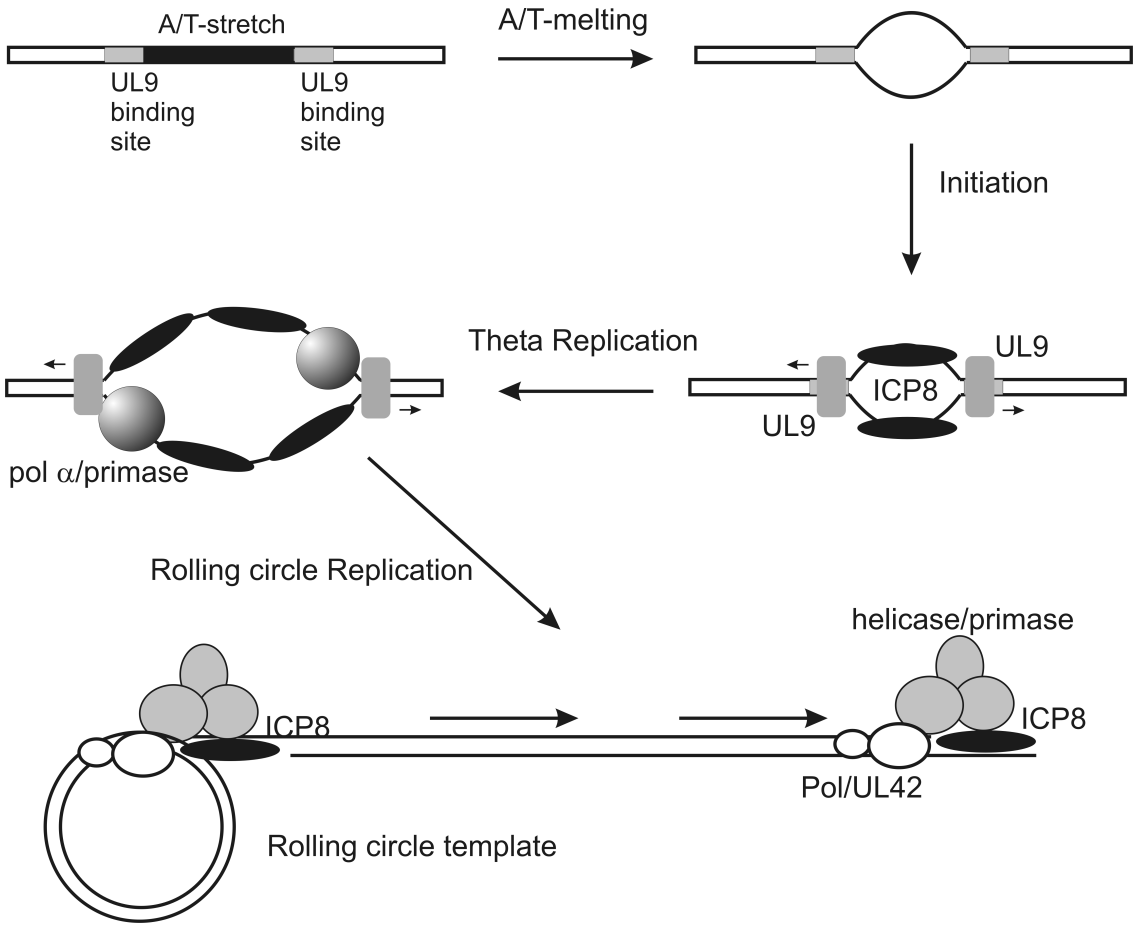

Figure 10: Model of HSV-1 DNA replication. The A/T stretch in the HSV-1 ORI is at 37°C sufficiently single-stranded to permit ICP8 binding. The ORI becomes sufficiently unwound by ICP8 to allow UL9 binding. Next UL9 and ICP8 together unwind the complete ORI which in turn allows assembly of the pol α/primase complex on the ORI. The initial phase of *theta* replication is followed by a rolling circle mode, the predominant mode of HSV-1 DNA replication.

has been proposed because ICP8 has been shown to catalyze homologous pairing and strand transfer on M13 DNA (15, 137). The conformation the ICP8-ssDNA complex is similar to that found for recombinases in their active form. Both its helix destabilization and unwinding characteristics suggest a similar role for ICP8 in catalyzing homologous recombination (64). Recently the role of ICP8 in recombination has been confirmed *in vitro (137).*

4.3.2 Phage T4 gene 32 protein, gp32

The phage T4 gene 32 product is a 33 kDa protein that binds cooperatively to ssDNA although gp32 cannot actively unwind dsDNA at physiological salt concentrations. Gp32 facilitates strand displacement synthesis by binding to transiently unwound regions of the duplex (138). The gp32 protein contains three functional domains. The N-terminal 17 residues are important for the gp32:gp32 cooperativity on ssDNA, whereas the C-terminal 46 residues are important for interactions with other proteins. The remaining core domain of gp32 (aa 21 to 254) contains the ssDNA binding domain (160). DNA replication by phage T4 consists of a leading and lagging strand synthesis.

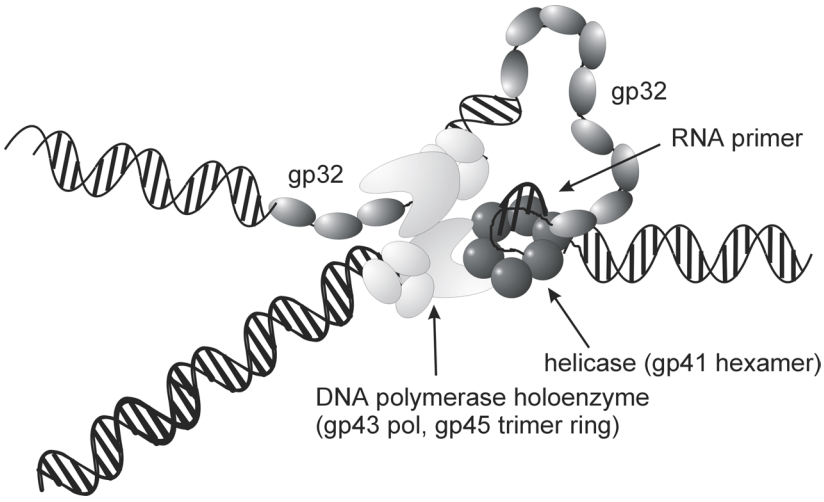

Figure 11: A functional model of the phage T4 DNA replication fork with gp32 positioned on the ssDNA regions

Initiation of replication by T4 requires the host RNA polymerase (RNAP) to synthesize a primer RNA (112). Subsequently, T4 polymerase attaches approximately 70 nt to the RNA and then pauses, resulting in the displacement of the non-template strand. This in turn provides the priming site for the primase (gp61)(138). The helicase (gp41) then interacts with the gp61-DNA complex, only in the presence of the helicase loading protein gp56 (8), and a so-called primosome is formed. It should be noted that, although this is a well studied mode of initiation for T4, the majority of T4 replication appears to be the result of host RNA polymerase-independent pathways, that involves recombinational initiation (41, 95, 112). The DNA replication on the leading and lagging strands is catalyzed by T4 polymerase, gp43, which is a relatively non-processive enzyme (139). The processivity of T4 polymerase is increased by gp32, which destabilizes the secondary structure in ssDNA templates. The second function of gp32 is its stimulation of the assembly of T4 polymerase and its accessory proteins (a clamp loader complex, containing gp44 and gp62 and a clamp, gp45), on the primer template of the lagging strand (24). T4 polymerase alone is not capable of strand displacement it requires the function of several accessory proteins such as the gp45 trimer and gp32 together with the T4 helicase (gp41) (140). An additional role of gp32 is its function in the coordination of the reactions of the leading and lagging strands, where it regulates the length of the Okazaki fragments together with the accessory proteins (83) (for review see, (138)). Figure 11 shows the involvement of gp32 at the T4 replication fork.

Phage T4 SSB, gp32, is essential in recombination, where it facilitates the renaturation of homologous ssDNA. T4 gp32, together with the T4 helicase and gp59, catalyzes the strand transfer (92, 93), a function important in homologous recombination.

4.3.3 Bacteriophage ϕ29 protein p5, ϕ29 SSB

The bacteriophage ϕ29 p5 SSB protein (ϕ29 SSB) has a molecular weight of 13 kDa. ϕ29 SSB binds to ssDNA as a monomer. Although the proteins do not self-interact, multiple proteins cover the ssDNA forming a protein chain with high cooperativity. ϕ29 SSB is not actively involved in unwinding, but ssDNA is immediately bound by ϕ29 SSB and

thereby protected from degradation. Furthermore, secondary structures formed in ssDNA parts of ϕ29 replicative intermediates, are removed by ϕ29 SSB.

ϕ29 DNA replication is related to adenovirus DNA replication, since both systems use a terminal protein to initiate replication (152). ϕ29 SSB does not seem to be involved during the initiation of ϕ29 DNA replication. The requirement of the ϕ29 SSB is particularly important during strand-displacement DNA synthesis. First, ϕ29 SSB increases the rate of elongation by removing the secondary structures from the ssDNA and preventing the displaced strand from renaturation and nuclease attacks. Second, it increases the amount of dNTPs that are incorporated. It is proposed that these functions are related to the helix-destabilizing properties of ϕ29 SSB (117, 158). Functional protein-protein interactions between ϕ29 SSB and other proteins of the DNA replication machinery have not been reported.

4.3.4 Escherichia coli single-stranded binding protein, *E. coli* SSB

The Escherichia coli SSB (EcoSSB) is an 18.8 kDa protein that binds ssDNA as a homotetramer. EcoSSB binds to ssDNA in three different modes. Each mode is defined by the number of nucleotides that are bound by each homotetramer, referred to as Eco SSB_n (where (*n*) is the number of nucleotides occluded by each bound tetramer). At least three binding modes have been identified with n=35 ± 2, n=56 ± 3 and n=65 ± 3 nucleotides per tetramer, respectively (21, 110). Two of the four subunits interact with ssDNA in the Eco SSB_{35} mode, whereas all four subunits interact with ssDNA in the $EcoSSB_{65}$ mode (22, 23). Only the $EcoSSB_{35}$ binding mode plays a role in helix destabilization. $EcoSSB_{35}$ tetramers bind with high cooperativity to locally unwound ssDNA regions, thereby keeping the duplex DNA open (111).

The mechanisms of *E. coli* chromosomal DNA replication is rather complex, especially the initiation reactions (for review see (116). Only the most important functions of the EcoSSB will be discussed here. DNA replication in *E. coli* is primed at a specific sequence (*ori*C) by the function of RNAP (98) at the ORI (OriC) (142). The *E. coli* ORI contains a DnaA protein binding site, which consists of four copies of an A/T-rich sequence, flanked by series of three A/T-rich 13-mers. This A/T-stretch plays an important role in destabilizing the ORI (58, 59). First, DnaA recruits a protein complex containing DnaB-DnaC to the origin. Subsequently, DnaB unwinds the A/T-rich 13-mers with the aid of EcoSSB and DNA gyrase (6, 7). After initiation the DNA replication continues via leading and lagging strand synthesis, as described for T4 and T7 DNA replication. The ssDNA can be found in the replication fork between the Okazaki fragment, and EcoSSB covers this ssDNA. In addition, a helicase unwinds the parental duplex and ssDNA is generated on the lagging strand. EcoSSB coats this ssDNA thereby removing hairpin blocks to stimulate polymerase progression.

EcoSSB has also been found to play a rather elegant role in the primase to polymerase switch (Fig 12) (196). During lagging strand synthesis, an RNA primer is used by polymerase to initiate lagging strand synthesis (Fig 12A), however this requires a switch where the primase needs to be replaced by the polymerase. EcoSSB is used by the primase for its grip on the primed site. The primase-SSB contact has to be

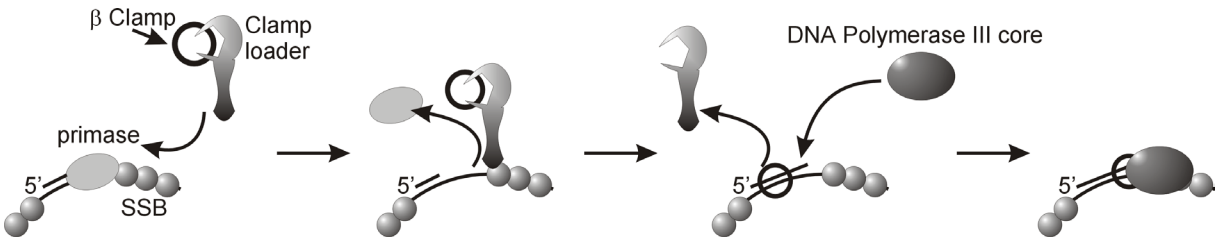

Figure 12: Model of the primase to polymerase switch (Source: (196) **A**: The primase requires contact with EcoSSB to remain bound to the RNA primer. **B**: The clamp loader binds to SSB disrupting primase-SSB contact leading to primase displacement. **C**: The clamp loader assembles the clamp onto the primed site and dissociates from the clamp. **D**: DNA polymerase III core associates with the clamp to form the processive polymerase III.

destabilized in order for DNA polymerase to bind. This is mediated via the χ subunit of the pol III holoenzyme. The χ subunit is one of the six subunits of the clamp assembly apparatus (DnaX complex, $\tau_2\gamma_2\delta_1\delta'_1\chi_1\psi_1$) (86), which loads the β_2 sliding clamp processivity factor (196). Binding of the χ subunit to EcoSSB leads to the dissociation of the primase (Fig. 12B). Subsequently the DNA polymerase III core complex can associate with the β_2 the sliding clamp (Fig. 12C-D) to form the processive polymerase III complex.

The function of the *E. coli* SSB protein in *E. coli* recombination is restricted to the binding of ssDNA formed by the RecBCD helicase complex, thereby preventing degradation of the ssDNA by nucleases, (for review see (94)).

4.3.5 Human replication protein A, RPA

RPA was identified as an essential component of the SV40 replication machinery. The human single-stranded DNA binding protein, RPA, is a heterotrimeric protein complex, consisting of the RPA70, RPA34 and RPA14 (70-, 34- and 14 kDa, respectively) subunits. Binding of RPA to ssDNA exhibits high cooperativity when the target sites are not longer than approximately 30n, whereas binding of longer ligands show weak cooperativity (81, 194). RPA has an intrinsic DNA-unwinding activity, and can unwind fragments up to 1000 bp (60) (175). Binding of the RPA complex is a three-step process, consisting of three different binding modes. First RPA binds approximately 8 to 10 nucleotides with low affinity and high cooperativity (10). Second, an intermediate mode has been observed in which 13-14 nucleotides are bound (13, 99). Finally, approximately 30 nucleotides are bound by RPA with high affinity for ssDNA but with low cooperativity (87). The transition between the first and the third mode is thought to be a functionally important event implicated in DNA unwinding (10).

The function of human RPA in the DNA replication of the simian virus 40 (SV40) has been well studied. SV40 initiation of DNA replication starts with the binding of the SV40 large T-antigen (Tag) to the ORI. There it opens the ORI and becomes a true helicase that unwinds the SV40 ORI. Subsequently, opening of the ORI enables RPA to bind to the formed ssDNA, where it unwinds the replication origin in the presence of Tag and topoisomerase I/II. In this first stage, any other SSB protein can substitute RPA. This suggests that the

ssDNA binding function suffices for extending the open region and preventing it from renaturation. During later stages of the replication RPA exhibits specific contacts with SV40 Tag and can no longer be substituted by any other SSB (47). After opening of the origin, Tag recruits the DNA polymerase α-primase complex to the replication fork (47). It has been demonstrated that the DNA polymerase α-primase complex interacts with RPA (101) and that this is necessary for the initiation of SV40 replication (39), possibly to increase the stability of the complex (113). Recently it has been demonstrated that human RPA along with the DNA replication protein Cdc45p is involved in the recruitment of polymerase α-primase to the chromosomal origin of replication (186). Subsequently, RPA stimulates pol α, pol δ and pol ε activity during elongation to extend the primed template DNA (88). It should be noted that although RPA is a SSB protein its function is far more complicated than that of SSB proteins from viral or bacterial origins. The functions of RPA are wide spread, and often involve specialized protein-protein interactions, like the interaction with SV40 Tag (47), with polymerase α-primase (101), with proteins of the nucleotide excision repair pathway (XPA, XPG, XPF) (72, 100, 118, 119, 188), with recombination specific proteins (62), and with transcriptional activators (GAL4, VP16, p53, RBT1) (33, 48, 71, 107, 121). The three subunits of RPA are likely to play different roles in all these DNA transactions.

RPA binds to ssDNA fragments present during recombination. It facilitates destabilization of dsDNA, a property shared with Ad DBP, but moreover it stimulates the annealing of complementary ssDNA. The stimulation of strand annealing is a result of the function of Rad51, which is targeted to a complex of RPA with ssDNA via Rad52 (123, 134). In this last example the SSB protein functions as a recruitment factor for recombination specific proteins, a function found for many other SSB proteins.

4.3.6 General conclusion

In the previous sections I have tried to show some functional similarities and differences of a few proteins from the SSB group. SSBs share the ability to bind cooperative and sequence independent to ssDNA with high affinity. These DNA binding properties are used to destabilize dsDNA and in some cases, like with DBP, gp32, hRPA and ICP8 the SSBs are even able unwind duplex DNA in an ATP-independent manner. This characteristic property of SSB is used during the DNA replication process, where in some cases the SSB functions as a helicase-like protein, e.g. DBP, or where the SSB functions in concert with a helicase to keep the unwound DNA duplex in an open form. The helix destabilizing property of the SSB is also used to enhance the processivity of the replicative complex, where the SSB removes the secondary structures from the ssDNA template.

Although the SSB proteins have a common role during DNA replication, additional properties have been acquired by the SSBs, resulting in species specific differences. The differences in cooperativity, ssDNA binding affinity and even binding-site sizes might play a important role in their specific characteristics, e.g. both ICP8 and DBP can unwind dsDNA without the addition of ATP, but are also able to renature homologous strands of ssDNA. For ICP8 this might be an important function in its

role during homologous recombination. On the other hand, the SSBs functioning in eukaryotic of even higher eukaryotic systems are used even in more specialized systems. RPA, for example, is has been found to interact with a large set of proteins. In this way RPA is able to function during DNA replication, homologous recombination, DNA repair and so on. Here the SSB functions as a recruiting molecule that targets the proteins that need to be present near or at ssDNA sites. Table II summarizes the shared and separate functions of the SSBs discussed in this section.

5. Outline of this thesis

Adenovirus DNA replication is the main subject of this thesis. A well defined *in vitro* system with purified adenovirus replication proteins provides a useful tool to investigate the role of the separate replication proteins in DNA replication. The emphasis of this thesis is on the DNA binding protein DBP and its specific function in DNA replication and recombination.

Two crystal structures of DBP have been resolved. Both structures were almost identical except for the orientation of their C-terminal arms, suggesting that this part of the protein is flexible. Based on this observation mutants were designed with an altered flexibility in this C-terminal arm. Chapter 2 describes the impact of the alterations in the C-terminal arm of DBP on helix destabilizing activity and as a consequence on the elongation phase of the adenovirus DNA replication. A second role of DBP during adenovirus DNA replication is its ability to stimulate initiation. In Chapter 3 an in-depth investigation is presented on the function of DBP in stimulating initiation of DNA replication. Previous investigations suggested a direct protein-protein interaction between DBP and the pTP-pol complex or alternatively an indirect stimulation of initiation could be caused by DBP through structural alterations of the origin DNA template. We prove that the latter alternative, where DBP alters the DNA conformation, is the proper explanation.

After adenovirus infection of a eukaryotic cell, recombination levels increase. Previously it was shown that adenovirus DNA replication and the occurrence of recombination are interrelated. A role for DBP in recombination was predicted for many years by circumstantial evidence. However, an *in vitro* assay to examine the replication proteins in this process was not available until recently. Chapter 4 focuses on the role of DBP in the strand exchange reaction, an important step of homologous recombination. For the first time a clear role for DBP in strand exchange could be demonstrated. In chapter 5 a prospective for future DBP research is discussed.

