Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis

Job Dekker¹, Panagiotis N.Kanellopoulos²,
Ate K.Loonstra¹,
Joost A.W.M.van Oosterhout¹,
Kevin Leonard², Paul A.Tucker² and
Peter C.van der Vliet^{1,3}

¹Laboratory for Physiological Chemistry, Utrecht University,
Universiteitsweg 100, 3584 CG Utrecht, The Netherlands and

²Structural Biology Programme, European Molecular Biology
Laboratory, D-69012 Heidelberg, Germany

³Corresponding author

In contrast to other replication systems, adenovirus DNA replication does not require a DNA helicase to unwind the double-stranded template. Elongation is dependent on the adenovirus DNA-binding protein (DBP) which has helix-destabilizing properties. DBP binds cooperatively to single-stranded DNA (ssDNA) in a non-sequence-specific manner. The crystal structure of DBP shows that the protein has a C-terminal extension that hooks on to an adjacent monomer which results in the formation of long protein chains. We show that deletion of this C-terminal arm results in a monomeric protein. The mutant binds with a greatly reduced affinity to ssDNA. The deletion mutant still stimulates initiation of DNA replication like the intact DBP. This shows that a high affinity of DBP for ssDNA is not required for initiation. On a single-stranded template, elongation is also observed in the absence of DBP. Addition of DBP or the deletion mutant has no effect on elongation, although both proteins stimulate initiation on this template. Strand displacement synthesis on a double-stranded template is only observed in the presence of DBP. The mutant, however, does not support elongation on a double-stranded template. The unwinding activity of the mutant is highly reduced compared with intact DBP. These data suggest that protein chain formation by DBP and high affinity binding to the displaced strand drive the ATP-independent unwinding of the template during adenovirus DNA replication.

Keywords: adenovirus/DNA replication/DNA unwinding/
helix-destabilizing proteins

Introduction

The single-stranded DNA-binding protein DBP is expressed in large amounts in adenovirus-infected cells. DBP is essential for DNA replication (for reviews, see Chase and Williams, 1986; Hay *et al.*, 1995; van der Vliet, 1995) and is involved in many steps in the viral life cycle, like transformation (Ginsberg *et al.*, 1974), transcriptional control (e.g. Cleghon *et al.*, 1989; Zijderfeld *et al.*, 1994)

and assembly of the virus (Nicolas *et al.*, 1983). DBP binds cooperatively to single-stranded DNA (ssDNA) in a non-sequence-specific fashion. Binding to double-stranded DNA (dsDNA) is weak and not cooperative (Stuiver and van der Vliet, 1990). The protein can be cleaved into two parts by mild chymotrypsin treatment. The C-terminal part of DBP, amino acids 174–529, contains the DNA-binding domain and is sufficient to function in adenovirus DNA replication *in vitro* (Ariga *et al.*, 1980; Friefeld *et al.*, 1983; Tsernoglou *et al.*, 1985). The adenovirus DNA polymerase (pol) forms a complex with the precursor terminal protein (pTP) that serves as a primer during initiation. During initiation, the first nucleotide, a dCMP residue, is covalently coupled to pTP. Initiation is stimulated by two cellular transcription factors, nuclear factor I (NFI) and Oct-1 (for reviews, see van der Vliet, 1991, 1995). Initiation of replication starts at position 4 of the linear template, followed by formation of a trinucleotide intermediate, pTP-CAT (King and van der Vliet, 1994). This pTP-CAT intermediate jumps back to position 1–3 of the template strand. Elongation proceeds via a strand displacement mechanism.

DBP has several functions during adenovirus DNA replication. It stimulates initiation indirectly by increasing the binding of NFI (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990) to the origin and directly by lowering the K_m for dCTP (Mul and van der Vliet, 1993). Furthermore, DBP plays an essential role during elongation. DBP enhances the processivity of the polymerase (Lindenbaum *et al.*, 1986) and, by cooperative binding to the displaced strand, it protects it from nuclease digestion. Strand displacement synthesis does not require a helicase and is ATP independent (Pronk *et al.*, 1994). Since DBP has been shown to have helix-destabilizing properties (Monaghan *et al.*, 1994; Zijderfeld and van der Vliet, 1994), it has been suggested that DBP is required to unwind the double-stranded template. Finally, DBP enhances the renaturation of complementary displaced strands (Zijderfeld *et al.*, 1993).