Table II

Shared and separate functions of the SSBs discussed in this section

	OB-fold	ssDNA binding	Destabilization	Active Unwinding	Protein interaction	Function in Recombination
DBP	no	yes	yes	yes (>5000bp)	AAV rep78/rep68 SrCap	possibly
HSV ICP8	unknown	yes	yes	yes (~2500bp)	UL9 & primase/helicase	yes
T4 gp32	putative	yes	yes	only under low salt conditions	T4 pol & gp41	yes
φ29 SSB	unknown	yes	yes	no	none	unknown
E. coli SSB	yes	yes	yes	no	primase & χ-subunit	yes
hRPA	yes	yes	yes	yes	SV40 Tag & DNA pol α/primase and several other proteins	yes

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2

The Formation of a flexible DBP protein chain is required for efficient DNA unwinding and Adenovirus DNA chain elongation

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The formation of a flexible DBP protein chain is required for efficient DNA unwinding and Adenovirus DNA chain elongation

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The adenovirus DNA binding protein (DBP) binds cooperatively to single-stranded DNA (ssDNA) and stimulates both initiation and elongation of DNA replication. DBP consists of a globular core domain and a C-terminal arm that hooks onto a neighboring DBP molecule to form a stable protein chain with the DNA bound to the internal surface of the chain. This multimerisation is the driving force for ATP-independent DNA unwinding by DBP during elongation. As shown by X-ray diffraction of different crystal forms of the C-terminal domain, the C-terminal arm can adopt different conformations, leading to flexibility in the protein chain. This flexibility is a function of the hinge region, the part of the protein joining the C-terminal arm to the protein core .
To investigate the function of the flexibility, proline residues were introduced in the hinge region and the proteins were purified to homogeneity after baculovirus expression. The mutant proteins were still able to bind ss- and dsDNA with approximately the same affinity as wild type and the binding to ssDNA was found to be cooperative. All mutant proteins were able to stimulate the initiation of DNA replication to near wild type levels. However, the proline mutants could not support elongation of DNA replication efficiently. Even the elongation up to 26 nucleotides was severely impaired. This defect was also seen when DNA unwinding was studied. Binding studies of DBP to homo-oligonucleotides showed an inability of the proline mutants to bind to poly(dA)$_{40}$, indicating an inability to adapt to specific DNA conformations. Our data suggest that the flexibility of the protein chain formed by DBP is important in binding and unwinding of DNA during adenovirus DNA replication. A model explaining the need for flexibility of the C-terminal arm is proposed.

Introduction

Adenovirus DNA replication can be reconstituted *in vitro*, using three viral proteins, adenovirus DNA polymerase (pol), precursor terminal protein (pTP) and the DNA binding protein (DBP). Optimal replication efficiency is obtained when two cellular transcription factors are added, Nuclear Factor I (NFI) and Oct 1 (for reviews;(8, 15, 31)

The adenoviral dsDNA genome contains two terminal proteins (TP) covalently linked to the 5' ends. The inverted terminal repeats contain the origins of

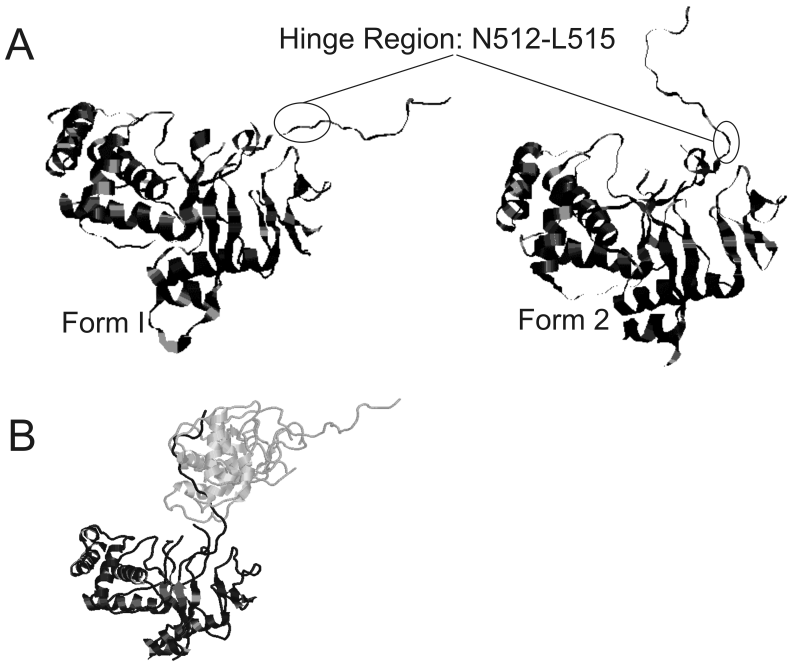

Figure 1: Structure of DBP.
A: Crystal structures I and II of the C-terminal fragment of DBP (aa 174-529, ΔN-DBP) are shown with different orientations of their C-terminal arm. The difference lies in the arrangement of residues V512-L515 (encircled), called the hinge region. **B**: DBP monomers form a multimer. The C-terminal arm of one monomer hooks into the hydrophobic cleft of the second monomer and so on leading to a protein chain. Only the dimer is shown (17).

replication. pTP and pol are tightly associated in solution. During initiation of replication pTP functions as a primer to which the first nucleotide, dCTP, is covalently coupled. Both NFI and Oct-1 stimulate the initiation by recruiting the pTP-pol complex to the origin of replication (3, 4, 7, 12). Initiation starts opposite position 4 of the template strand. After formation of a pTP-trinucleotide (pTP-CAT) (19), the complex jumps back and CAT becomes paired with template residues 1-3. Shortly after jumping back, the polymerase dissociates from pTP and elongation proceeds via strand displacement (18).

DBP has several functions during the adenovirus lifecycle. Besides DNA replication, the protein is involved in transcriptional control and mRNA stability (6, 33), transformation (26), virion assembly (24) and determination of the host range (1, 14).

DBP performs several functions in DNA replication. During initiation, it stimulates directly the formation of a pTP-CAT intermediate by lowering the Km of the reaction (23), possibly via a direct interaction with the pTP-pol complex. Indirectly, DBP stimulates initiation by increasing the binding of NFI to the origin (5, 21, 27). The stimulation of initiation by DBP is most pronounced at low pTP-pol concentrations, suggesting a role of DBP in recruitment of the pTP-pol complex to the origin. Furthermore DBP plays an essential role during the elongation phase of DNA replication, where it helps to unwind the parental strand (35) and enhances the processivity of the polymerase (21). This is achieved by cooperative binding to the displaced strand during replication, thereby protecting it from nuclease digestion and facilitating strand displacement. Strand displacement is ATP independent and requires only the helix DNA unwinding activity of DBP, unlike helicase activity, which does require ATP (22, 25, 35). Finally, DBP enhances the renaturation of

complementary displaced strands (34).

The crystal structure of the C-terminal domain of DBP (amino acids 174 to 529, ΔN-DBP) has been solved (29). This region contains the DNA binding domain and is functional in *in vitro* assays (2, 13, 28). The protein is mainly globular except for the last 17 amino acids, which form a protruding arm. This C-terminal arm binds to a hydrophobic cleft of an another DBP molecule, allowing protein chain formation which is essential for the function of DBP in elongation and cooperative binding on ssDNA as shown by deletion studies (10) (Figure 1B). A second crystal structure of ΔN-DBP has been described, which superimposes except for the C-terminal arm (Figure 1) (16). Comparison of the structure of the last 17 amino acids of the crystal forms 1 and 2 demonstrates a difference in the arrangement of residues N512-L515, called the hinge region. Further investigations have revealed amino acids N512 and S514 to be mainly responsible for the conformational changes. The ability of the C-terminal arm to adopt several orientations, suggests that the DBP protein chain is flexible and can adopt different arrangements. This effect could explain different estimations of the length of the binding sites found for different homopolynucleotides (16, 30).

Here we have studied the role of the flexibility of the C-terminal arm in ssDNA binding and DNA replication using DBP with mutations in the hinge region which are expected to lead to reduced flexibility. We find that the proline mutations severely impair the capability of DBP to sustain elongation and to unwind DNA, although protein chain formation is still possible. This indicates that flexibility of the protein chain is essential for its function, possibly by enabling adaptation to different DNA conformations.

Experimental procedures.

Construction of mutants

The hinge mutants were prepared by PCR in two steps from the adenovirus5 ΔN-DBP (amino acids 174-529) gene (17) in the pVL1392 baculovirus vector. In the first step the 5' primer was

TTTAGATCTTCATGAGTGTGCCGATCGTGTCTGC

and the 3' primers were

CGCGCATCGCTATGCGCCACTGGCAGGGACACGGGGCGATACT

CGCGCATCGCTATGCGCCACTGGCAGGGACAGGTTGCGATA

CGCGCATCGCTATGCGCCACTGGCAGGGGAGGTGGGCGATACT

for the N512P,V513L and NVS512-514PPP mutants (changes in bold) respectively. In the second PCR step the generated PCR product was, in each case, annealed with the 3' end of a common 3' primer

GGCACGAATTCTCAAAAATCAAAGGGGTTCTGCCGCGCATCGCTATGCGCCAC and the DNA amplified using the same 5' primer as above.

In each case the constructs were prepared by isolating, purifying and annealing three DNA fragments: a) the pVL1392-ΔN-DBP vector processed with *Eag*I (cleavage site at position 128) and opened 3' with *Eco*RI, b) this fragment further cut with *Ban*I (cleavage site at position 897) to yield the *Eag*I-*Ban*I piece and c) the second PCR product digested with *Ban*I and *Eco*RI. The third fragment (containing the mutations) was sequenced to check the correctness of the construct.

ΔC-DBP (aa 174-511) was constructed and purified as described by Dekker et al. (10),

Purification of ΔN-DBP and mutant DBP from baculovirus infected cells.

Monolayers of SF9 Cells were infected with recombinant baculoviruses at 28°C for 72 hr. The titer giving optimal protein expression levels was determined beforehand in pilot experiments. The purification of wt and mutant DBP was as follows. Cells were harvested, washed twice with PBS and resuspended in 50mM Tris-Cl (pH 8.0), 5mM KCl, 1mM DTT, 1 mM PMSF, 500mM NaCl and

0.5mM $MgCl_2$ followed by homogenizing using a Dounce homogenizer. The solution was clarified by centrifugation at 60,000g for 30 min at 4°C. The supernatant was diluted in DEAE buffer, 25mM Tris-Cl (pH 8.0), 1mM DTT, 0.1 mM PMSF, 1mM EDTA, 20% glycerol, to lower the salt concentration to 100mM. The diluted supernatant was loaded on a DEAE-FF-sepharose column equilibrated in DEAE buffer containing 200mM NaCl. The flow through was applied to a ssDNA-cellulose column equilibrated in 10mM Tris-Cl (pH 8.0), 1mM DTT, 0.1mM PMSF, 20% glycerol and 50mM NaCl (ssDNA buffer). The column was washed in buffer containing 500mM NaCl and the protein was eluted with buffer containing 2M NaCl. The protein was dialyzed to 100mM against 25mM Hepes-KOH (pH 8.0), 100mM NaCl. To concentrate the protein, the solution was loaded on a monoS1 FPLC column equilibrated with 25mM Hepes-KOH (pH 8.0), 20% glycerol and 80mM NaCl and developed with a linear gradient of 80 to 600mM NaCl. The proteins eluted around 200mM and were shown to be at least 95% homogeneous as judged by SDS-PAGE and Coomassie staining.

DNA binding assays

For the ssDNA electric mobility shift assay (EMSA), a 114 bp EcoR1/XbaI fragment from pHRI was Klenow end-labeled in the presence of [α-32p]dCTP and denatured by boiling. The dsDNA assays were performed with a 50nt dsDNA oligonucleotide (TD50), containing the first 50 base pairs of the Ad5 origin.

Binding assays were performed in a final volume of 20μl buffer containing 20mM Hepes-KOH (pH8.0), 100mM NaCl, 4mM $MgCl_2$, 0.4 mM DTT, 4% Ficoll, 1μg BSA, 0.05ng denatured DNA or dsDNA and the indicated amounts of ΔN-DBP or mutants. Bound and free DNA's were separated on a 10% polyacrylamide gel at room temperature. The running buffer contained 0.5 TBE and 0.01% NP40. Gels were dried and quantified using a Phosphor Imager Storm 820 of Molecular Dynamics with ImagequaNT 4.2a, Build 13 software. The concentration at which 50% of the ssDNA is complexed with DBP is used as a measure of the ssDNA binding affinity. A more accurate calculation as described by Verrijzer et al. (32), was not possible since binding of DBP to ssDNA is cooperative and does not fit a normal Scatchard plot.

DNA unwinding

DNA unwinding assays were performed using a partially double stranded oligonucleotide consisting of the last 50 bases of the template strand of the adenoviral origin of replication, hybridized with an oligonucleotide containing the complementary bases 15-50 from the displaced strand, thereby creating a dsDNA oligonucleotide with an 3' (template) 15 base pair single stranded overhang. Both strands were 5' labeled prior to hybridization. DNA (0.5ng) and indicated amounts of DBP or mutant forms of DBP were incubated for 1 h at 30°C in a total volume of 25μl in a buffer containing 25mM HEPES-KOH (pH 8.0), 1mM DTT, 0.1mM PMSF, 20% glycerol, 0.02% NP40, 0.5mM EDTA, 1 μg BSA and 100mM NaCl. Reactions were stopped by addition of 5μl 40% sucrose, 1.2 % SDS, and 0.1% bromophenol blue and 0.1% Xylene cyanol. Products were analyzed on a 12.5% SDS-polyacrylamide gel using a running buffer containing 1 TBE and 0.2% SDS. Gels were dried and quantified using a Phosphor Imager.

DNA replication on TP-DNA

The pTP-pol complex was purified as described (19). Terminal protein containing Ad5 DNA (TP-DNA) was obtained as described (9). Adenovirus DNA replication was performed in a final reaction volume of 25μl in the presence of 25 mM HEPES-KOH (pH 7.5), 50mM NaCl, 1.5 mM NaCl, 1.5mM $MgCl_2$, 1mM DTT, 500nM [α-^{32}P]-dCTP and 40 μM of dATP, dTTP, dGTP and 30ng TP-

DNA cut with XhoI. 140ng pTP-pol was added to the reaction. The amounts of ΔN-DBP and mutants are indicated in the legends. After incubation for 45 min at 37°C reactions were stopped by addition of 2.8 μl stop mix (40% sucrose, 1% SDS, 0.1% bromophenol blue, 0.1% Xylene cyanol). Replication products were analyzed on a 1% agarose gel. Gels were dried followed by autoradiography. Replication products were quantified by densitometric analysis using a Phosphor imager.

Initiation and partial elongation of DNA replication

Initiation of replication on TP-DNA was performed in a final reaction volume of 25μl in the presence of 25mM HEPES-KOH (pH7.5), 50mM NaCl, 1.5mM $MgCl_2$, 1mM DTT, 50nM [α-^{32}P]-dCTP, 90ng Ad5 TP-DNA and 50ng pTP-pol. When partial elongation was performed extra

1μM dCTP, 1μM dATP, 1μM dTTP and 5μM ddGTP together with 100ng pTP-pol and 26nM [α-^{32}P]-dCTP were added. The amounts of ΔN-DBP and mutants are indicated in the legends. Reactions were performed at 37°C for TP-DNA and at 30°C when a synthetic oligonucleotide was used. After 45 min the reactions were stopped by addition of 80mM EDTA. The samples were precipitated with 20% TCA for 30 min on ice. Precipitates were washed with 5%TCA, resolved in sample buffer and analyzed on a 7.5% polyacrylamide/SDS gel and autoradiographed. Initiation products were quantified by densitometric analysis using a Phosphor imager.

Results

The hinge mutants bind ss- and dsDNA with approximately wild type like affinity

ΔN-DBP (wild type) and the mutants N512P (P-DBP), V513L (L-DBP) and NVS512-514PPP (PPP-DBP) were purified and assayed for their ability to bind to ss- and dsDNA as is shown in Figure 2. All proteins bound cooperatively to ssDNA resulting in fully saturated protein-DNA complexes without intermediate complexes (Figure 2A). Multiple intermediate complexes can be seen when ΔC-DBP was used in this assay (Figure 2B). ΔC-DBP lacks the C-terminal arm and is therefore not able to form multimers on ssDNA, resulting in the loss of cooperative binding (10).

Protein	50% shift (nM)	σ
ΔN-DBP	0.15	0.07 (n=7)
L-DBP	0.17	0.05 (n=5)
P-DBP	0.23	0.06 (n=5)
PPP-DBP	0.25	0.3 (n=5)

Table 1: Binding affinity of ΔN-DBP and mutants on ssDNA.

To estimate the binding affinity, the concentration required to shift 50% of the probe was determined after quantification (Table 1). All proteins bound to ssDNA with approximately wild type affinity. The slight differences in binding affinity for the different mutants are not significantt in view of the scattering of the data around the 50% shift point. The binding of the mutants to a 50 base pair dsDNA probe is shown in Figure 2C. All three mutants were able to bind to dsDNA with wild type affinity. Binding to dsDNA is not cooperative for DBP and therefore intermediate complexes can be found in this assay. We assume that the band with the slowest migration is fully saturated whereas the fastest migrating band contains only 1 monomer. However we have not studied the stoichiometry of the bands in detail. The lack of cooperativity also leads to a lower binding affinity which is reflected by the higher concentration of proteins needed to obtain a shifted complex (20).

The hinge mutants, in particular PPP-DBP, are defective in stimulating DNA replication.

We tested the hinge mutants for their activity to support DNA replication in an *in vitro* assay, see Figure 3A. Ad5 DNA isolated from virus particles and containing the terminal protein was digested with XhoI and used as template. The reaction was carried out in the presence of pTP-pol, NFI, Oct-1, radio labeled dNTPs and increasing amounts of wt or mutant DBP. Analysis of the products on agarose gels shows specific labeling of two restriction fragments, B and C, containing the origin of replication. In addition two labeled fragments migrating with higher mobility are observed which contain ssDNA.

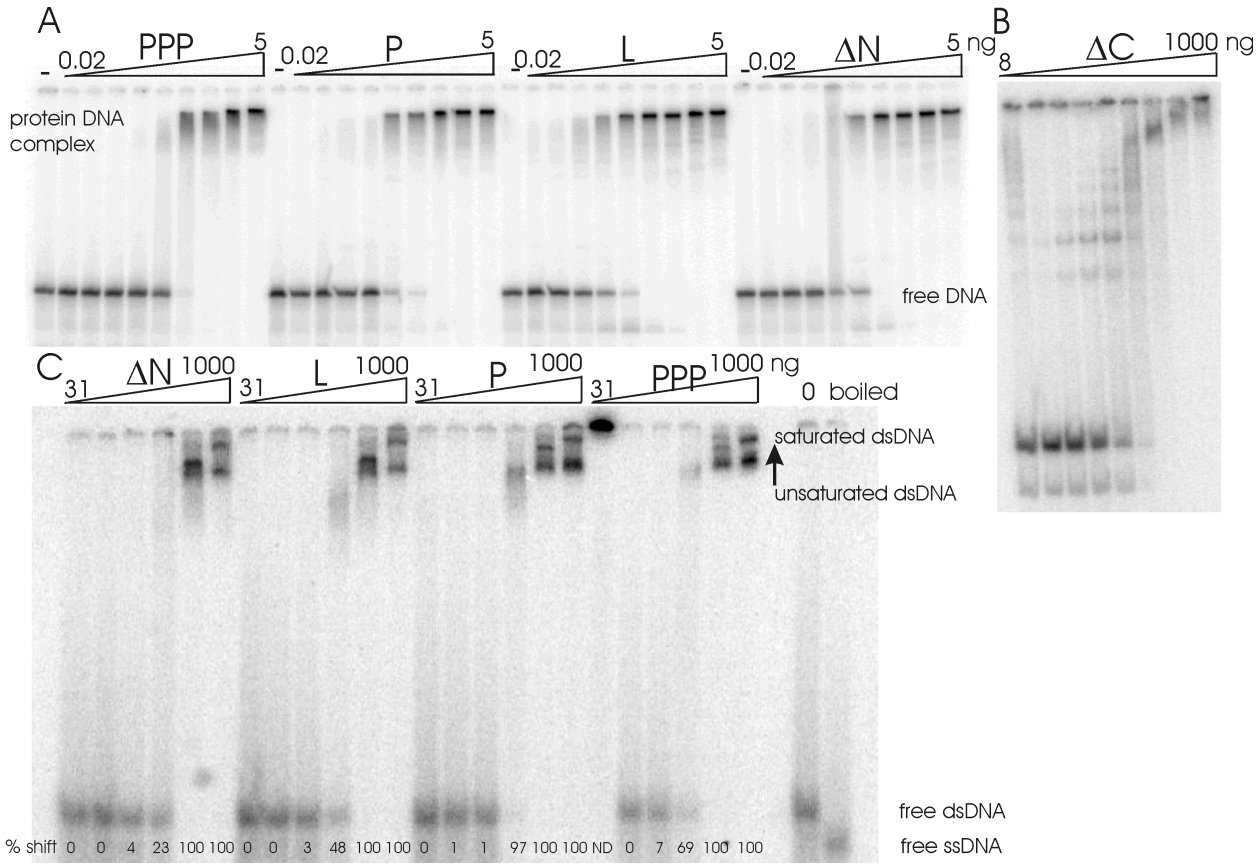

Figure 2: The hinge mutants have a wild type like ss- and dsDNA binding affinity.
A: Gel retardation assay on a 114nt ssDNA probe. The highest amount of DBP and mutants was 5ng. Proteins were diluted by successive 2-fold steps. The percentage of complexed DNA was quantified and the calculated values of the 50% shift concentration are shown in table 1. **B**: ΔC-DBP on a 114nt ssDNA probe. The highest concentration of protein is 1000ng and is diluted by successive 2-fold steps. **C**: Gel retardation assay on a 50bp dsDNA probe. The highest amounts of DBP and mutants were 1000ng. Proteins were diluted by successive 2-fold steps.

These originate from second rounds and further of displacement synthesis, indicative of effective template usage (Figure 3B). Quantitation of the total DNA replication activity, the 1st as well as further rounds together, was performed and compared for the 500 (3,7,11 and 14) and 1000ng (4,8,12 and 15) lanes. P-DBP had a slighly lowered replication activity (4.2- to 1.1 fold), whereas PPP-DBP had a 100 to 50 fold decrease in activity and could hardly support any replication. L-DBP behaved wild type like with a slight decrease in activity (1.4 fold) at 500ng.
The differences in activity are most prominent at low protein concentrations.
To test whether the loss of activity for PPP-DBP is due to the presence of inhibitors in the protein solution, a control was performed by mixing in PPP-DBP in a ΔN-DBP replication (lane 18). Inhibition of DNA replication was not detected. Lane 17, the negative control (no DBP), contains some background bands, caused by aspecific labeling of all XhoI TP-DNA fragments not related to protein-primed replication.

The hinge mutants stimulate initiation but are defective in early elongation.

To test whether the observed decrease in replication efficiency is due to reduced

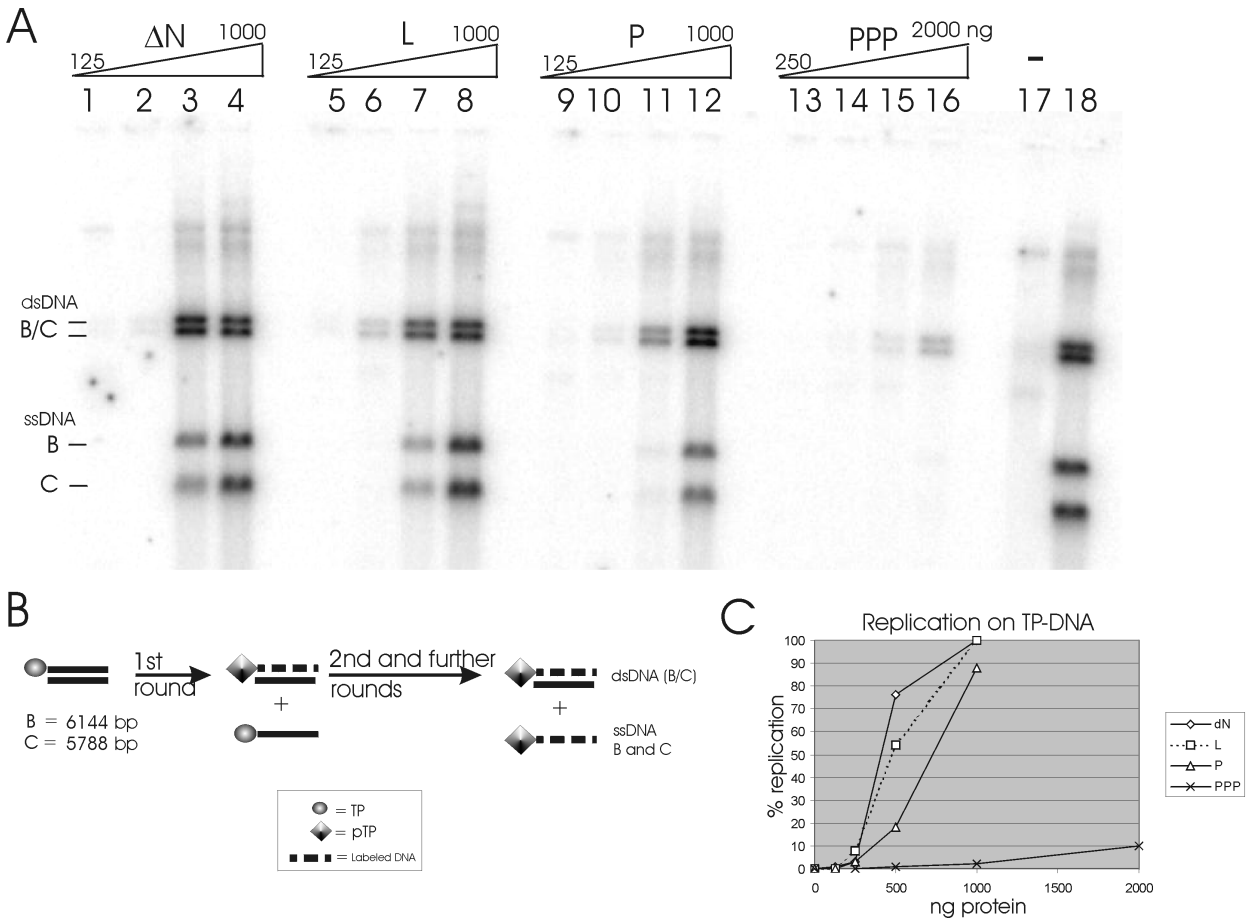

Figure 3: PPP-DBP can not support replication.
A: DBP proteins were added at the indicated amounts to the reaction as described in materials and methods. Lane 17 contained no DBP and in lane 18 ΔN-DBP and PPP-DBP (500ng each) were mixed to check for inhibitors in the PPP-DBP preparation. The autoradiogram shows the double stranded B/C bands containing the origins of replication as well as the single stranded bands B and C originating from second and further rounds of displacement synthesis. **B**: The scheme represents the origin containing fragments B, right origin, or C, left origin during the first and further rounds of DNA replication. The labeled fragments are depicted as striped lines and are the visible products (bands) on the autoradiogram. **C**: The replication products are quantified and presented in a line graph. The total amount of activity of the bands (B/C and B and C) in lane 4 is set as 100%.

initiation levels, an initiation assay wasperformed. The first step in initiation is the covalent coupling of dCTP to the pTP of the pTP-pol complex. This reaction is enhanced considerably by DBP when the pTP-pol concentration is low (10).

A low amount of pTP-pol (50ng) was incubated together with TP-DNA and dCTP. The stimulation of pTP-C formation was determined for two DBP concentrations (250ng and 500ng) (Figure 4A). Without DBP only a low level of initiation was observed (1%). L-DBP and P-DBP stimulated the initiation like wild type with activities ranging from 86 to 116%. For a quantitation see Figure 4A. The stimulation by PPP-DBP was slightly lower (25-36%), but still considerable.

To distinguish between defects during the initiation and elongation phase, we performed a partial elongation. This leads to the formation of a pTP-CAT initiation intermediate (19) as well as a pTP-26 nucleotide product indicative of

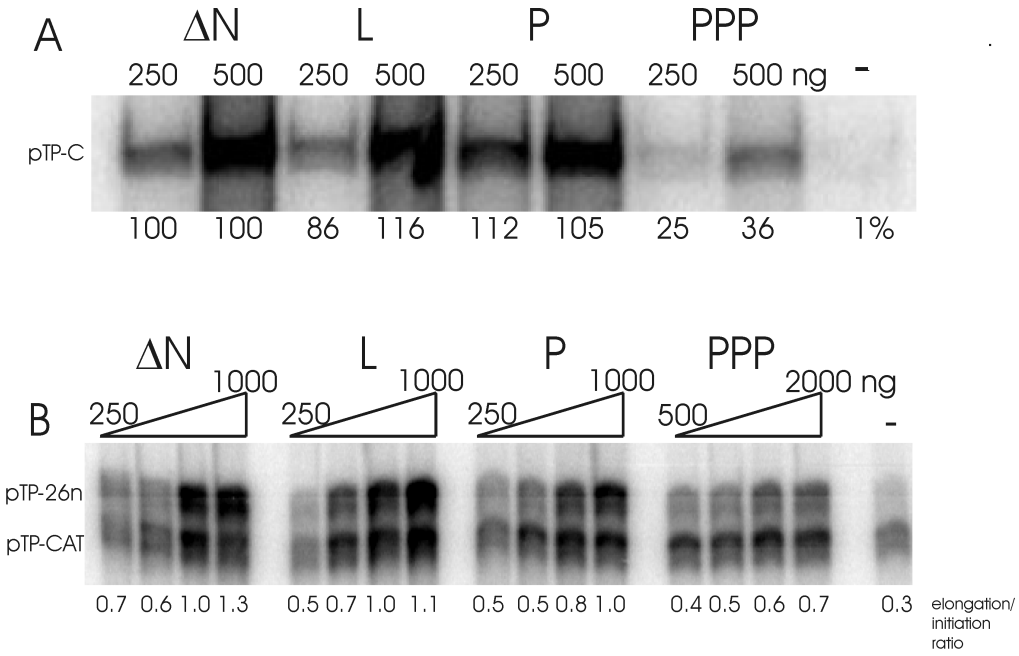

Figure 4: PPP-DBP still stimulates initiation but cannot support elongation.
A: pTP-C formation as a measure of initiation was tested with two amounts of DBP, 250ng and 500ng. The assay was performed at a low pTP-pol concentration (50ng) at which stimulation is most pronounced. The activities at 250 and 500ng of ΔN-DBP were set as 100% and compared to the activities determined for the other mutants. **B**: Partial elongation (pTP-26n formation) was tested as a function of DBP. The assay was performed at high pTP-pol concentration (100ng) at wich stimulation of the initiation (pTP-CAT) is independent of the DBP concentration. Under the condition chosen also the pTP-CAT initiation intermediate is observed. The elongation/initiation ratio was quantified. The protein concentration ranges are 250, 500, 750 and 1000 ng for ΔN-, L- and P-DBP and 500, 1000, 1500 and 2000 ng for PPP-DBP

early elongation (Figure 4B). The elongation to initiation ratio was calculated after quantitation (Figure 4B). This ratio is indicative of the ability to stimulate the elongation by DBP. Whereas ΔN-DBP as well as L-DBP and P-DBP were all able to stimulate elongation efficiently, PPP-DBP was deficient indicating that stimulation of elongation is already inhibited at an early stage of elongation.
The pTP-CAT formation is slightly stimulated by all mutant DBP's, but the absolute rate of stimulation is lower that in Figure 4A, due to the higher pTP-pol (100ng) concentration and for PPP-DBP the increase is minimal as this mutant is already less efficient stimulating initiation (Figure 4A).
For ΔN-DBP the pTP-CAT formation decreases at the highest amount of protein (1000 ng), due to efficient elongation of this intermediate. For L- and P-DBP however, no decrease in pTP-CAT formation was observed, presumably since higher protein concentrations are required for optimal stimulation of elongation by these mutants.
A small band below the pTP-C and pTP-26n products was also observed, presumably due to degradation of pTP. Unwinding of DNA correlates with the reduction in DNA replication activity
During elongation, DBP destabilizes dsDNA and facilitates elongation of the DNA polymerase. The unwinding of

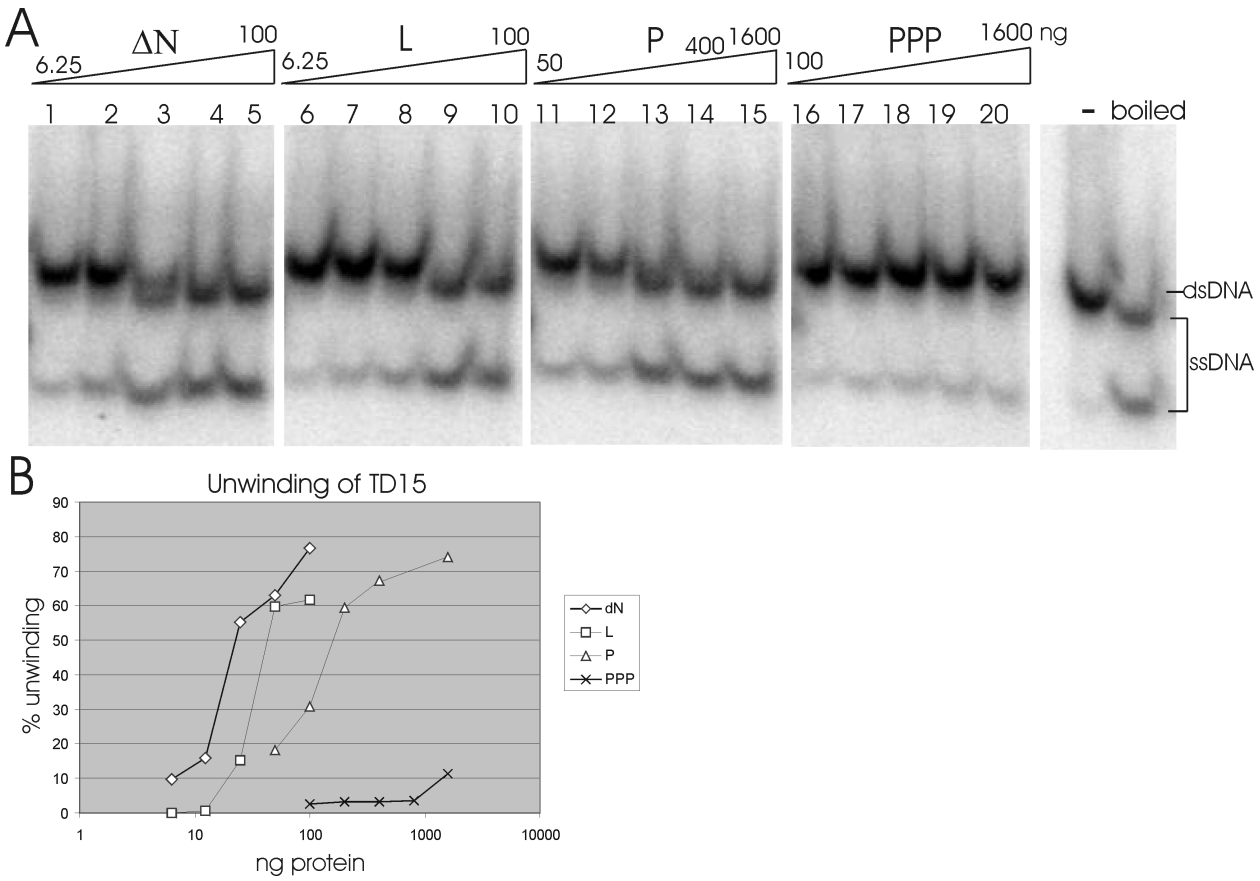

Figure 5: The proline mutants have a reduced unwinding activity.
A: DBP was incubated with a partial duplex DNA (TD15, see materials and methods). The concentration was increased with 2-fold successive steps except for lanes 14 and 15 where a 4-fold increase was used. **B**: The percentage of the unwinding was quantified and presented in a graph. The – lane was used to determine 0% of unwinding and the boiled lane was used to dertermine the 100% unwinding value.

dsDNA is ATP independent and only DBP is required, cooperative binding of the DBP monomers to the displaced strand being the driving force (22, 25, 35). Defects in unwinding will therefore result in diminished, or loss of, replication activity. An unwinding assay was performed with a partial duplex DNA (TD15) to investigate the unwinding activity of DBP and the hinge mutants (Figure 5A). The percentages of unwinding were calculated and precented in Figure 5B. All proteins were able to unwind TD15 but the activity of the hinge mutants was diminished as higher protein concentrations were required. The differences in unwinding activity were calculated from the slope of the curves. Most prominent was the decrease of unwinding activity for PPP-DBP (125 fold) whereas the reductions for L- and P-DBP were 1.25-fold and 23-fold respectively (Figure 5B). The decrease in unwinding activity for PPP-DBP can account for the large decrease found in the DNA replication activity during the elongation phase.

Binding to poly(dA)$_{40}$ is diminished for P- and PPP-DBP.

Both the unwinding- and replication assays show large differences in activity for the hinge mutants, with L-DBP behaving like wild type, P-DBP having

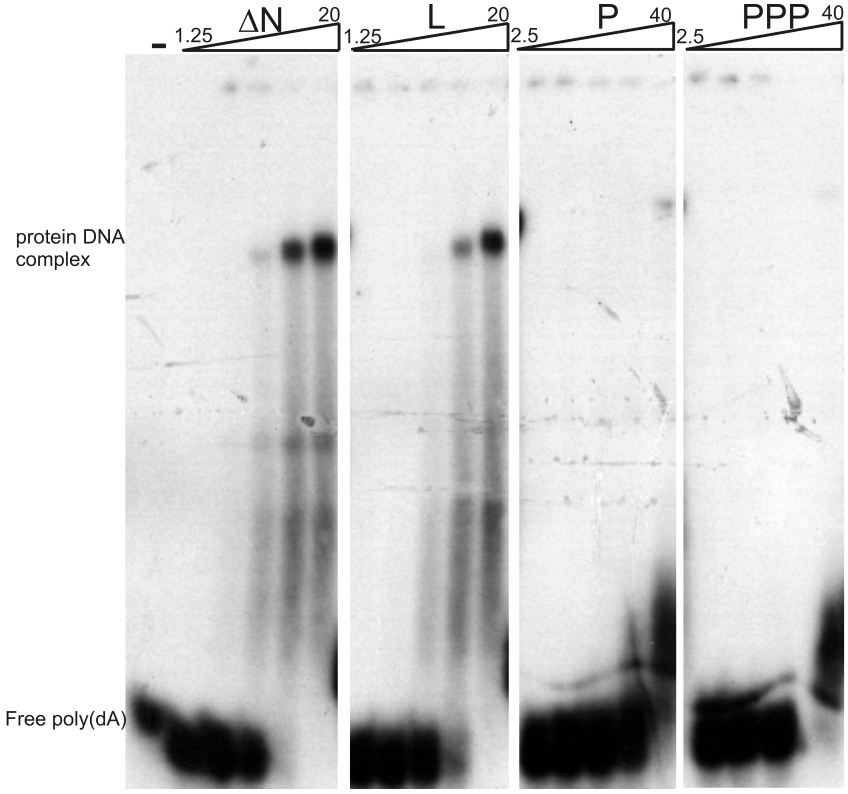

Figure 6: Gel retardation assay on a poly(dA)$_{40}$ homo oligonucleotide.
Indicated amounts of DBP or mutants were mixed with poly(dA)$_{40}$. The proteins were diluted by 2-fold successive steps.

an intermediate effect and PPP-DBP being most severely impaired. An explanation for these results could be that the flexibility of the C-terminal arm is gradually reduced. This could lead to a change in the multiprotein chain making adaptation to rigid or irregularly shaped DNA more difficult. To investigate this, we tested the proteins for binding to homo-oligonucleotides containing either 40 A, T, C or G residues. Binding to poly(dT)$_{40}$, (dC)$_{40}$ and (dG)$_{40}$ showed only small differences and are not shown. In contrast, P- and PPP-DBP are unable to bind to poly(dA)$_{40}$ efficiently (Figure 6). The highest concentration used in Figure 6 was 40 ng for P- and PPP-DBP. No binding of P- and PPP-DBP could be found up to a 1000 ng (data not shown). This suggests that reduction of the flexibility of the C-terminal arm can result in loss of the ability to bind particular sequences, possibly caused by the presence of aberrant secondary or tertiary structures in these homopolymers.

Discussion

Reduction of the flexibility of the C-terminal arm does not result in loss of ss- and dsDNA binding.

The ss- and dsDNA binding capabilities of the hinge mutants were not significantly reduced. This is in contrast with deletion of the C-terminal arm (ΔC-DBP), which leads to a 100-fold reduction in ssDNA binding, whereas no change in dsDNA binding is detectable (10, 11). This has been explained by the lack of cooperativity in ΔC-DBP. Since the cooperative binding is not lost in the hinge mutants this strongly suggest that the hinge mutants are still able to form multimers on DNA. Direct assays to test this (electron microscopy with negative staining and dynamic light scattering) were inconclusive perhaps due to aggregation problems.

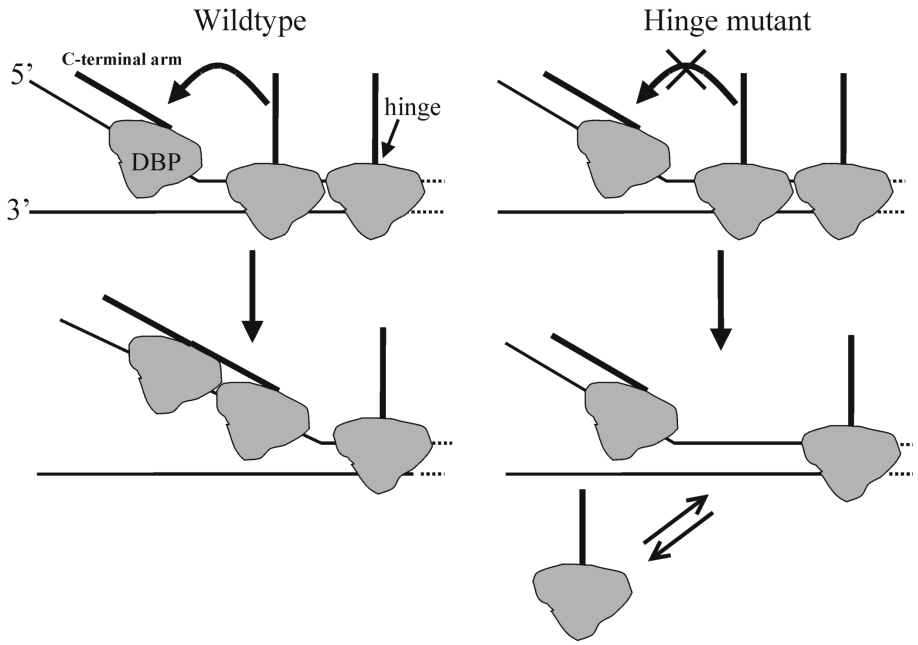

Figure 7: A model explaining the need for flexibility of the C-terminal arm.
We propose that the transition of DBP from dsDNA to ssDNA upon a striding replication fork requires flexibility of the C-terminal arm. In hinge mutants this step is blocked leading to dissocciation from dsDNA caused by the high offrate (11). DBP molecules bound to dsDNA are drawn as separate molecules for clarity reasons, but may well form a multimer with a conformation different from that of the DBP-ssDNA complexes.