The crystal structure of the C-terminal part of DBP (ΔN -DBP) has been solved (Tucker *et al.*, 1994; Kanellopoulos *et al.*, 1996) and shows that the protein is mainly globular with a 17 amino acid long C-terminal arm (Figure 1A). This arm hooks on to a second DBP monomer, which results in the formation of long protein chains. In the DBP-(dT)₁₆ crystal, DBP again forms long protein chains via the C-terminal hook (Kanellopoulos *et al.*, 1995, and in preparation). This hook is clearly not directly involved in DNA binding. In agreement with deductions based on the uncomplexed DBP structure, this preliminary analysis of the crystals of DBP in complex with (dT)₁₆ (Kanellopoulos *et al.*, 1995) shows that sheet 3 and the loop region containing residues 450–467 are in close proximity to electron density that can be interpreted

Fig. 1. (A) Crystal structure of the C-terminal fragment of DBP (Δ N-DBP, amino acids 174–529). This fragment is sufficient for cooperative DNA binding and DNA replication. Two monomers are shown that are bound to each other in the orientation found in crystal form II (Kanellopoulos *et al.*, 1996). The C-terminal arm of the first monomer hooks on to the hydrophobic cleft of the second monomer. This results in the formation of long protein chains. The ssDNA is thought to be wrapped around this chain. The 17 amino acid long C-terminal extension of the second monomer is indicated. (B) A schematic representation of the deletion mutants and a silver-stained SDS–polyacrylamide gel showing 50 ng of purified DBP, Δ N-DBP and Δ N Δ C-DBP. DBP was purified from adenovirus-infected HeLa cells; Δ N-DBP and Δ N Δ C-DBP were purified from Sf9 cells infected with recombinant baculoviruses. Some degradation products of DBP are visible.

as DNA (Kanellopoulos *et al.*, in preparation). This area of the protein contains several well conserved positively charged residues that may interact with the phosphate backbone. In the proximity of this sheet, several hydrophobic residues are found that can stack with the bases of the ssDNA.

In this study, we analyzed the requirement for multimerization of DBP for its function in adenovirus DNA replication. Deletion of the C-terminal arm abolished chain formation by DBP. Analysis of this deletion mutant provides evidence that oligomerization and, consequently, high affinity binding to ssDNA is required for unwinding double-stranded templates during strand displacement synthesis, whereas for initiation of replication monomeric DBP is sufficient. Since many helix-destabilizing proteins, like phage T4 gp32, herpes simplex virus ICP8 and phage ϕ 29 p5, form oligomers on DNA, multimerization and cooperative binding to DNA may be a more general mechanism to drive unwinding of DNA duplexes.

Results

The C-terminal arm of DBP is required for oligomerization

The crystal structure of DBP shows that oligomerization by DBP is caused by interactions between the 17 amino

acid long C-terminal arm and a hydrophobic pocket of the adjacent DBP molecule in the protein chain (Tucker *et al.*, 1994; Kanellopoulos *et al.*, 1996) (Figure 1A). To study whether chain formation is essential for the function of DBP, we expressed the intact DNA-binding domain (Δ N-DBP) and the DNA-binding domain with the C-terminal extension deleted (Δ N Δ C-DBP) using the baculovirus expression system. The proteins were purified to near homogeneity as shown in Figure 1B. The oligomeric state of the proteins was studied by electron microscopy using negatively stained specimens (Figure 2). The intact DBP and Δ N-DBP formed small aggregates. Deletion of the C-terminal arm abolished multimerization, and very small particles, probably corresponding to a distribution of monomeric protein molecules, were observed. DBP, Δ N-DBP and Δ N Δ C-DBP were also tested for their polydispersity in the absence of DNA by dynamic light scattering. In the case of intact DBP and Δ N-DBP, the photon counts were extremely high and no accurate estimation of the particle size or molecular weight could be made. This clearly indicates that these two proteins are polydisperse in solution. Δ N Δ C-DBP, however, is monomeric in solution, with the hydrodynamic radius of the particles in a concentrated protein solution corresponding to that expected for a monomer (not shown).