A small decrease in ssDNA binding was found for P- and PPP-DBP. A low resolution crystal structure of DBP complexed with ssDNA shows that although N512 approaches the DNA it is unlikely to make any interaction with the phosphate backbone (16). Rather than a loss of direct contacts, the slight decrease in binding affinity for ssDNA could be due to the reduced flexibility of the C-terminal arm. Possibly, the mutant protein chain is not able to adapt to certain secondary structures or conformations in the ssDNA strand as indicated by the inability to bind polydA efficiently.

Elongation of replication is dependent on the flexibility of the C-terminal arm

P-DBP, and in particular PPP-DBP have a reduced replication activity. No strong differences in stimulation of initiation were detected and direct assays show that the main defect lies in elongation. This is in agreement with the reduced unwinding. A similar effect was observed upon deletion of the C-terminal arm (10). Like PPP-DBP, the ΔC-DBP mutant was still able to stimulate initiation with a slight reduction in efficiency while being unable to support replication or DNA unwinding. From this we have concluded earlier that oligomerization of DBP is the driving force of ATP independent DNA unwinding during the elongation phase. While this may be true, multimerization apparently is not the only requirement for DBP to function effectively in elongation. Previously we showed the need for an intact flexible loop located between aa K296 and aa S332 that ensures high affinity binding to ssDNA (11). Here we suggest yet another requirement, i.e. the need for flexibility in the protein chain even in the presence of an intact flexible loop and multimerization

Model

What could be the function of the flexibility of the C-terminal arm during elongation and replication fork destabilization? We propose (Figure 7) that reduction of the flexibility of the C-terminal arm leads to an inability to hook into a neighboring DBP molecule when bound to the replication fork. In particular we assume that a conformational change is required to accomodate the transition of DBP bound to the double stranded parental strands to that when bound to the displaced single strand, which is situated at the ssDNA part of the replication fork. The high offrate of DBP on dsDNA coupled to an impairment of the transition will prevent unwinding at the replication fork. Why is multimerization possible when bound on single stranded DNA and not in the replication fork? Presumably less mobility exists in the replication fork, compared to single stranded DNA.

As an addition to the model, binding of a less flexible DBP protein to the displaced strand could also lead to difficulties when AT tracts, hairpins or other secondary DNA structures are encountered. This might be reflected by the problems in binding polydA which has an abberant structure and a different binding site for DBP (20, 30).

The model might explain the lack of unwinding by hinge region mutants during elongation. This situation might also apply to the early stages of elongation. For stimulation of initiation DBP monomers suffice (10), but even early in elongation multimerization is required. Although the conformation of the preinitiation complex and the changes occuring during transition of initiation to early elongation are unknown, we assume that the same flexibility of DBP is needed at this stage. Alternatively we could envisage the dsDNA breathing and the irreversible steps that occur when DBP binds cooperatively to the part unwound by breathing to be slowed down with the mutants. However we consider this to be less likely because this effect would mainly influence unwinding of long stretches of DNA and we observe already a block in unwinding with a 35bp probe.

Acknowledgements

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3

Adenovirus Type 5 DNA Binding Protein Stimulates Binding of DNA Polymerase to the Replication Origin

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Adenovirus type 5 DNA binding protein stimulates binding of DNA polymerase to the replication origin.

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The adenovirus (Ad) DNA binding protein (DBP) is essential for the elongation phase of Ad DNA replication by unwinding the template in an ATP-independent fashion employing its capacity to form multimers. DBP also enhances the rate of initiation, with the highest levels obtained at low concentrations of Ad DNA polymerase (pol). Here, we show that stimulation of initiation depends on the template conformation. Maximal stimulation, up to 15-fold, is observed on double-stranded (ds) or viral TP-containing origins. The stimulation is reduced on partially single-stranded (ss) origins and DBP does not enhance initiation anymore when the origin is completely unwound. This suggests a role for DBP in origin unwinding, comparable to its unwinding capacity during elongation. However, mutant DBP proteins defective in unwinding and elongation can still enhance initiation on ds templates. DBP also stimulates the binding of nuclear factor I (NFI) to the origin and lowers the Km for coupling of the first nucleotide to the precursor terminal protein (pTP) by pol. Mobility shift experiments reveal that DBP stimulates the binding of pol on ds origin and non-origin DNA, but not on ssDNA. This effect is specific for DBP and is also seen with other DNA polymerases. Our results suggest that, rather than by origin unwinding, DBP enhances initiation by modulating the origin conformation such that DNA polymerase can bind more efficiently.

Introduction

DNA binding protein (DBP) plays an important role in the adenovirus (Ad) life cycle where it is involved in DNA replication, transcriptional control and mRNA stability (8, 36), host-range specificity (1, 13, 20), transformation (12) and virus assembly (26).

Ad5 DBP is a 529 amino acids (aa) protein with a molecular weight of 59,049 Da, which binds nucleic acids with high affinity. DBP binds cooperatively to ssDNA with a binding site size of approximately 12 nucleotides in a sequence independent manner (21). Mild chymotrypsin treatment cleaves DBP into two domains, an N-terminal part (aa 1-173) and a C-terminal part (aa 174-529) that is well conserved among different serotypes and harbors most of the biological functions ascribed to DBP, including its nucleic acid-binding and replication functions (32). The outermost C-terminal part of DBP (aa 510-529) forms a protruding C-terminal arm and contains a hook (33). This C-terminal arm is involved in the formation of a DBP protein chain, where the C-terminal arm

hooks into another DBP molecule to form a multi-protein complex. DBP binds to ssDNA in a cooperative manner, whereas binding to dsDNA is non-cooperative (11). Two crystal structures of the C-terminal part of DBP have been resolved, showing a remarkable difference in the orientation of this C-terminal arm, suggesting flexibility around the so-called hinge region (aa 512-515) (15). Deletion of the C-terminal arm results in loss of cooperative DBP binding to ssDNA and also abolishes the DNA unwinding activity of DBP (11). Recently we determined that the flexibility of the DBP molecule is important for the DNA unwinding activity of DBP as it functions to adapt the ssDNA and dsDNA structure present at the replication fork during initiation (34).

The role of DBP during adenovirus DNA replication is well established. Efficient DNA replication can be reconstituted with five proteins only. Three of these proteins are of viral origin, i.e. precursor terminal protein (pTP), polymerase (pol) and DBP. pTP and pol form a tight heterodimer in solution and bind to the origin located at the molecular ends of adenovirus DNA (23, 28, 31). Two other proteins, nuclear factor I (NFI) and Oct-1, originate from the host cell. NFI and Oct-1 are not absolutely required for *in vitro* DNA replication, but addition of these two transcription factors results in a 200-fold stimulation of DNA replication (for reviews see (10, 14, 35)).

Two phases can be distinguished in Ad DNA replication. During initiation the first nucleotide, a dCTP residue, is coupled opposite the fourth base of the template strand to the serine(580)-OH of pTP. Subsequently the pTP-C product is elongated to form the trinucleotide intermediate pTP-CAT (19). Then pTP-CAT shifts in position and jumps back to residues 1 to 3 of the template strand. During or right after the jumping back step, pTP and pol dissociate, after which further elongation can take place (18).

DBP is essential during the elongation phase of DNA replication. Here, DBP is responsible for the unwinding of the dsDNA template and enhancing the processivity of pol via the removal of secondary structures (11, 22).

DBP enhances the initiation of DNA replication in at least two ways. It enhances the binding of NFI to its recognition site within the auxiliary origin (7, 30), which in turn results in the stimulation of initiation by NFI (3). Also, DBP lowers the K_m for the coupling of the first nucleotide to pTP (24). This suggests a specific DBP-pol interaction as was also suggested from protection of pol by DBP against thermal inactivation (22). However, no direct interaction could be established by immunoprecipitation experiments (data not shown). In this paper we have examined other alternatives that can explain the specific function of DBP during the initiation of adenovirus replication. DBP may facilitate origin unwinding, thereby allowing more efficient binding of the pTP-pol complex. Alternatively, DBP may increase binding of pTP-pol in another way, either via a direct interaction of DBP with pol, or indirectly via a change in dsDNA structure. Our results show that DBP is involved in the stimulation of pol binding to the origin, employing the latter mechanism.

Materials and Methods

DNA templates and oligonucleotides

All oligonucleotides were purchased from Amersham Pharmacia Biotech. T50; 5'-CTCATTATCATATTGGCTTCAATCCAAAATAA GGTATATTATTGATGATG-3', representing the

first 50 nucleotides of the template strand of the Adeno type 5 genome, and D50; 5'-CATCATCAATAATATACCTTATTTTGGATTGA AGCCAATAT-GATAATGAG-3', representing the complementary (displaced) strand of T50. T50 and D50 were hybridized to form TD50. TDΔ5, TDΔ10, TDΔ15 and TDΔ20 are all derivatives from TD50 in which the number behind the Δ represents the amount of nucleotides deleted from the 5' end of the D50 complementary strand. TD50fork was created after hybridization of T50 and D50fork: 5'-GTAGTAGTTATTATATGGAAAT-TTTGGATTGAAGCCAATATGATAATGAG-3' to form a partially dsDNA template in which the first 20 nucleotides are non-complementary to create a forked structure. TD50random was formed after hybridization of T50random; 5'-TGGCTTGCTTGG-TGGTCGTCTTCTATGTTGTCTCCACTCCGCTA GTCATA-3' and D50random; 5'-TATGACTAGCGGAGTGGAGACAACATAGAAG ACGACCACCAAGCAAGCCA-3' to create a non adenovirus dsDNA template.

Labeling of the oligonucleotides was performed with T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ-^{32}P]ATP. All oligonucleotides (ss and ds) were purified by 12.5% polyacrylamide-Tris-Borate-EDTA (TBE) gel electrophoresis.

Proteins

The adenovirus proteins ΔN-DBP, PPP-DBP, pol, pol exo mutant (D422A) and pTP were expressed in baculovirus-infected SF9 cells and purified to near homogeneity as checked by silver staining as described previously (5, 6, 9, 34). Phage T4 gp32 and T4 polymerase were purchased from Amersham Pharmacia Biotech. Rabbit polyclonal antibodies were raised against Ad5 DBP (αDBP) (a kind gift of J. Dekker) and Ad5 polymerase (αpol) (6), and used in EMSA studies.

Initiation assay

Initiation of replication assays on origin based templates were performed in a final reaction volume of 25μl containing 25 mM Hepes-KOH (pH 7.5), 50 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 50 nM [α-^{32}P]dCTP, 0.3 pmol template, 0.35 pmol pol and 0.35 pmol pTP. The amounts of ΔN-DBP are indicated in the figure legends. Reactions were performed for 45 min. at 37°C and were stopped by addition of 80 mM EDTA. The samples were precipitated with 20% trichloroacetic acid (TCA) for 30 min. on ice. Precipitates were washed with 5% TCA, redissolved in sample buffer, and analyzed on an SDS-7.5% polyacrylamide gel and autoradiographed. Data were quantified by densitometric analysis using a PhosphorImager.

Electrophoretic mobility shift assay (EMSA)

For the EMSAs, ss or dsDNA [γ-^{32}P]-ATP labeled probes (approximately 0.05 ng) were incubated with purified proteins in 25 mM HEPES-KOH (pH 7.5), 4 mM $MgCl_2$, 0.4 mM DTT, 4% Ficoll and 50 mM NaCl, in a total volume of 25 μl for 30 min. at 4°C. Bound and unbound DNA were separated on a 9% polyacrylamide gel at 4°C in TBE and 0.01% Nonidet P-40 and analyzed by autoradiography or densitometric scanning using a phosphorimager.

Results

Stimulation of initiation by DBP depends on template conformation

Previously it was shown that the C-terminal part of DBP (aa 174-529) containing the DNA binding domain is sufficient to function in Ad DNA replication *in vitro* (2). The DBP deletion mutant ΔN-DBP lacking the first 173 aa will be referred to as DBP in this paper.

Because stimulation of *in vitro* initiation was shown to be dependent on the pol concentration (25), we first determined the optimal pol concentration to analyze stimulatory effects of additional components. For 0.3 pmol dsDNA or ssDNA templates the optimal concentration of pol for stimulation of initiation was determined to be 0.35 pmol. pTP was added to the initiation reactions in a 1:1 molar ratio with pol, to produce pTP-pol complexes necessary for initiation. Addition of higher pTP concentrations, up to 5-fold molar excess over pol, did not influence the initiation reaction. Under the given conditions, the basal level of initiation produces a detectable signal. Optimal conditions for stimulation of initiation by DBP were determined using a dsDNA Ad5 origin consisting of the first 50

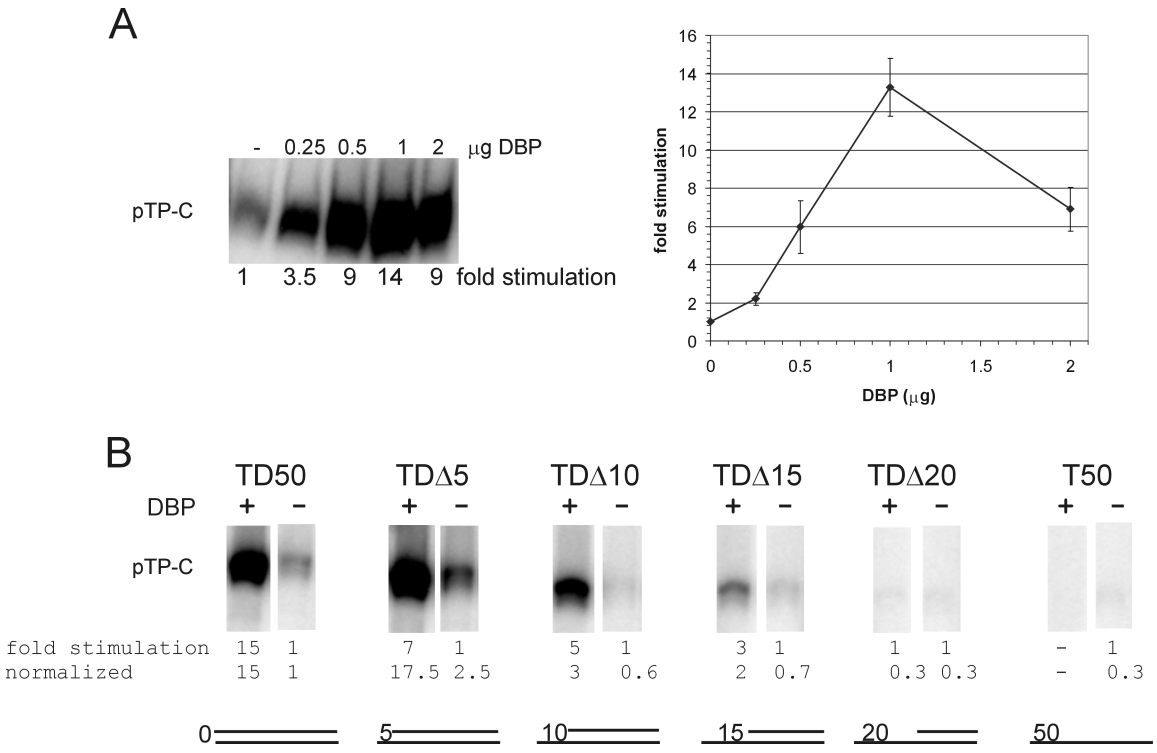

Figure 1: A: Initiation assay on 0.3 pmol of the TD50 template with various concentrations of DBP. A representative initiation assay is shown. The average level of initiation based on three independent experiments is graphically represented.
B: Initiation assay on origin containing templates with increasing 5'-gaps. Equimolar amounts of DNA templates, 0.3 pmol, were used. The fold stimulation of initiation by DBP of a representative experiment is shown. The pTP-C signal without the addition of DBP was set to 1 for each template.
The basal level of initiation (no DBP) on TD50 was set to 1 and the basal level of initiation of the other templates was determined: TDΔ5, 2.5; TDΔ10, 0.6; TDΔ15, 0.7; TDΔ20, 0.3 and T50, 0.3.

basepairs (TD50) as a template. A DBP concentration range from 0 to 33.8 pmol (0-2 μg) was tested in an initiation assay (Fig. 1A). A linear range of stimulation of initiation is observed up to 1μg of DBP, whereas higher amounts inhibit the initiation. At 1 μg of DBP the maximal level of stimulation was 14-fold (± 1.5). Similar titrations were performed for all templates used in the initiation assays, all showing optimal stimulation with 1 μg of DBP. Furthermore, the levels of stimulation of initiation by DBP were comparable for both TP-DNA and TP-less DNA templates (11).
Since DBP is a helix destabilizing protein, it may be capable of unwinding the dsDNA terminus of the origin, thereby allowing efficient binding of the pTP-pol complex, leading to stimulation of initiation. The partial unwinding of the terminus can be mimicked by creating templates with progressive deletions of the displaced strand. Assuming that the major role of DBP is unwinding of the DNA terminus, one would expect that the stimulation of initiation by DBP is lost when the opening is large enough to facilitate efficient binding of the pTP-pol complex to the origin.
To test this possibility partially ssDNA templates were designed with progressive 5'-deletions. The stimulation of initiation in the presence or absence of DBP was determined for templates

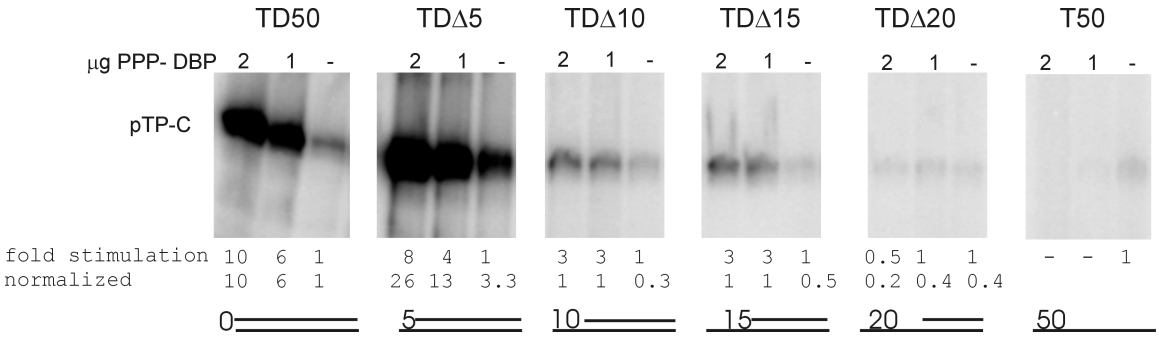

Figure 2: Initiation assay with the unwinding defective PPP-DBP mutant on 0.3 pmol origin containing templates. Indicated are the 5'-gaps ranging from 0 to 50 nts. The amount of stimulation of initiation is shown. The pTP-C signal without the addition of PPP-DBP was set to 1 for each template.
The basal level of initiation (no PPP-DBP) on TD50 was set to 1 and the basal level of initiation of the other templates was determined: TDΔ5, 3.3; TDΔ10, 0.4; TDΔ15, 0.3; TDΔ20, 0.3 and T50, 0.4.

with an ss moiety ranging from 5 to 20 nucleotides, as well as for the completely ss template strand (Fig. 1B). The stimulation of initiation by DBP was optimal when the DNA template is in a complete ds configuration. Upon increasing deletions of the displaced strand, the stimulation of initiation by DBP gradually decreases and beyond 15 nucleotides of ssDNA the stimulation by DBP is lost completely (Fig. 1B). This indicates that the role of DBP in stimulating the initiation requires the presence of dsDNA. An obvious possibility is that this is related to the unwinding capability of DBP.

It should be noted that the basal level of initiation in the absence of DBP, is higher for the TDΔ5 template than for the TD50. Similar observation of enhanced basal activity with partially ssDNA templates were reported by Kenny et al. (16). The most likely explanation for this observation is that the pTP-pol complex can bind more efficiently to the already partially unwound template.

Helix destabilization by DBP does not play a role in stimulation of initiation

To investigate if DNA unwinding by DBP is the determining factor that stimulates initiation, a similar experiment with partially ssDNA was performed with an unwinding-negative DBP mutant called PPP-DBP (34). In PPP-DBP three amino-acids (512N, 513V and 514S) in the hinge region of the flexible C-terminal arm are substituted by proline thereby destroying the flexibility, which in turn results in loss of unwinding activity on TD50 and smaller dsDNA templates, while binding of PPP-DBP to ss- and dsDNA was only slightly affected (34). This mutant was still capable of stimulating initiation on the various templates (Figure 2). Although the stimulation on TD50 by PPP-DBP is slightly lower than that by DBP in this experiment, this is due to the variation of individual experiments. An average of three independent experiments showed that PPP-DBP stimulated initiation on TD50 is 12.3 $\pm$ 1.7 fold and the average of DBP stimulated initiation is 13.0 $\pm$1.5 (n=3), demonstrating that the stimulation of initiation is not affected by the PPP-DBP mutant. It should be noted that due to the slightly lower DNA binding affinity of PPP-DBP, a two-fold higher concentration is required to obtain optimal stimulation of initiation (34). Therefore we conclude that stimulation

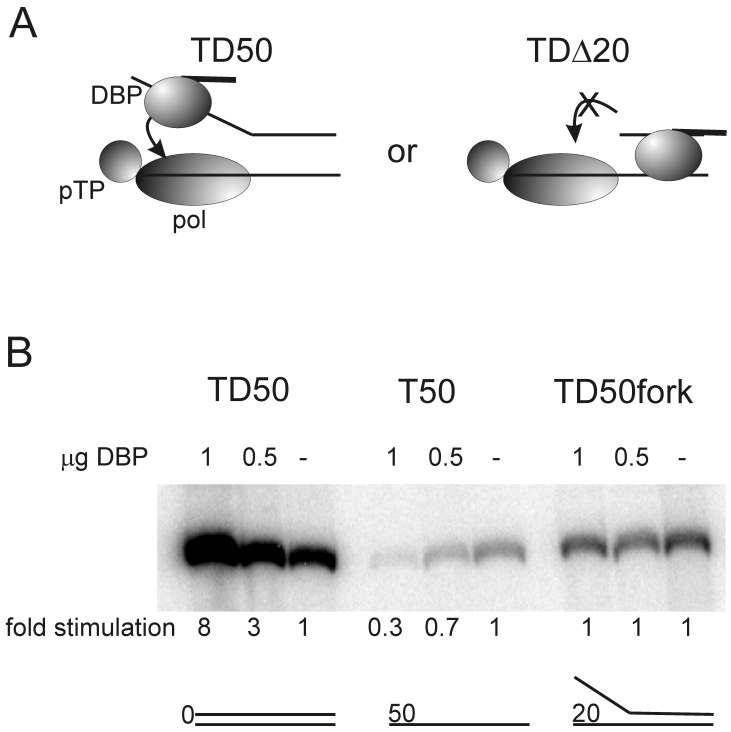

Figure 3: **A**: Model for transient DBP-pol interaction via the displaced strand. Left; DBP is able to contact pol via the displaced strand and thereby stimulates the initiation. Right; DBP is unable to contact pol when the ssDNA gap becomes too large, such as in TDΔ20, hence no stimulation of initiation can be observed. **B**: Initiation assay on 0.3 pmol origin containing templates. Assay conditions are as described in Figures 1 and 2. The fold stimulation of initiation by DBP is shown.

of initiation is independent of the unwinding activity. Apparently, optimal stimulation of initiation requires the first 15 nt of the origin to be in a double-stranded form.

Binding of DBP to the displaced strand does not stimulate initiation

Since the unwinding of dsDNA by DBP does not seem to play a major role in stimulation of initiation, we considered an alternative mechanism. The stimulatory effect of DBP on initiation could be caused by binding of DBP to the displaced strand, which might lead to a transient interaction of DBP with the pTP-pol complex, thus stimulating initiation. At partially ss templates such as TDΔ20, the distance between the pTP-pol complex on the template strand and DBP on the displaced strand might become too long, preventing this putative interaction and leading to loss of the stimulation (Fig. 3A). To test this hypothesis, a forked template was constructed similar to TD50, but with the first 20 nucleotides of the displaced strand non-complementary to the template strand (TD50fork). Stimulation of initiation by DBP of the forked-template was compared with the stimulation of TD50 and with a completely ss template strand, T50 (Fig. 3B). A clear stimulation of initiation by DBP is observed on the TD50 template, whereas DBP had a slightly inhibiting effect on the T50 ss template strand. When using the TD50-fork, initiation of replication could still take place, but DBP did not stimulate the reaction. This suggests that mere binding of DBP to the displaced strand does not determine

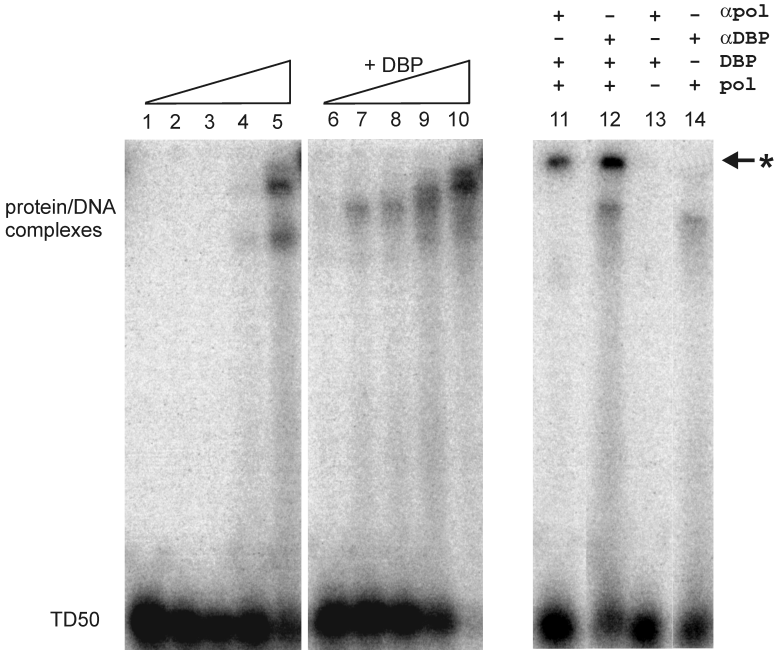

Figure 4: EMSA with TD50 and increasing pol concentrations (0, 25, 50, 100 and 200 ng). Lanes 1-5 contain no DBP and show DNA-polymerase complexes only. The reactions in lanes 6-10 contain an additional 20 ng of DBP. Supershifted complexes with antibodies against pol (lane 11) or DBP (lane 12) are indicated by an asterisk. (+) represents the addition of DBP (20 ng), pol (50 ng) or antibodies.

the stimulation of initiation.

DBP stimulates binding of pol to the dsDNA origin

Since stimulation of initiation was neither dependent on the binding of DBP to the displaced strand, nor on unwinding of the displaced strand, we examined another possibility. Does DBP bind to the dsDNA template in such a way that it facilitates the binding of the pTP-pol complex to the dsDNA origin? One indication could be that a similar mechanism was described for the effect of DBP on binding of NFI to its cognate site in the auxiliary origin. This is accompanied by DBP-induced conformational changes in the dsDNA as observed by hydroxyl radical footprinting and circular dichroism experiments (29). In the case of NFI, DBP alters the conformation of dsDNA leading to more efficient NFI binding. To examine whether DBP is able to stimulate pol binding, an electrophoretic mobility shift assay (EMSA) was performed with TD50 DNA and increasing amounts of polymerase, in the presence and absence of DBP (Fig. 4, lanes 1-10). Complex formation between TD50 and polymerase was visible from 100 ng of polymerase onwards (Fig. 4, lane 4). Increasing amounts of polymerase showed additional complexes containing multiple polymerase molecules bound to one TD50 template (Fig. 4, lane 5), as was previously described (6). In the presence of DBP a protein-DNA complex is already visible when only 25 ng of polymerase was used in the reaction (Fig. 4, lane 7). Interestingly, the complex seems to run at a slightly higher mobility when DBP is present. This suggests that DBP is included in the DNA-protein complex. A similar experiment was performed to determine if this stimulation also occurs when pTP-pol is used instead of pol alone. Indeed, binding of DNA by pTP-pol is also stimulated in the presence of DBP (data not shown).

To determine the composition of the multiprotein complex, supershift experiments were performed under

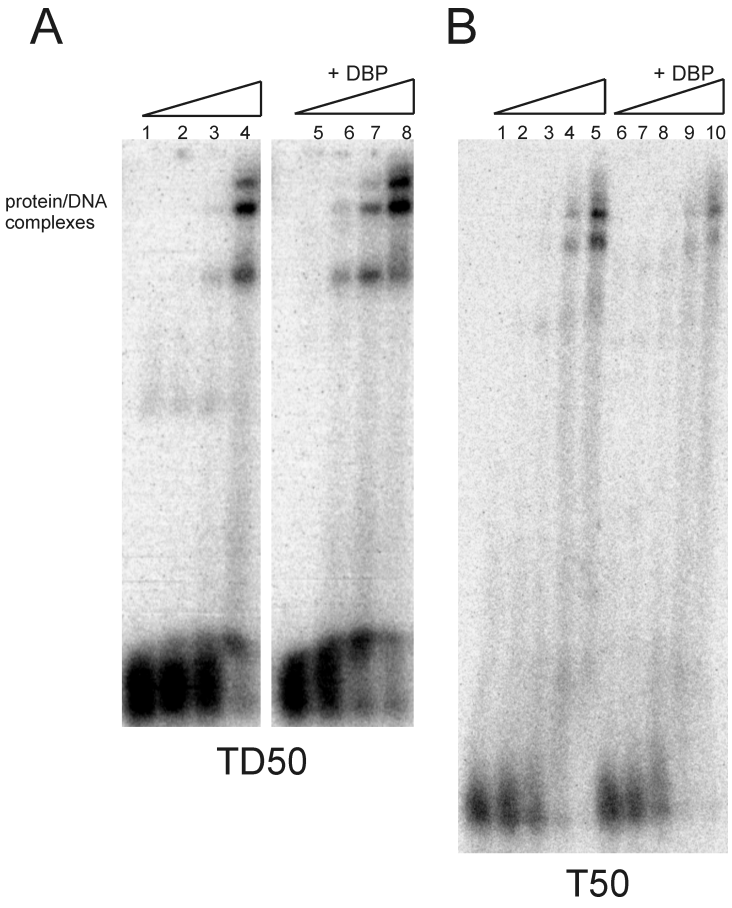

Figure 5: **A**: EMSA with TD50 and increasing pol (exo mutant) concentrations (0, 25, 50, and 100 ng). Lanes 1-4 contain no DBP and show DNA-polymerase complexes only. The reactions in lanes 5-8 contain an additional 15 ng of DBP.
B: EMSA with increasing pol (exo mutant) concentrations (0, 25, 50, 100, 200 ng) preincubated with T50 ssDNA, without DBP (lanes 1-5) and with 0.6 ng DBP (lanes 6-10). Please note that the DBP concentration is lower in EMSAs on ssDNA because the affinity of DBP for ssDNA is much higher than that for dsDNA.

conditions comparable to lane 8, using antibodies specifically raised against Ad5 polymerase or DBP. When using the anti-pol antibody (αpol) (lane 11) a supershift was observed. Likewise, when using the anti-DBP antibody (αDBP) (lane 12) a supershift with similar mobility was observed. In addition complexes with a higher mobility are present in lanes 12 and 14 that are most likely caused by contaminants in the αDBP sample. Two conclusions can be drawn from these experiments. First, binding of pol to the dsDNA origin is significantly stimulated by DBP. Second, DBP and pol form a multiprotein complex at the dsDNA origin. Although we show that both proteins are bound, we do not know if this is due to a direct pol/DBP interaction or if both proteins bind to separate positions on dsDNA. Since under replication assay conditions we were not able to show a direct interaction between DBP and pol in immunoprecipitation assays (data not shown), we favor the model in which pol and DBP bind to separate dsDNA regions.

Enhanced binding of polymerase correlates with enhanced stimulation of initiation

Is the enhanced stimulation of initiation by DBP, as observed in Fig. 1, reflected in enhanced polymerase binding? To address this issue we compared templates TD50 and T50, showing the two outermost situations in stimulation, in an EMSA (Fig. 5). To examine the specificity of the stimulation of pol binding by DBP on the ssDNA template T50, we used an exonuclease deficient

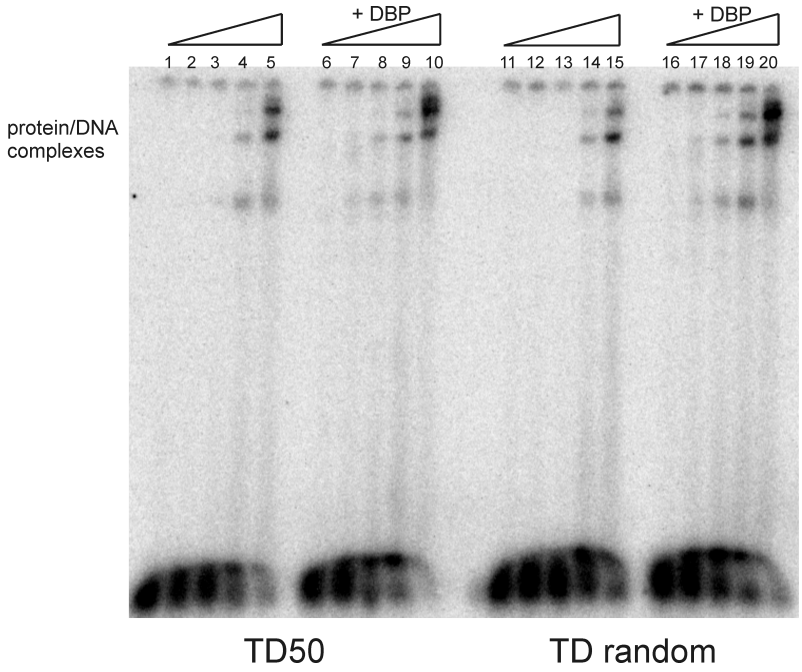

Figure 6: EMSA with increasing pol concentrations (0, 12.5, 25, 50, 100 ng) preincubated either with TD50 or a 50 bp random dsDNA probe (TD random), without DBP (lanes 1-5 and 11-15) or with 15 ng DBP (lanes 6-10 and 16-20).

polymerase mutant (pol exo mutant, D422A), because wild-type polymerase is known to degrade ssDNA in the absence of dNTPs (5). Stimulation of pol binding to TD50 with the pol exo mutant was similar to wild-type polymerase binding, compare Fig. 5A, lanes 1-8 with Fig. 6, lanes 1-10. However, incubation of T50 with the pol exo mutant, in the absence or presence of DBP revealed no stimulation of polymerase binding (Fig. 5B, lanes 1-10). In fact, a decreased polymerase binding is observed in the presence of DBP, possibly due to competition between polymerase and DBP for ssDNA binding.

Specificity of DBP stimulated polymerase binding

How specific are the interactions between DNA, polymerase and DBP? To address this issue we compared binding of Ad5 polymerase and DBP to another dsDNA template. Incubation of the origin containing dsDNA template TD50 and polymerase in the absence and presence of DBP resulted in stimulation of polymerase binding when DBP was present (Fig. 6, lanes 1-10). Because it is known that polymerase can bind non-specifically to any dsDNA fragment (31), we examined if the DBP stimulation was based on the nucleotide sequence of the adenovirus origin. Using a 50 bp random dsDNA probe, TD random, the experiment was repeated. In the absence of DBP polymerase bound to the dsDNA non-specifically, as was expected (Fig. 6, lanes 11-15). Addition of DBP still stimulated polymerase binding to random dsDNA (Fig. 6, lanes 16-20). The experiment was also performed with other 50 bp dsDNA templates that were either GC-rich or TA-rich and again polymerase binding was stimulated by DBP (data not shown). Our results suggest that the enhanced binding of polymerase in the presence of DBP is not dependent on the specific sequence of the template.

We further examined the specificity of DBP for another polymerase. TD50 template was incubated with Ad5 polymerase or with phage T4

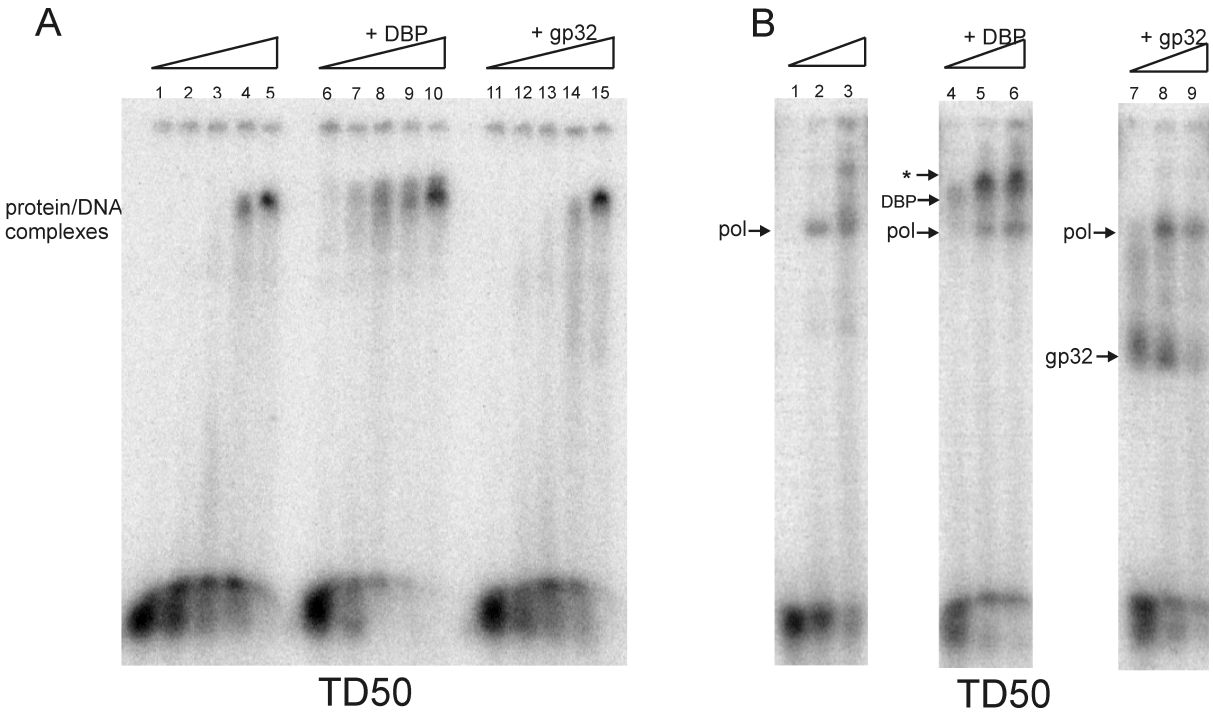

Figure 7: **A**: EMSA of the template TD50, preincubated with increasing phage T4 pol concentrations (0, 3.5, 7, 14, 28 ng), without SSB (lanes 1-5); in the presence of 15 ng DBP (lanes 6-10); or in the presence of 40 ng phage T4 gp32 (lanes 11-15). **B**: EMSA of the template TD50 preincubated with increasing Ad5 pol concentrations (0, 50 and 100 ng), without SSB (lanes 1-3); in the presence of 15 ng DBP (lanes 4-6); or 40 ng phage T4 gp32. Arrows indicate the single protein DNA complexes. Only in lanes 5 and 6 a complex consisting of DNA, DBP and pol is present (*).

polymerase, in the absence or presence of DBP (Fig. 7). In the absence of DBP T4 polymerase can bind to TD50 (Fig. 7A, lanes 1-5), and in the presence of DBP the binding of T4 polymerase is stimulated (Fig. 7A, lanes 6-10). The results indicate that the effect of DBP is not specific for Ad5 polymerase.

Finally we examined the specificity of DBP and compared its stimulatory properties with that of phage T4 SSB, gp32. TD50 was incubated with Ad5 polymerase in the absence or presence of gp32 (Fig. 7B lanes 1-3 and lanes 7-9), or with DBP (lanes 4-6). Furthermore, we tested if enhanced polymerase binding could occur with phage T4 DNA polymerase and its native SSB gp32 (Fig. 7A, lanes 11-15). No stimulation of phage T4 polymerase binding to TD50 was detected when gp32 was added. In addition, no stimulation of Ad5 polymerase was detected, since no gp32/pol/DNA complexes with lower mobility than the pol/DNA complex were observed (Fig. 7B, lanes 7-9). This suggests that the enhanced polymerase binding is DBP specific and that functionally related SSB proteins such as gp32 are not capable of stimulation of polymerase binding to dsDNA.

Discussion

The major role of DBP in adenovirus type 5 DNA replication is helix destabilization of the dsDNA template (11, 37). Previously, an indirect role of DBP during initiation was described, showing that DBP stimulates NFI binding to the origin of replication (7, 30). Here we describe an additional role for DBP during initiation of DNA replication based

on the fact that initiation can also be stimulated by DBP without NFI. Two possible models for the stimulation of initiation by DBP were investigated in detail.