Deletion of the C-terminal extension reduces the affinity for single-stranded DNA

DNA binding was studied using a 114 nucleotide long ssDNA molecule (Figure 3). As expected, intact DBP and Δ N-DBP bound cooperatively to DNA with nearly identical affinities. The ssDNA was saturated with DBP and no intermediate complexes were detected. Deletion of the C-terminal extension resulted in a 100-fold reduction of the affinity for ssDNA. This protein also bound cooperatively since saturated ssDNA– Δ N Δ C-DBP complexes were detected without intermediate complexes. In contrast to the intact DBP, no binding of Δ N Δ C-DBP to dsDNA was observed (not shown). The C-terminal arm is involved in most protein–protein interactions between two adjacent monomers and therefore it was surprising that deletion of this arm did not abolish cooperative binding. This may indicate that upon DNA binding, protein–protein interactions that do not involve the C-terminal arm are formed as a result of structural changes in the DBP molecule or that these interactions are stabilized by DNA binding. It is clear, however, that deletion of the C-terminal arm abolishes chain formation in the absence of DNA and this results in a highly reduced affinity for ssDNA.

Δ N Δ C-DBP can perform some but not all functions of DBP during DNA replication

The effect of deletion of the C-terminal arm on the function of DBP was tested in an *in vitro* replication system containing purified pTP-pol and *Xho*I-digested Ad5 terminal protein containing DNA. As shown in Figure 4A, wild-type DBP supported the preferential labeling of the two origin-containing restriction fragments (indicated as the B and C fragments). Baculovirus-expressed Δ N-DBP also stimulated origin-specific replication, in agreement with earlier results obtained with proteolytic fragments of DBP containing the C-terminal domain (Ariga *et al.*, 1980; Friefeld *et al.*, 1983; Tsernoglou *et al.*,

Fig. 2. Oligomeric state of DBP, Δ N-DBP and Δ N Δ C-DBP studied by electron microscopy. DBP and Δ N-DBP form aggregates while Δ N Δ C-DBP is monomeric.

Fig. 3. Deletion of the C-terminal arm reduces the affinity for ssDNA without complete loss of cooperativity. A gel retardation assay was performed using a 114 nucleotide ssDNA probe. The highest concentrations of DBP, Δ N-DBP and Δ N Δ C-DBP were 44 (80 ng), 50 (50 ng) and 2108 nM (2000 ng) respectively. Proteins were diluted by successive 2-fold steps. The first lanes contained no protein. The percentage of free DNA was quantified and plotted.

1985). Since the Δ N-DBP preparation contained some nuclease activity, we were not able to add sufficient amounts of Δ N-DBP to obtain optimal levels of replication. Therefore, the intact DBP was used instead of Δ N-DBP in the experiments described below. Deletion of the C-terminal extension from Δ N-DBP had a drastic effect on replication (Figure 4A, upper panel, lanes 9–16). Not only were much higher concentrations required for labeling of the DNA but, more importantly, the origin-containing B/C fragments were not labeled preferentially. The affinity of Δ N Δ C-DBP for ssDNA is 100-fold lower compared with intact DBP. We could not, however, add 100 times more Δ N Δ C-DBP than intact DBP in a replication assay

due to the fact that the salt concentration may not exceed 55 mM. DBP has multiple functions in DNA replication, during initiation as well as during elongation. To examine whether Δ N Δ C-DBP was able to perform one of these functions or whether it was impaired in both steps, the activity of Δ N Δ C-DBP was tested in the presence of a suboptimal concentration (25 ng) of intact DBP. This low amount of DBP was not sufficient to support origin-specific replication by itself (compare lane 1 of the lower panel of Figure 4A with lanes 2 and 3 of the upper panel). If Δ N Δ C-DBP is defective in some but not all functions, the intact DBP might complement the replication defect. Indeed, in the presence of 25 ng of intact DBP, origin-specific replication was stimulated by Δ N Δ C-DBP (Figure 4A, lower panel, lanes 9–16). Similar results were obtained when low concentrations of Δ N-DBP were added instead of intact DBP (data not shown). The single-stranded DNA-binding protein (SSB) of bacteriophage T4, gp32, did not stimulate replication in the presence or absence of DBP, which indicates that the effects of Δ N Δ C-DBP are specific and not simply due to the addition of a SSB. In Figure 4B, the amount of DBP was titrated in the absence or presence of 1 μ g of Δ N Δ C-DBP. The maximum level of replication was the same, but in the presence of Δ N Δ C-DBP the amount of DBP required was considerably lower. These results show that DBP performs multiple functions during *in vitro* replication and that Δ N Δ C-DBP can perform some but not all of these functions.