The first model proposes that DBP partially unwinds the termini, thereby creating a partially unwound duplex origin required for optimal pTP-pol binding. This model is based on an observation by Kenny et al., (16, 17) who showed that optimal initiation activity of Ad5 replication was dependent on the function of a 5' -> 3' exonuclease, factor pL. Factor pL, purified from non-infected HeLa cells, was shown to degrade the 5'-end of the non-template (displaced) strand of the Ad5 origin. This partially duplex DNA is an efficient template for initiation of DNA replication (16).

We hypothesized that the helix destabilization activity of DBP could create a partially unwound origin, that similarly would stimulate initiation of DNA replication. In this model DBP would destabilize the dsDNA origin in such a way that it allows optimal access of the replication complex, consisting of pTP-pol, NFI and Oct-1.

Initiation assays on partially ssDNA templates were performed with DBP or with a DBP mutant defective in its dsDNA destabilization activity (Figs. 1 and 2). No helix destabilizing activity of DBP was required for stimulation of initiation. In fact, binding of pTP to dsDNA inhibits unwinding (de Jong, unpublished results), strengthening our hypothesis that during initiation unwinding of the dsDNA template by DBP does not play a role. In addition, it was shown with a forked template that mere binding of DBP to the displaced strand is also not sufficient for stimulation of initiation (Fig. 3). From these results we can conclude that initiation of replication is neither dependent on the binding of DBP to the displaced strand, nor is it dependent on destabilization of the origin.

It might be argued that the conformational differences among the various partially dsDNA templates influence the kinetics of the initiation assays and thus the stimulation by DBP. Although we cannot exclude this completely, we have no indications that the template conformation changes the kinetics. The incubation time of 45 min is well beyond the reaction time. Incubation at shorter time points up to 30 min gave a similar linear response for all templates tested.

In a second model we hypothesized that DBP can bind to dsDNA in such a way that it alters the dsDNA structure thereby facilitating binding of the pTP-pol complex to the origin. This assumption was based on experiments of Stuiver et al. showing that DBP can change the structure of dsDNA upon binding (29). Due to DBP binding the dsDNA becomes more rigid and adapts a more regular conformation devoid of tertiary structures, as was shown by hydroxyl radical footprinting and circular dichroism. The effect of DBP binding on dsDNA results in stimulation of NFI binding to the dsDNA origin, which in turn leads to stimulation of initiation.

The specific role of DBP in initiation thus could be the stimulation of polymerase binding to the origin in a similar fashion as described for NFI (29). To examine DBP stimulated polymerase binding, electrophoretic mobility shift assays were performed with polymerase and DBP using the Ad5 origin as a dsDNA probe. Upon addition of DBP, polymerase binding to dsDNA was clearly enhanced, strongly suggesting that DBP can stimulate polymerase binding to the

origin (Fig. 4).

We wondered if the enhanced stimulation of initiation by DBP is reflected in stabilization of pol binding to dsDNA or if it is due to enhanced polymerase binding. After pol binding in the presence or absence of DBP in competitor experiments to study the stability of the pre-formed complex, no changes in stability of the complex were observed (data not shown). In addition, when comparing templates TD50 and T50 in an EMSA, stimulation of pol binding was only observed with the dsDNA template (Fig. 5). This strengthens the assumption that the origin needs to be in a dsDNA conformation and that DBP changes this conformation in such a way that polymerase binding is enhanced. An obvious next step would be to also test the partial duplex templates for their ability to function in the DBP stimulated pol binding to dsDNA. However, due to the non-specific nature of pol and DBP binding to dsDNA and the fact that all partially ss-templates also contain a dsDNA region, these templates are able to sustain enhanced pol binding by DBP.

Additional experiments were performed to examine the specificity of DBP stimulated polymerase binding. Enhanced polymerase binding was not dependent on the specific sequence of the template (Fig. 6). In addition, the binding of phage T4 polymerase to dsDNA is also enhanced by DBP (Fig. 7A).

The specificity and affinity of polymerase for the adenovirus dsDNA origin is predominantly determined by two cellular transcription factors NFI and Oct-1 that are part of the preinitiation complex. Both NFI and Oct-1 target the pTP-pol complex to the origin and then dissociate from the pTP-pol complex early during initiation. In addition increased specificity of the pTP-pol complex for the origin was reported (31). We report an additional function of DBP, which is the stimulation of the polymerase binding affinity to the origin.

Stimulation of polymerase binding to dsDNA was only observed with DBP. When a related SSB, phage T4 gp32 was used no stimulated polymerase binding was detected (Fig. 7A, lanes 12-15 and Fig. 7B). This suggests that the conformational changes induced by DBP on dsDNA are specific for DBP. These specific changes in the dsDNA structure might increase the binding affinity of proteins such as polymerase and NFI to dsDNA.

One could argue that the increased polymerase binding is caused by a direct interaction between DBP and polymerase (22). Interactions between SSBs and their cognate polymerase have been described in other systems such as phage T4 polymerase and gp32 (27). However, we do not favor this alternative because we have never been able to demonstrate a direct interaction between DBP and Ad5 polymerase. In addition, since stimulation of polymerase binding by DBP was also shown for other polymerases it is unlikely that such a specific interaction exists between pol and DBP. Functionally, such a direct interaction could keep the pTP-pol complex trapped to the origin since direct binding of DBP to polymerase might interfere with elongation. Similarly, no direct interaction could be demonstrated between DBP and NFI, also suggesting that direct interaction would interfere with elongation (4, 29).

Based on the presented data we propose the following model for the role of DBP in the initiation of DNA replication. *In vivo* DBP is present in

large quantities and consequently it can cover the entire adenovirus genome. DBP binding changes the dsDNA structure. First, DBP stimulates binding of NFI to its dsDNA recognition site in the adenovirus origin. Since NFI is part of a multiprotein complex consisting of pTP-pol, Oct-1, and NFI, stimulated NFI binding enhances the recruitment of pTP-pol to the origin. Second, due to the DBP induced altered dsDNA structure of the origin also the pTP-pol complex binds with higher affinity to its binding site. Once the entire complex is formed on the DNA, initiation can take place. During elongation DBP employs its helix destabilizing activity and unwinds the dsDNA template ahead of polymerase.

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4

The Role of the Adenovirus DNA Binding Protein in homologous Recombination

Manuscript in preparation

The role of the adenovirus DNA binding protein in homologous recombination

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A putative role of DBP in adenovirus recombination has been suggested for many years. So far, direct evidence for a role of DBP in recombination has not been obtained. This is due to the fact that adenovirus recombination is critically dependent on viral DNA replication. Mutations disturbing DBP function in replication also affect recombination, rendering it difficult to distinguish between the functions of DBP in each separate process. A crucial step in homologous recombination is the strand exchange reaction. Using *in vitro* strand exchange assays it is now possible to directly study the function of the adenovirus DBP in the strand exchange reaction of homologous recombination, independent of its function in replication. In this chapter we demonstrate for the first time that DBP is functional in the strand exchange reaction. A model is presented for the function of DBP in homologous recombination.

Introduction

Homologous recombination is a fundamental biological process. In addition to its role in meiosis, it serves to repair DNA damage or to reinitiate DNA replication stalled at replication forks. In mammalian cells an efficient recombination machinery is present. This was demonstrated when partially overlapping gene fragments were transfected into cells leading to the formation of functional genes (7). Upon adenovirus infection the viral DNA is also subject to homologous recombination. Interestingly, the frequency of recombination of adenovirus DNA is much higher than that of the host cell DNA as was demonstrated with adenoviruses of the same serotype, but with different restriction maps. This lead to the hypothesis that adenovirus could encode additional recombination enzymes (8).

An important step in homologous recombination is the strand exchange reaction. Promoting strand exchange requires two activities, helix destabilization (unwinding) of the ds donor DNA and renaturation of the two homologous DNA strands. This has been described in detail for RecA in *E. coli* (for reviews see; (5, 22)). Many organisms, ranging from bacteriophages to yeasts to metazoans encode their own RecA-like proteins such as T4 UvsX, archaeal RadA, yeast Dmc1 and Rad51 (2, 24, 25, 29). In contrast, members of the *Herpesviridae* and other viruses such as bacteriophage T7 do not encode a RecA-type protein. An alternative mechanism for strand exchange is present in these viruses, as was shown for bacteriophage

T7 and HSV-1 (12, 16). In addition such a mechanism has been suggested for adenoviruses (30)

In T7, two DNA replication proteins, the T7 SSB gp2.5 and helicase primase gp4 perform pivotal roles in catalyzing strand exchange (9, 11). Helix destabilization and renaturation activities are also present in HSV that encodes a HSV-helicase-primase protein as well as the protein ICP8 that serves multiple functions. ICP8 is a sequence-independent DNA binding protein, which is able to both unwind dsDNA as well as stimulate renaturation of two homologous DNA strands. Recently, it was demonstrated that ICP8 can promote strand exchange *in vitro*. In addition, a more efficient strand exchange takes place when ICP8 acts in concert with HSV helicase-primase (17).

Studies performed by Young et al. (30, 32) suggested that recombination in adenoviruses is interrelated with adenovirus DNA replication. Presumably the formation of displaced ssDNA products during replication plays an important role in this process. Efforts to localize regions encoding adenovirus proteins involved in recombination showed that mutations in the E1A and E4 regions had no effect on recombination frequencies (6). The involvement of the E2 products is difficult to investigate directly by deletion analysis. Three important adenovirus replication proteins are encoded in the E2 region, e.g. pTP and pol in the E2b and DBP in the E2a region. Because recombination is interrelated with replication E2 mutants result in replication deficient viruses thereby also affecting recombination *in vivo* (30, 31). Although many speculations have been expressed on the function of adenovirus proteins in recombination, in particular for the ssDNA binding protein DBP, no direct evidence has been presented.

The adenovirus DNA binding protein possesses both the unwinding and renaturation properties. Therefore it was suggested that in addition to its indispensable role in DNA replication, DBP might play also an important role in adenovirus recombination. Here we investigate the putative role of DBP in the strand exchange step of homologous recombination, and present data that DBP is capable of promoting strand exchange.

Materials and Methods

DNA and proteins

M13 replicative form and M13 ssDNA were a kind gift of Dr. P. Baas, UMC Utrecht. The 39 kDa N-terminal deletion mutant of the adenovirus DNA binding protein (ΔN-DBP, aa. 174-529, was purified to near homogeneity from baculovirus infected SF9 cells as was described in (27). ΔN-DBP as shown to be functional in replication in a similar fashion as full length DBP.

Helix destabilization assay

Helix destabilization assays were performed using a partially double-stranded oligonucleotide consisting of 50 bases of the template strand of the adenoviral origin of replication, hybridized with an oligonucleotide containing the complementary bases 15–50 from the displaced strand, thereby creating a dsDNA oligonucleotide with a 15-base pair single-stranded overhang (TDΔ15). In addition a 30 bases A stretch was hybridized to a 30 bases T stretch, thereby creating a 30b nt ds A/T dsDNA template (AT30). All strands were [γ-^{32}P]-labeled with T4 polynucleotide kinase prior to hybridization. DNA (0.5 ng) and indicated amounts of ΔN-DBP were incubated for 1 h at 30 °C in a total volume of 25 μl in a buffer containing 25 mM Hepes-KOH (pH 8.0), 1 mM DTT, 0.1 mM PMSF, 20% glycerol, 0.02% Nonidet P-40, 0.5 mM EDTA, 1 mg of bovine serum albumin, and 100 mM NaCl. Reactions were stopped by addition of 5 μl of 40% sucrose, 1.2% SDS, and 0.1% bromophenol blue and 0.1% xylene cyanol. Products were analyzed on a 12.5% SDS-polyacrylamide gel using a running buffer containing 1 x TBE and 0.2% SDS. Gels were dried and quantified using

a PhosphorImager Storm 820 from Molecular Dynamics with ImageQuaNT 4.2a, Build 13 software.

Electrophoretic mobility shift assay (EMSA) /renaturation assay

A 114-nt *EcoRI/XbaI* DNA fragment from pHRI was labeled with Klenow enzyme in the presence of [α-^{32} P]dCTP and denatured by boiling. Renaturation assays were performed with 0.05 ng of denatured DNA and the indicated amounts of ΔN-DBP, in a final volume of 20 μl of buffer containing 20 mM Hepes-KOH (pH 8.0), 100 mM NaCl, 4 mM $MgCl_2$, 0.4 mM DTT, 4% Ficoll, 1 μg of bovine serum albumin. Bound and free DNAs were separated on a 10% polyacrylamide gel at room temperature. The running buffer contained 0.5 x TBE and 0.01% Nonidet P-40. Gels were dried and quantified using a PhosphorImager.

***In vitro* recombination assay**

Joint molecule formation and strand exchange between homologous DNA molecules consisting of linear M13 dsDNA (donor DNA) and circular M13 ssDNA (acceptor DNA) was detected using an electrophoretic mobility shift assay. Donor DNA was produced from M13 replicative-form, which was digested with *Dra*I to create a blunt end and with *Tsp*RI to generate a 2516 bp linear dsDNA with a 9 nt 3'-overhang. The strand possessing the overhang was complementary to viral circular M13 ssDNA. The ds donor DNA was [γ-^{32}P]-labeled with T4 kinase.

Unless otherwise stated, reactions were performed as follows; dsDNA (5 μM) was preincubated with 2μg DBP in 20 mM HEPES-KOH, pH 7.5, 5% glycerol, 50 mM NaCl and 1 mM dithiothreitol for 30 min at 37 °C. The reaction was supplemented with M13 ssDNA (10 μM), 2.5 mM $MgCl_2$ and further incubated for 30 min at 37 °C. Reactions were quenched by the addition of termination buffer (final concentration: 1% SDS, 25 mM EDTA, and 0.1 mg/ml proteinase K) followed by incubation for 10 min at 37 °C. The reaction mixtures were resolved by electrophoresis through 1% agarose-Tris acetate EDTA, pH 8.3, gels at 1.5 V/cm for 12 h. The gels were dried onto DE81 chromatography paper (Whatman) and analyzed and quantified using a PhosphorImager.

Results

DBP functions in dsDNA unwinding.

A prerequisite for homologous recombination is the strand exchange reaction, where the ds donor DNA becomes unwound due to the activity of a helix destabilizing protein. This is followed by renaturation of the ss acceptor DNA with one of the unwound donor DNA strands. In order to test if adenovirus DBP is capable of performing strand exchange, the unwinding and renaturation properties of DBP were tested using artificial adenovirus origin DNA templates.

Helix destabilization by DBP was tested with an adenovirus origin containing a 50 nt partially ds DNA template, TDΔ15, containing 15 nucleotides of ssDNA to allow optimal DBP binding to the template (38). Unwinding of the partially ds TDΔ15 template was tested using increasing amounts of DBP (Fig. 1A). As a control, end labeled dsDNA TDΔ15 template is present in lane 1. To indicate the position of the individual single strands, which represent fully unwound TDΔ15, the template was heat denatured and quickly cooled on ice (lane 2). When increasing amounts of DBP were incubated with TDΔ15 an increase in unwinding activity was detected (Fig. 1A lanes 3-7). Accumulation of the ss products is visible at higher DBP concentrations.

To determine if a ssDNA region is required for unwinding of a dsDNA probe by DBP and in addition, to determine if unwinding is sequence dependent, an analogous experiment was performed with AT30 template. The 30nt dsDNA template and the 30nt ssDNA fragments are shown in Fig. 1B lanes 1 and 2, respectively. Various DBP concentrations were incubated with AT30 showing an accumulation of ssDNA products with increasing amounts of DBP (Fig. 1B lanes 3-7). From these results

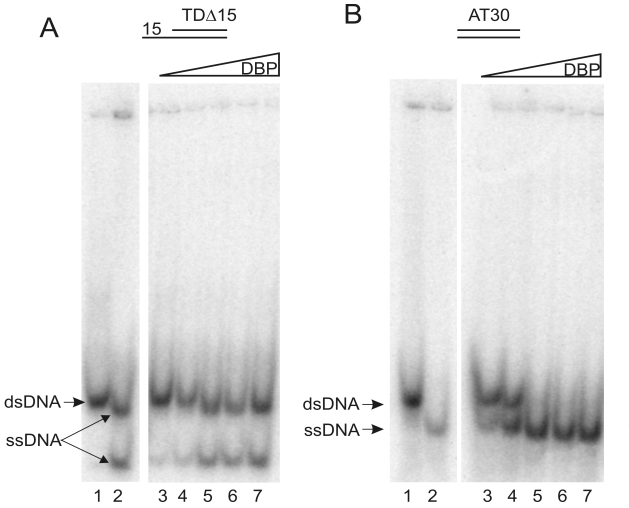

Figure 1: Unwinding assay at 30°C of dsDNA templates with DBP on TDΔ15 (**panel A**) and AT30 (**panel B**). The positions of (partially) ds- and unwound- DNAs are shown in lane 1 (dsDNA template) and lane 2, (template boiled at 100°C), respectively. Lanes 3 to 7; 6.25, 12.5, 25, 50 and 100 ng of DBP were added to the reaction mixture.

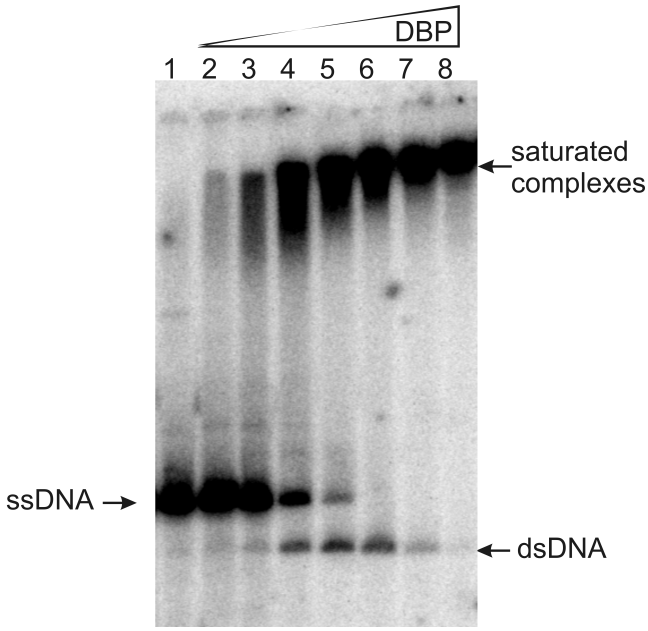

Figure 2: Renaturation assay with DBP on an 114bp boiled dsDNA fragment containing the adenovirus origin. Lane 1; DNA only. Lanes 2-8; 0.08, 0.16, 0.32, 0.64, 1.25, 2.5, 5 ng DBP. Maximum renaturation is found at 1.25 ng of DBP (lane 6). Saturated complexes consits of ssDNA bound cooperatively by DBP and, when the K_d for dsDNA binding has been reached, dsDNA is bound by DBP as well.

we can conclude that DBP can efficiently unwind fully dsDNA and in addition, unwinding of dsDNA by DBP is sequence independent. Interestingly, DBP can unwind the AT30 template with a higher efficiency than that of TDΔ15. Most likely this is due to the lower melting temperature of AT30.