Level of stimulation of initiation by DBP depends on the pTP-pol concentration

The role of DBP in initiation of replication has been the subject of some debate. Various levels of stimulation by DBP have been described (Nagata *et al.*, 1982; Rosenfeld *et al.*, 1987; Stuiver and van der Vliet, 1990; Mul and van der Vliet, 1993). Figure 5 shows that at the low pTP-pol concentration we used here to study replication (Figure 4), DBP strongly stimulates initiation. At this concentration of pTP-pol, we did not detect any initiation in the absence

A

B

Fig. 4. Δ NΔC-DBP can only stimulate replication when subsaturating concentrations of intact DBP are present. (A) Upper diagram: DNA replication was performed with increasing amounts of DBP, Δ NΔC-DBP and gp32. The amounts of DBP and Δ NΔC-DBP added were 0, 16, 32, 63, 125, 250, 500 and 1000 ng. The amounts of gp32 added were 0, 16, 32, 63, 125, 250 and 500 ng. The lower diagram shows the same experiment, but in this case all reactions were performed in the presence of 25 ng extra of DBP. (B) DNA replication was performed with various amounts of DBP (0, 6, 12, 25, 50, 100, 200 and 400 ng) in the presence or absence of 1 μ g of Δ NΔC-DBP. The bands indicated as B/C, the origin-containing fragments, were quantified and plotted.

Fig. 5. Stimulation of initiation by DBP is optimal at low pTP-pol concentrations. Initiation of replication was tested using increasing concentrations of pTP-pol (5, 10, 20, 40 and 80 nM) in the absence (lanes 1–5) or in the presence (lanes 6–10) of 1 μ g of DBP.

of DBP (Figure 5, compare lane 1 with lane 6). At higher concentrations of pTP-pol, initiation was also observed in the absence of DBP. However, the level of stimulation dropped from 10-fold at 10 nM pTP-pol to 2-fold at 80 nM pTP-pol. These results may explain the different values of stimulation of initiation by DBP reported in the literature. We conclude that under *in vitro* replication conditions, and in the absence of NFI and Oct-1, DBP is required for high levels of initiation.

Δ NΔC-DBP stimulates initiation

As shown in Figure 4, Δ NΔC-DBP only stimulates DNA replication in the presence of suboptimal amounts of intact DBP, suggesting that it is not active either in initiation or in elongation. The effect of DBP and Δ NΔC-DBP on initiation was tested at two pTP-pol concentrations. As a negative control we tested gp32. Δ NΔC-DBP stimulated initiation up to 8-fold at the lowest pTP-pol concentration (10 nM), but stimulation dropped to 3-fold when the pTP-pol concentration was raised (Figure 6). The level of stimulation was slightly lower compared with intact DBP. This shows clearly that the replication defect of Δ NΔC-DBP is not at the level of initiation.

Δ NΔC-DBP stimulates formation of the pTP-CAT intermediate but is defective in elongation

To test whether Δ NΔC-DBP also stimulates replication after pTP-dCMP formation, replication was performed in the presence of all nucleotides except dGTP. Elongation then stops at position 26 of the template. Under these conditions, the pTP-CAT intermediate is also detected (King and van der Vliet, 1994). Figure 7 shows that Δ NΔC-DBP stimulates pTP-CAT formation since the smallest replication product that runs at the same position as pTP-dCMP can be labeled with dCMP, dAMP and dTMP.