DBP stimulates renaturation

An additional activity required for strand exchange is the ability of a protein to stimulate the renaturation of two homologous ssDNA strands. In the renaturation assay a 114 nt dsDNA fragment is boiled and cooled on ice to obtain two complementary ssDNA strands. DBP was added in various concentrations to the ssDNA in the reaction mixture and the formation of renaturation products was compared with a reaction without DBP (Fig. 2). Several products migrating with distinct mobilities are visible. Since the assay is performed under non-denaturing conditions, the dsDNA renaturation products migrate with the highest mobility (Fig. 2, lanes 3-7), whereas the ssDNA probe is visible at a slightly lower mobility (Fig. 2, lane 1). Second, complexes consisting of DBP complexed to ssDNA are also visible (Fig 2. lanes 2-8). Formation of the dsDNA renaturation product is DBP concentration dependent. Optimal renaturation is obtained at saturating DBP concentrations (Fig. 2, lanes 5 and 6). At these concentrations DBP is not bound to the dsDNA product due to its lower binding affinity for dsDNA than for ssDNA. At higher DBP concentrations

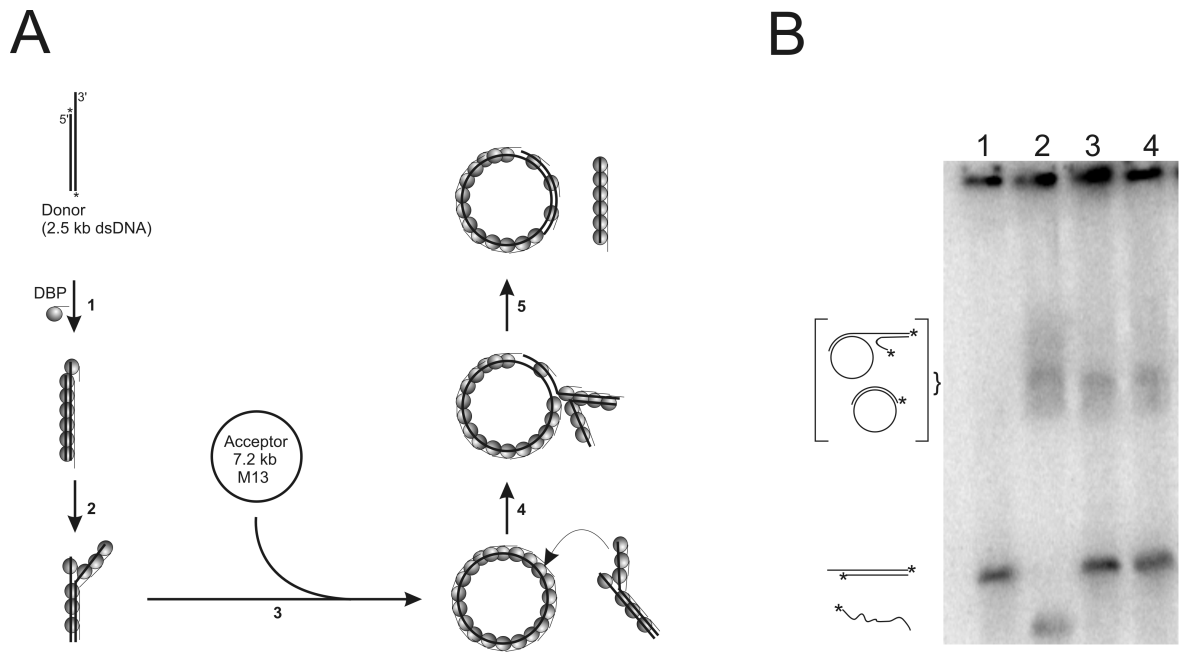

Figure 3: A. Schematic representation of the strand exchange reaction. **1;** The 2.5 kb ds M13 DNA fragment with a 9 nt 3'-overhang (Donor) is incubated with DBP. **2;** DBP partially unwinds the dsDNA donor. **3;** The 7.2 kb acceptor M13 ssDNA is added to the reaction and becomes bound by DBP. **4;** The ssDNA region complementary to that of the acceptor renatures. For clarity only one DBP molecule on the newly formed dsDNA duplex is drawn. **5;** Products formed after complete strand transfer. **B.** *In vitro* strand exchange assay. Lane 1; Linear duplex 2.5 kb M13 donor DNA. Lane 2; Products formed by gradual cooling of denatured dsDNA (donor) and circular 7.2 kb M13 ssDNA (acceptor). Lane 3; Complete reaction with 1 μg DBP. Lane 4; Complete reaction with 1 μg DBP and 10 μg BSA.

the K_d for dsDNA binding by DBP is reached and a dsDNA-DBP complex will be formed running at the same mobility as the ssDNA-DBP complex (Fig. 2, lanes 6 to 8). From these data we can conclude that DBP is functional in stimulating the renaturation of two complementary ssDNA products, which is in agreement with previously published results (34).

Strand exchange is mediated by DBP

To function in recombination, DBP has to be able to unwind a dsDNA duplex as well as stimulate renaturation of the two complementary ssDNA strands simultaneously. A recombination assay was recently developed by (18) for HSV-1 SSB ICP8. To examine the putative role of DBP in strand exchange, we performed similar experiments for DBP. An *in vitro* strand exchange reaction was performed with labeled 2.5 kb ds-M13 DNA, which serves as a donor, providing ssDNA with a sequence complementary to the circular 7.2 kb M13 ssDNA that serves as an acceptor. End-labeled donor DNA was preincubated with 1 μg of DBP, followed by addition of the ss acceptor DNA. The amount of 1 μg DBP was selected because this was shown to be the optimal concentration at which DBP acts in DNA replication (26). As a control for strand exchange product formation, donor DNA and acceptor DNA were mixed and heat denatured and gradually cooled in order to obtain strand exchange products (Fig. 3B, lane 2). The products of the complete reaction in the presence of DBP are shown in Fig. 3B,

lanes 3 and 4 and exhibit a mobility identical to those formed by gradual cooling of denatured ds and ss DNA (Fig. 3B, lane 2). Substrates and reaction products are schematically indicated in Fig. 3A. They represent hybrids between the 7.2 kb M13 acceptor ssDNA and the 2.5 kb (partially) unwound donor dsDNA. The ssDNA reaction product that remains after complete unwinding, which is visible in lane 2 can not be detected in lanes 3 and 4, possibly due to the low amount that is formed in the reactions (Fig. 3B).

From these results we can conclude that DBP plays an active role in strand exchange reactions and thus in homologous recombination. In order to examine additional factors that may influence the efficiency of strand exchange in the presence of DBP, several variations in reaction conditions were tested (Fig. 4). First, the level of spontaneous strand exchange was determined. Donor and acceptor DNAs were incubated in the absence of DBP. No strand exchange products were observed (Fig. 4, lane 1), indicating that no spontaneous strand exchange takes place under the reaction conditions used. In order to verify any effect of non-specific protein interactions on strand exchange, an excess of BSA was added to the ssDNA and dsDNA mixture, similar to the reaction in lane 1. Again no strand exchange products are formed (Fig. 4, lane 2). To determine if a two-fold higher concentration of DBP would enhance the formation of strand exchange products in a linear fashion, 1μg and 2μg of DBP were tested in the complete reaction (Fig. 4, lanes 3 and 4). No stimulating or inhibiting effect was detected when the concentration was raised, indicating that saturating amounts of DBP are used in the reactions. Furthermore, we also

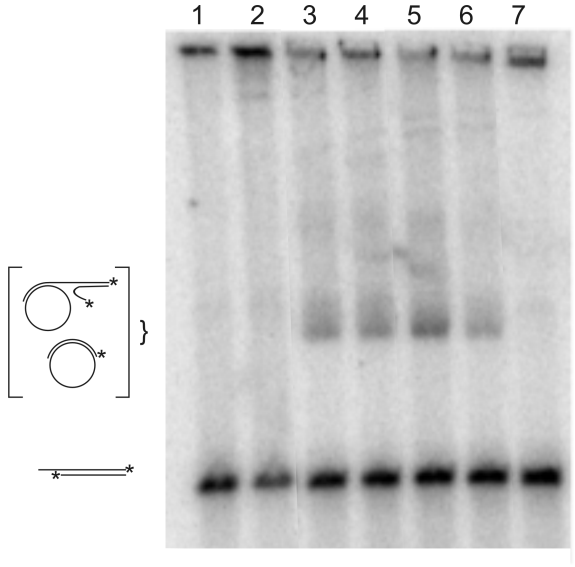

Figure 4: Strand exchange assay. Lane 1; Reaction without DBP. Lane 2; Reaction with 10 μg BSA and without DBP. Lane 3; Complete reaction with 1 μg DBP. Lane 4; Complete reaction with 2 μg DBP. Lane 5; Complete reaction after preincubation with $MgCl_2$. Lane 6; Complete reaction at room temperature. Lane 7; Complete reaction with 2 μg heat denatured DBP.

tested 500 ng of DBP and this concentration was still saturating (data not shown). Under the reaction conditions used, Mg^{2+} ions are only present after addition of the acceptor DNA to stabilize the renaturation products. Because Mg^{2+} ions are also known to stabilize dsDNA conformation and inhibit dsDNA unwinding(37), we determined if addition of $MgCl_2$ during the complete reaction would inhibit strand exchange by DBP (Fig. 4, lane 5). Surprisingly, strand exchange was not inhibited by $MgCl_2$, but in fact was stimulated 1.4-fold when $MgCl_2$ was present during the whole reaction. Apparently, inhibition of unwinding and stimulation of the renaturation by $MgCl_2$ is in favor of the strand exchange reaction. This suggests that the unwinding- and renaturation activities of DBP are coupled. To test if unwinding could be inhibited when lower reaction

temperatures were used, incubation at room temperature was compared with incubation at 37^{o} (Fig. 4, lane 6). Reactions at room temperature reduced the amount of strand exchange products to 70%, suggesting that unwinding of the donor DNA by DBP is required for strand exchange. Finally, when DBP was heat inactivated before it was supplemented to the reaction mix, no strand exchange products are visible (Fig. 4, lane 7), suggesting that DBP is responsible for the formation of the strand exchange products.

Discussion

The strand exchange reaction is an important step in homologous recombination. During this step a dsDNA region called the donor DNA is unwound. Subsequently, the formed ssDNA strand "searches" for its complementary region, called the acceptor, and renatures to a heteroduplex. Functional requirements for recombination proteins should include the ability to unwind or destabilize dsDNA regions as well as the ability to promote pairing between two complementary DNA regions. These two functions can be executed by two separate proteins within an organism as was shown for bacteriophage T7 proteins T7 gp4 (helicase-primase) and T7 gp2.5 (SSB) (10). Similarly, the HSV-1 protein ICP8 together with the HSV helicase-primase can exert efficient strand exchange. In the absence of the HSV helicase-primase, ICP8 is still able to stimulate strand exchange, although with a lower efficiency (19)

In this paper we show that the adenovirus type 5 DNA binding protein is also functional in strand exchange. In Fig. 1 we demonstrate that DBP can catalyze the unwinding of dsDNA. In addition, DBP can promote the renaturation of two complementary ssDNA strands (Fig. 2). Although these two activities are in contrast to each other, an equilibrium should exist for both unwinding and renaturation. They can complement each other to facilitate strand exchange, which requires both activities to be exerted simultaneously. We have demonstrated in an *in vitro* strand exchange assay that DBP is active in unwinding a 2.5 kb donor dsDNA fragment, while at the same time it facilitates the renaturation of the unwound product with the 7.2 kb acceptor ssDNA (Fig. 3 and 4).

Previously, Young proposed two models for homologous recombination in adenovirus (30). In one model a pool of displaced strands, produced by the adenovirus DNA replication machinery, is available for recombination. In this model recombination can only take place after one complete round of replication. However, it was shown that recombination already takes place at the onset of replication, rendering this model rather unlikely. The other model suggests that DNA replication starts from the molecular ends of the adenovirus genome and produces ssDNA of one duplex, the donor, that invades a second non-replicating duplex (recipient). Based on our results that DBP acts in strand exchange, we favor the latter model. Figure 5 illustrates a model for adenovirus recombination and the function of DBP in the various steps. A single strand DNA displaced by DBP during replication of one duplex, the donor, invades a nonreplicating duplex, the acceptor. In this model replication and recombination are linked processes. In addition, the model is supported by the observation that polarity exists i.e. there is a gradient of recombination from the ends of the adenovirus genome (15).

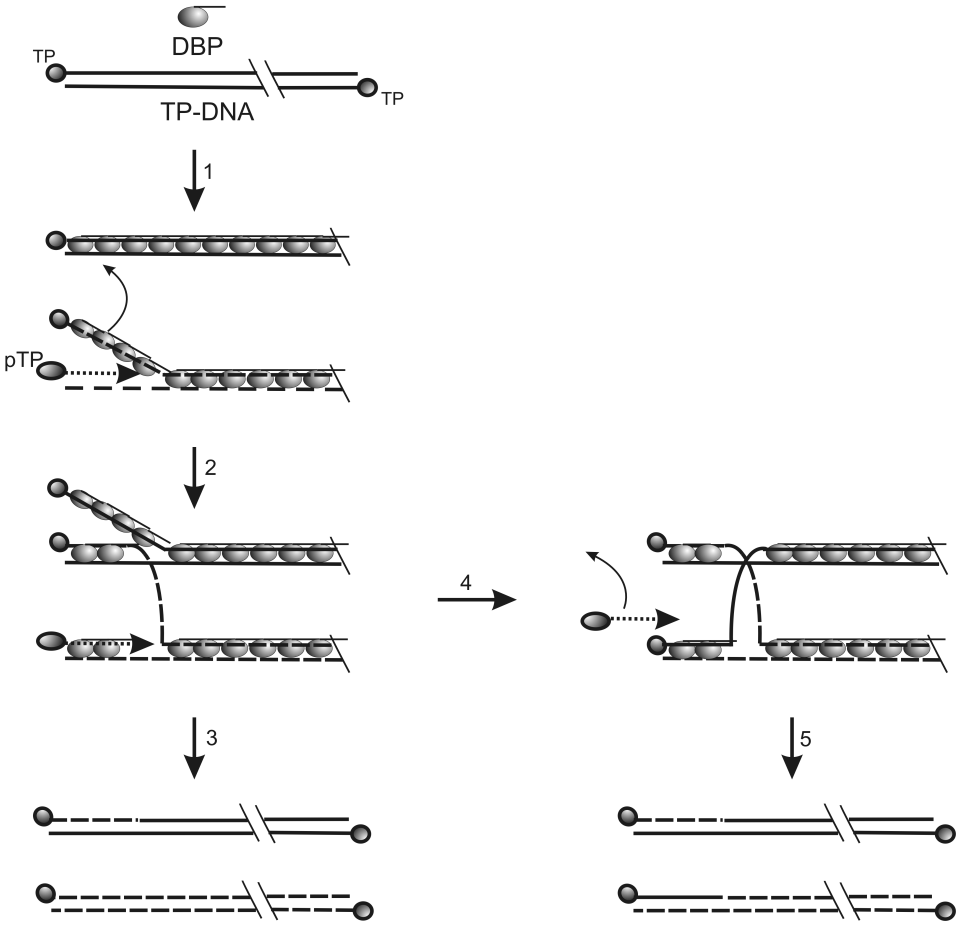

Figure 5: Model for DBP function in adenovirus recombination, based on the model presented by Young (30). The 36kb ds genomic DNA of adenovirus is represented. TP (terminal protein) is covalently attached to the 5'-ends of each strand. The dotted line with the rightward pointing arrow indicates the direction of replication. **1;** All DNA is bound by DBP. **2;** A single strand DNA displaced by DBP during replication of one duplex, the donor (dashed line) invades a nonreplicating duplex, the acceptor (black line). **3;** The single strands from both donor and acceptor are cut and rejoined by DNA ligase. Two end products are formed in which only one duplex has recombined. **4;** Alternatively, the strand that was displaced as a result of the strand invasion can in turn invade and displace the replicating strand of the donor, and a Holliday structure is formed. **5;** Resolving the Holliday structure results in the classic single-strand crossover products.

Although adenovirus DBP is capable of strand exchange, the *in vitro* efficiency of this reaction is around 15%. This is similar to what has been found in related systems. For example, in HSV-1 the efficiency observed for strand exchange by ICP8 was calculated between 10% and 20% (20). This activity could be enhanced to almost 90% when HSV helicase-primase was added to the reaction. Often a protein with helicase activity is implicated in homologous recombination. E.g. the *E. coli* recombination protein RecA uses the branch migration enzymes RuvAB and RecG, which exhibit helicase activity, for homologous recombination (13, 14). Similarly, in bacteriophage T4 branch migration is catalyzed by the SSB gp32 that functions together with the replicative helicase gp41 (1, 23).

Adenovirus type 5 does not encode a helicase, therefore unwinding of dsDNA depends on DBP alone or alternatively an additional viral or cellular protein is required. Although we cannot exclude

the activity of an additional protein *in vivo,* other explanations are also possible. For example, adenovirus DNA replication requires unwinding of large regions of dsDNA and this is very efficient without a helicase (3, 4). DBP and adenovirus polymerase together can unwind and replicate products up to 5 kb. Furthermore, it was demonstrated that DBP alone can only unwind dsDNA of up to 200 nt long (36). Therefore, we suggest that for the unwinding of larger regions of dsDNA during replication, DBP acts in concert with DNA polymerase. Possibly, a similar mechanism for unwinding large dsDNA region applies to the strand exchange reaction. The possibility of such a mechanism is subject for future investigations.

The addition of $MgCl_2$ during the unwinding step in the strand exchange assay stimulated product formation 1.4-fold. In addition, it was previously shown that unwinding of dsDNA by DBP is inhibited upon addition of $MgCl_2$, whereas renaturation is stimulated (33, 35). The strand exchange reaction does not seem to be affected by addition of $MgCl_2$, possibly unwinding plays a subordinate role when compared to renaturation in the

strand exchange reaction. This is in contrast to the observations of Nimonkar et al, (21), who showed that $MgCl_2$ inhibits strand exchange by ICP8. The reason for this difference remains to be investigated. Possibly, the balance between the unwinding and renaturation properties of DBP are less dependent on $MgCl_2$ than for ICP8.

A detailed understanding of the mechanism of adenovirus recombination is important because, adenovirus has been implicated as a potent vector for gene therapy. It is relatively easy to obtain high titers in cell cultures, adenoviruses have a broad host range and they can infect both dividing and non-dividing cells (28). For gene therapy one has to use a recombinant adenovirus that is replication incompetent. One of the risks may be that recombinant adenovirus can become replication competent again once the host cells are also infected with wild-type adenovirus. Homologous recombination between wild-type and replication deficient adenovirus could result in viable viruses.

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5

General Discussion

General discussion

In this thesis we have reported and discussed new results on the function of DBP in adenovirus DNA replication and recombination. Some "old" questions on the function of DBP have been solved. The function of DBP in initiation (Chapter 3) is to modulate the DNA structure in such a way that polymerase can bind better to its origin of replication, thereby stimulating initiation of replication. A longstanding question about the putative function of DBP in recombination has now been addressed (Chapter 4). DBP is required for the strand-exchange reaction in the recombination event. Still, new questions have surfaced over the past years that could not be answered yet. This chapter will discuss new areas of research on the functions of DBP.

DBP protein interaction with pol might play a role in DNA replication

One subject for investigation on the function of DBP in adenovirus DNA replication has been the possibility of a protein-protein interaction between DBP and polymerase. Several indications for such an interaction are available. Upon purification of adenovirus type 4 DNA polymerase co-elution of adenovirus type 4 DBP is observed (13). Also, the sensitivity of pol for inhibitors is increased in the presence of DBP (10). Finally, DBP cannot be replaced in adenovirus DNA replication by another SSB (2). Although these observations suggest that an interaction between DBP and pol exists, evidence for a direct protein-protein interaction between DBP and pol has never been obtained. We have tried to investigate this direct protein-protein interaction employing immunoprecipitation- (IP) and ELISA assays (van Breukelen et al., unpublished results) however were unable to demonstrate an interaction between DBP and pol in these assays. This implies that although evidence suggest that such an interaction exists it is not detectable using the conventional assays for direct interactions. A logical explanation could be that the interaction between DBP and pol might be transient or weak rendering it difficult to detect in our assays. Alternative methods will have to be used to address this question in the future.

One method to investigate the putative DBP-pol interaction is to determine a protein interaction domain with pol on the DBP surface, applying mutagenesis studies. Although this seems to be straightforward, one lacks the readout to determine if such an interaction is indeed affected. Since no interaction between DBP and pol can be detected in an IP, a mutation which shows an effect in adenovirus DNA replication cannot be correlated to a loss of interaction. Therefore it is virtually impossible to discriminate between an effect due to the loss of protein interaction between DBP and pol or due to altered function of DBP. A way to circumvent this problem is to find other interaction partners for DBP. Recently, an interaction between DBP and the SNF2-Related CBP activator protein (SrCap) has been reported. SrCap is a member of the SNF2 protein family of

DNA-dependent ATPases and functions amongst other in chromatin remodeling, DNA repair and regulation of transcription. DBP binds to and inactivates SrCap thereby inhibiting SrCap mediated transcription in the host cell (15).

In addition, an interaction between DBP and the adeno associated virus (AAV) Rep68 protein has been demonstrated in our laboratory (van Breukelen et al., and Weitzman et al., unpublished results). Rep68 binds to its Rep recognition site in the AAV origin of replication and facilitates unwinding with its helicase activity. In AAV, DBP stimulates the processivity of AAV DNA replication. Possibly Rep68 recruits DBP to its origin of replication to stimulate DNA replication.

These two proteins, SrCap and Rep68, are good candidates to map the region on the surface of DBP responsible for protein-protein interaction. Of course, for this setup we assume that the protein interactions with DBP are located within a specific domain of the protein. At present, mutagenesis studies with deletion mutants of DBP have been initiated in our laboratory. We have constructed four N-terminal deletion mutants as well as 1 C-terminal deletion mutant. Adenovirus DBP contains 7 alpha helices, A to G. The first four helices were determined to be accessible for protein interactions while the other helices were either involved in the DBP protein-protein chain formation (helices E and G) or buried (helix F). For each N-terminal deletion mutant an increasing number of helices were deleted, e.g. A, AB, ABC and ABCD.

Our data suggest that the core DNA binding domain of DBP between aa. 174 and 512 is involved in the DBP-Rep68 protein-protein interaction. In addition preliminary data on the deletion mutagenesis show that the region between aa. 228 and 251 is responsible for the protein-protein interaction with Rep68 (van Breukelen et al., unpublished results). After determination of this protein-protein interaction region on DBP, specific mutations disrupting this interaction can be designed. These mutants can be tested for their integrity in DNA binding (EMSA) and helix destabilization assays. Subsequently they can be used in the *in vitro* replication assay to determine their phenotype.

Possibly new insights on the function of DBP in DNA replication will arise as well. These mutants might well show that, in addition to the published functions of DBP in unwinding the dsDNA helix at the replication fork (Chapter 2), an additional interaction between DBP and pol is required. This might explain why DBP cannot be replaced by another SSB during elongation. Several reports regarding SSB protein interactions with other replication proteins have been presented for other systems (4, 9, 12, 16). In Chapter 1 we have discussed several of such SSBs and their interactions with other proteins in DNA replication.

DBP in adenovirus recombination

We have barely scratched the surface for the function of DBP and the other replication proteins in adenovirus recombination. In Chapter 4, we demonstrate that DBP plays a role in the strand exchange step of homologous recombination. Although DBP is required for strand exchange, it is not very efficient by itself in vitro. Future investigations could focus on additional factors that stimulate the strand exchange reaction. For the herpes

simplex virus (HSV) the function of its SSB, ICP8, in strand exchange has been described (11). Strand exchange was shown to be sub-optimal when only ICP8 was used. However, when in addition to ICP8 also the HSV helicase was added to the strand exchange reaction, efficient strand exchange was observed (11). A similar mechanism might also apply to adenovirus. Adenovirus does not encode for a helicase and in addition, a helicase is not required for DNA replication. For DNA replication *in vitro* only DBP, pTP and pol are required. Unwinding of the 5kb dsDNA fragments during DNA replication is performed by DBP alone. This is remarkable since it was shown in an *in vitro* helix destabilization assay that DBP unwinds up to 200bp at the most (17). This observation suggests that DBP can unwind longer products when it acts in concert with pol. We therefore suggest a similar mechanism for unwinding in the strand exchange reaction. However, we cannot exclude that the host cell provides an additional protein required for optimal strand exchange, e.g. a cellular helicase.

In Chapter 4 we have demonstrated that the unwinding and renaturation properties of DBP are required for strand exchange. Especially the unwinding activity of DBP plays an important role in adenovirus DNA replication. It would be interesting to find DBP mutants that are defective in strand exchange while its function in DNA replication remains intact. Such DBP mutants could provide useful information on the *in vivo* function of DBP in adenovirus recombination. In addition, adenoviruses defective in recombination are interesting vectors for gene therapy. A risk in gene therapy is that recombinant adenovirus can become replication competent again once the host cells are also infected with wild-type adenovirus. Homologous recombination between wild-type and replication deficient adenovirus could result in viable viruses again. Therefore detailed information on adenovirus recombination might provide useful new strategies for adenovirus vector design.

Final remarks: Functional overlap between various SSBs

Many organisms encode SSB proteins. These proteins are generally used to destabilize ssDNA and protect ssDNA strands against degradation by nucleases and therefore often play a role in DNA replication and recombination where ssDNA products are generally formed.

Adenovirus DBP has been the subject of research for many years. The function of DBP in unwinding and renaturation finds its similarities with SSBs originating form various sources. The similarities in functions of the various SSBs might suggest that they are able to substitute each other in several processes. Indeed this has been observed for example with RPA in the stimulation of SV40 large T antigen mediated DNA unwinding. In this reaction RPA can be replaced by other SSBs, including *E. coli* SSB, HSV ICP8 and DBP, suggesting that the function of RPA in this step is stabilization of the produces ssDNA regions (1, 3, 14). In Chapter 1 similarities among SSBs have been discussed extensively. In addition in Chapter 4 another example of SSBs performing similar activities is presented. In this chapter we propose a function for DBP in strand exchange that finds it similarities in mechanisms that have been published for HSV (11), Bacteriophage T7 and bacteriophage T4 (6-8). It may well be that although SSBs

are very dissimilar, structural similar domains exists. In Chapter 1 an example of such a structure, the OB-fold is presented. The OB-fold has been described as a DNA binding domain which is often found in SSB proteins.

It should be noted that absolute complementation between SSBs from different organisms is not observed. In addition some processes require specific protein interactions and therefore even closely related SSBs do not substitute each other, e.g. hRP-A cannot be substituted by other SSBs in stimulating pol α mediated chain elongation (5). It can be concluded that, although substitution is generally not possible when specific protein interactions are required, detailed knowledge on the structure and function of DBP might provide useful new insights and tools for the research on other SSB.

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Summary

Summary

Replication of adenovirus DNA in infected cells is an efficient process that, compared to cellular replication, has the use of a protein primer as a hallmark. The mechanism of this DNA replication process and especially the role of one of the replication proteins, the DNA binding protein DBP, is the main subject of this thesis.

Adenovirus DNA replication can be reconstituted *in vitr*o, using three viral proteins, adenovirus DNA polymerase (pol), precursor terminal protein (pTP), and DBP. Optimal replication efficiency is obtained when two cellular transcription factors are added, nuclear factor I (NFI) and Oct-1. The adenoviral dsDNA genome contains two terminal proteins (TP) covalently linked to the 5' ends. The inverted terminal repeats contain the origins of replication. pTP and pol are tightly associated in solution. During initiation of replication pTP functions as a primer to which the first nucleotide, dCTP, is covalently coupled. Both NFI and Oct-1 stimulate the initiation by recruiting the pTP-pol complex to the origin of replication. Initiation starts opposite position 4 of the template strand. After formation of a pTP-trinucleotide (pTP-CAT), the complex jumps back and CAT becomes paired with template residues 1–3. Shortly after jumping back, the polymerase dissociates from pTP and elongation proceeds via strand displacement.

The adenovirus DNA binding protein is an important player in adenovirus DNA replication, where it serves multiple functions. In the first step of adenovirus DNA replication DBP stimulates the coupling of the first nucleotide to pTP. Also binding of NFI to the origin is stimulated by DBP. Subsequently, during elongation DBP unwinds the dsDNA ahead of pol and removes secondary structures. Adenovirus DBP binds with high affinity and cooperativity to ssDNA, whereas binding to dsDNA is non-cooperative and with lower affinity. These differences in binding affinity are the driving force for dsDNA unwinding which is required for processive DNA chain elongation by pol.

Function of DBP in elongation of adenovirus DNA replication

DBP is a 529 amino acids long protein with a molecular weight of 59 kD. The structure of adenovirus DBP possesses a remarkable feature; the C-terminal arm (aa. 512-529), which is important for the cooperative binding of DBP to another DBP molecule. In addition, the C-terminal arm is flexible and can rotate around a fixed point, called the hinge-region (aa. 512-515). In Chapter 2 we present data on the function of the flexibility of the C-terminal arm of DBP and we discuss the implications of an altered flexibility on adenovirus DNA replication by mutating several aa in the hinge-region. We have demonstrated that unwinding of dsDNA was severely affected when the flexibility of the C-terminal arm was reduced. Surprisingly, binding of DBP to both dsDNA and ssDNA was not affected and binding of DBP to ssDNA was still cooperative. This led to the hypothesis that unwinding involves a conformational transition of DBP. It is at the replication fork that DBP has to switch from dsDNA binding to ssDNA binding thereby destabilizing the dsDNA duplex. We propose that the transition between dsDNA binding and ssDNA binding requires the flexibility of the C-terminal arm of DBP. Possibly the C-terminal arm

of a DBP molecule that is bound to the ssDNA part of the replication fork cannot accommodate the orientation that is required to bind to a DBP molecule on the dsDNA part of the replication fork. Eventually the inability of DBP to accommodate this transition would lead to a collapse of the replication fork, thereby stalling DNA replication.

Function of DBP in initiation of adenovirus DNA replication

An additional role of DBP in DNA replication is its ability to stimulate initiation of DNA replication. Previously it was demonstrated that DBP stimulates the initiation via lowering the K_m for the incorporation of the first nucleotide by DNA polymerase. Several suggestions were postulated to explain the role of DBP in stimulation of initiation, ranging from a protein-protein interaction between DBP and polymerase to an indirect effect of DBP on polymerase that might be mediated via the DNA template. The possibility of a direct interaction of DBP with polymerase was suggested because DBP cannot be replaced by a SSB originating from other sources in an *in vitro* DNA replication assay. Also, polymerase becomes more sensitive to inhibitors in the presence of DBP. In addition, when polymerase of adenovirus type 4 is purified, co-elution of adenovirus type 4 DBP is observed. In our study to explain the mechanism involved in the stimulation of initiation by DBP the possibility of a protein-protein interaction with DBP and DNA polymerase was investigated into more detail. Although indirect evidence suggest that such an interaction must exist, attempts to detect a DBP protein interaction employing immunoprecipitation assays and ELISA, were unsuccessful. This suggests that if such an interaction of DBP with pol exists, it is transient or weak.

In Chapter 3, we present data on the function of DBP in the stimulation of initiation. In contrast to a direct protein-protein interaction of DBP with pol we demonstrate that DBP stimulates the binding of pol to the dsDNA origin. We assume that the structure of the dsDNA template is changed by DBP in such a way that polymerase binds more efficiently to the dsDNA. This observation shows resemblance with the previously published mechanism of the stimulation by DBP of NFI binding to dsDNA. Stimulation of pol binding to dsDNA by DBP was shown to be sequence non-specific.

This does not pose a problem *in vivo,* because the specificity for pol binding to the origin is determined by three proteins: pTP, NFI and Oct-1. After recruitment of pol to the origin of DNA replication by NFI and Oct-1 specificity is not required anymore and both proteins dissociate.

Function of DBP in adenovirus recombination

Adenovirus homologous recombination and DNA replication are functionally interrelated processes, as was demonstrated with several adenovirus replication mutants and inhibitors of adenovirus DNA replication. The interrelation between recombination and replication makes it difficult to study adenovirus recombination separately. Already for many years a role for the adenovirus DBP in homologous recombination was suggested. This was based on the fact that DBP has two intrinsic activities, helix destabilization and renaturation. Both properties were shown to play an important role in the strand exchange reaction of homologous

recombination in other viral systems. Using an *in vitro* strand exchange assay, it was possible to study the role of adenovirus DBP in homologous recombination. In Chapter 4 we demonstrate that DBP functions in unwinding of the dsDNA donor and that it has an additional role in renaturation of the unwound donor with the complementary acceptor ssDNA. For the first time we were able to demonstrate a direct role for an adenovirus replication protein in homologous recombination. Although DBP seems to play an important role in the strand exchange mechanism, it is not very efficient *in vitro* since only a low amount of strand exchange products are formed. We suggest that for optimal strand exchange activity DBP functions together with another protein, such as a helicase or perhaps the adenovirus DNA polymerase itself. A similar mechanism has been demonstrated recently for the Herpes Simplex Virus SSB, ICP8, which requires the aid of a virally encoded helicase/primase for optimal strand exchange activity.

In Chapter 5 new questions, that have surfaced as a result of the research presented in this thesis, are discussed.

Samenvatting

Samenvatting

De verdubbeling (replicatie) van adenovirus DNA in geïnfecteerde cellen is een zeer efficiënt proces dat zich ten opzichte van de cellulaire replicatie kenmerkt door het gebruik van een eiwit als primer. Het mechanisme van dit DNA replicatie proces en met name van een van de virale eiwitten, het DNA bindend eiwit DBP, vormt het onderwerp van dit proefschrift.

Adenovirus DNA replicatie kan gereconstrueerd worden met behulp van drie virale eiwitten, het adenovirus DNA polymerase (pol), de precursor van het terminaal eiwit (pTP) en het DNA bindend eiwit (DBP). Optimale DNA replicatie wordt echter pas bereikt als er nog twee cellulaire transcriptie factoren worden gebruikt die afkomstig zijn van de gastheer, nuclear factor I (NFI) en Oct-1. Aan ieder 5' uiteinde van het adenovirus DNA, het genoom, zit het terminale eiwit (TP) covalent gekoppeld. De beide uiteinden van het genoom bevatten identieke DNA sequenties die de startpunten (origins) voor DNA replicatie bevatten. pTP en pol hebben een hoge affiniteit voor elkaar en vormen al in de oplossing een sterk eiwit complex. Tijdens de initiatie van de replicatie fungeert pTP als beginpunt (primer) waaraan het eerste nucleotide covalent wordt gekoppeld. Zowel NFI en Oct-1 stimuleren de initiatie van de DNA replicatie, omdat zij het pTP-pol complex naar de origins voor DNA replicatie brengen. De initiatie van adenovirus DNA replicatie start niet aan het begin van het genoom maar begint tegenover het vierde nucleotide van de te kopiëren DNA streng. Nadat drie nucleotiden aan het pTP eiwit gekoppeld zijn (pTP-CAT), springt het complex terug naar het begin van het adenovirus genoom. Kort hierna dissocieert het pTP van pol en wordt het pTP-CAT product verder verlengd (elongatie).

Het adenovirus DNA bindend eiwit (DBP) speelt een belangrijke rol in adenovirus DNA replicatie, waar het meerdere functies heeft. In de eerste stap van DNA replicatie, de initiatie, stimuleert DBP de koppeling van het eerste nucleotide aan pTP. Tevens wordt het binden van NFI aan het DNA van de origin gestimuleerd door DBP. Vervolgens zorgt DBP ervoor dat tijdens de elongatie het dubbel-strengs (ds) DNA ontwonden wordt en secondaire structuren worden verwijderd. DBP bindt met hoge affiniteit en cooperativiteit aan enkel-strengs (ss) DNA. Daarentegen bindt DBP met een lage affiniteit en niet coöperatief aan dsDNA. Juist deze verschillen in affiniteit zijn de stuwende kracht voor ontwinden van het dsDNA. Het ontwinden van het dsDNA is van belang voor het processief kopiëren van het DNA door pol.

De functie van DBP in de elongatie fase van adenovirus DNA replicatie

DBP is een 539 aminozuren (aa) lang eiwit met een moleculair gewicht van 59 kD. De structuur van het adenovirus DBP bevat een opmerkelijke C-terminale arm (aa 512-529), welke van belang is voor het coöperatief binden van het ene DBP molecuul aan het andere DBP molecuul. Deze C-terminale arm is flexibel en kan roteren om een vast punt dat ook wel als een scharnier gezien kan worden en de aa 512-515 omvat. In het tweede hoofdstuk van dit proefschrift worden de uitkomsten van het onderzoek naar de functie van deze flexibiliteit beschreven. Tevens wordt het effect van

een veranderde flexibiliteit van de C-terminale arm op de DNA replicatie bediscussieerd. Door middel van het muteren van aminozuren in het scharniergebied laten wij zien dat de ontwinding van dsDNA ernstig wordt verstoord als de flexibiliteit van de C-terminale arm wordt gereduceerd. Verrassend was dat noch de binding van DBP aan ssDNA noch die aan dsDNA was verstoord en dat de binding van DBP aan ssDNA nog steeds coöperatief was. Deze waarnemingen hebben geleid tot de hypothese dat, tijdens het ontwinden van dsDNA DBP een conformationele verandering moet ondergaan. Het is namelijk tijdens de ontwinding dat het dsDNA overgaat in ssDNA. Op dit punt moet een DBP molecuul, dat op het dsDNA zit, binden aan een DBP molecuul dat op het ssDNA zit. De overgang van het binden aan dsDNA naar het binden aan ssDNA heeft vermoedelijk de flexibiliteit van de C-terminale arm van DBP nodig. Waarschijnlijk kan de C-terminale arm van DBP, dat gebonden zit aan het ssDNA, niet de juiste oriëntatie aannemen welke nodig is om een DBP molecuul te binden dat aan het dsDNA gebonden zit. Uiteindelijk zal het onvermogen van DBP om deze transitie uit te voeren leiden tot een verstoring van de replicatie vork, wat tot gevolg heeft dat de DNA replicatie vast loopt.

De functie van DBP in de initiatie van de adenovirus DNA replicatie

Een andere rol van DBP in DNA replicatie is zijn vermogen om de initiatie van de DNA replicatie te stimuleren. Eerder was al aangetoond dat DBP de initiatie stimuleert door een verlaging van de K_m voor de koppeling van het eerste nucleotide door pol. Meerdere suggesties, variërend van een eiwit-eiwit interactie tussen DBP en pol tot de stimulatie van de initiatie door een indirect effect van DBP op pol, werden geopperd om de rol van DBP in de stimulatie van initiatie te verklaren. Een aantal waarnemingen wijzen in de richting van een directe interactie tussen DBP en pol. Ten eerste kan DBP tijdens een *in vitro* DNA replicatie experiment, niet vervangen worden door een ssDNA bindend eiwit (SSB) afkomstig van andere bronnen. Ten tweede wordt pol meer ontvankelijk voor de werking van specifieke remmers in de aanwezigheid van DBP. En ten derde, als pol uit adenovirus type 4 wordt gezuiverd dan wordt adenovirus type 4 DBP meegezuiverd (coelutie). Aangezien deze aanwijzingen een DBP-pol interactie suggereren, hebben wij getracht om deze interactie te detecteren met onder andere het gebruik van immunoprecipitatie en ELISA proeven, echter zonder resultaat. Dit wijst er op dat, als er al een DBP-pol interactie bestaat, deze erg zwak is of van erg korte duur.

In het derde hoofdstuk laten we de uitkomsten zien van ons onderzoek naar de rol van DBP in de stimulatie van initiatie. In tegenstelling tot een directe interactie tussen pol en DBP laten wij zien dat DBP het binden van pol aan de origin stimuleert. Het is waarschijnlijk dat DBP de structuur van het dsDNA zodanig verandert dat pol efficiënter aan het DNA kan binden. Deze observatie vertoont overeenkomsten met de stimulerende rol van DBP bij binding van NFI aan dsDNA. Stimulatie van het binden van pol aan dsDNA bleek niet specifiek voor de sequentie van het origin dsDNA. *In vivo* vormt dit geen probleem omdat de specificiteit voor pol voor het binden aan de origin wordt

opgelegd door drie eiwitten, te weten pTP, NFI en Oct-1. Nadat pol gerekruteerd is naar de origin is deze specificiteit niet meer nodig en dissociëren NFI en Oct-1.

De functie van DBP in adenovirus recombinatie

Homologe recombinatie en DNA replicatie zijn in adenovirus aan elkaar gerelateerde processen. Dit was eerder aangetoond met verschillende mutanten voor adenovirus DNA replicatie en met remmers voor adenovirus DNA replicatie. Omdat deze twee processen gerelateerd zijn aan elkaar is het moeilijk om beide processen afzonderlijk te bestuderen. Sinds vele jaren wordt er al een rol voor DBP in homologe recombinatie gesuggereerd. Dit was gebaseerd op het feit dat DBP twee activiteiten heeft, het ontwinden van dsDNA alsmede het stimuleren van de renaturatie van twee complementaire DNA strengen. Deze twee activiteiten spelen ook een belangrijke rol in de uitwisselingsstap van twee DNA strengen (strand exchange) bij homologe recombinatie in andere virale systemen. Door gebruik te maken van *in vitro* strand exchange proeven was het mogelijk de rol van DBP in homologe recombinatie te bestuderen (hoofdstuk 4). We laten zien dat *in vitro* DBP functioneert in het ontwinden van een dsDNA donor en dat DBP de renaturatie van de ontwonden donor met een complementaire acceptor stimuleert. Dit toont voor het eerst een mogelijk directe rol voor DBP in adenovirus recombinatie. Hoewel DBP een belangrijke rol lijkt te spelen in de “strand exchange” wordt echter maar een kleine hoeveelheid product gevormd. Waarschijnlijk is er voor een optimale “strand exchange” een samenwerking van DBP met een ander eiwit nodig, zoals bijvoorbeeld met een helicase of misschien met het adenovirus DNA polymerase zelf. Een dergelijk mechanisme is recent aangetoond voor het Herpes Simplex Virus (HSV) DNA bindend eiwit, ICP8, dat samen met een door HSV gecodeerd helicase/primase eiwit voor een optimale “strand exchange” zorgt.

In hoofdstuk 5 worden nieuwe vragen die opgekomen zijn naar aanleiding van het onderzoek beschreven in dit proefschrift nader besproken.

Curriculum Vitae

Curriculum Vitae

Bastiaan van Breukelen (Bas), werd op 24 maart 1974 te Arnhem geboren. In 1992 behaalde hij het diploma VWO aan de Thomas à Kempis College te Arnhem. In dat zelfde jaar begon hij aan zijn studie bioprocestechnologie (T34) aan de toenmalige Landbouw Universiteit Wageningen, nu Wageningen University and Research Center, te Wageningen. Het diploma bioprocestechnologie met als specialicatie dierlijke cel technologie werd in 1997 behaald. In september 1997 begon hij als assistent in opleiding bij de vakgroep fysiologische chemie aan het universitair medisch centrum te Utrecht onder leiding van Prof. Dr. P.C. van der Vliet. Tijdens deze AIO periode heeft hij gewerkt aan het Adenovirus DNA bindend eiwit en de rol van dit eiwit in het Adenovirus DNA replicatie systeem. De resultaten van het gedurende deze periode uitgevoerde onderzoek zijn beschreven in dit proefschrift. Sinds januari 2003 is hij werkzaam als postdoc aan de vakgroep Biologie, afdeling moleculaire genetica, op het project met als titel; Systeem biologie van Arabidopsis thaliana: ontwikkeling van transcriptionele netwerken en regulatoire modellen onder begeleiding van Prof. Dr. P.J. Weisbeek.

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Dankwoord

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Bas