Fig. 6. Δ N Δ C-DBP stimulates initiation of replication. Reactions were performed in the presence of 10 or 20 nM pTP-pol. Amounts of DBP, Δ N Δ C-DBP and gp32 added were 0, 62, 125, 250, 500 and 1000 ng.

At this pTP-pol concentration, DBP and Δ N Δ C-DBP stimulated pTP-CAT formation 2- to 3-fold. DBP lowers the K_m for dCTP during initiation (Mul and van der Vliet, 1993) and therefore stimulates especially at very low dCTP concentrations. The level of stimulation of initiation in this experiment is low since we used 1 μ M (instead of 0.05 μ M in Figures 5 and 6) of each nucleotide in order to obtain the same specific activity in each panel and to be able to observe elongation. Elongation to position 26 (pTP-26N) was absolutely dependent on DBP. At the highest concentration of DBP tested, 15% of the pTP-CAT formed was elongated. Δ N Δ C-DBP, however, did not stimulate pTP-26N formation at all. This shows that Δ N Δ C-DBP is defective in elongation.

DBP is not required for elongation on a single-stranded template

Adenovirus DNA replication employs a double-stranded template, but it can also be observed on ssDNA. Figure 8 shows a replication assay performed on a single-stranded origin. Also in the absence of DBP, elongation until position 26 is observed. Addition of DBP resulted in an increased level of initiation, but the percentage of pTP-dCMP molecules that were elongated did not change. Δ N Δ C-DBP also stimulated initiation, although not as strongly as DBP. No difference in the percentage of molecules that were elongated was observed. As a negative control gp32 was used. This protein did not have an effect on the levels of initiation and elongation. These results show that DBP is only required for elongation on double-stranded templates.

Δ N Δ C-DBP has a reduced helix-destabilizing activity

The unwinding activity of Δ N Δ C-DBP was tested. Figure 9 shows the unwinding of an 11 bp dsDNA molecule with a four base overhang on each strand. Δ N Δ C-DBP could unwind this dsDNA molecule, but the activity was at least 15 times lower compared with Δ N-DBP. The low helix-destabilizing activity of Δ N Δ C-DBP correlates with the elongation defect of the mutant on double-stranded templates and provides evidence that the helix-destabilizing activity of intact DBP is required to unwind the DNA during strand displacement synthesis.

Discussion

Deletion of the C-terminal extension results in a monomeric protein

DBP and Δ N-DBP form multimers already in the absence of DNA, as shown by the crystal structure (Tucker *et al.*, 1994; Kanellopoulos *et al.*, 1996), dynamic light scattering and electron microscopy. Cooperative binding by DBP is thought to be the result of these strong protein-protein interactions. Deletion of the C-terminal arm results in a monomeric protein. However, DNA binding by this protein is still cooperative since only free or fully saturated DNA-protein complexes are observed in a gel retardation assay. This indicates that protein-protein interactions can still occur although these interactions are too weak to be observed in the absence of DNA. Based on the crystal structure, it is very unlikely that the C-terminal arm is directly involved in DNA binding. Therefore, the 100-fold lower affinity for ssDNA is probably caused by a reduced cooperativity. Using bacterially expressed GST fusion proteins, we have shown previously that deletion of the C-terminus results in non-cooperative binding (Tucker *et al.*, 1994). The difference from the results that are presented here may be caused by the presence of the GST part which perhaps interferes with the weak protein-protein interactions between Δ N Δ C-DBP monomers. Multimerization via the C-terminal arm is clearly required for high affinity binding to ssDNA. Besides the C-terminal hook, other parts of the protein are probably also involved in protein-protein interactions.

Stimulation of initiation does not require the C-terminal arm of DBP

DBP stimulates initiation of replication in two ways. At subsaturating concentrations of NFI, DBP enhances the binding of NFI to the origin, which results in increased levels of initiation (Cleat and Hay, 1989; Stuiver and van der Vliet, 1990). DBP also stimulates initiation directly by lowering the K_m for incorporation of dCTP (Mul and van der Vliet, 1993). We show here that the level of stimulation also depends on the concentration of the pTP-pol complex. At a pTP-pol concentration of 5 nM, which is the concentration used for the complete replication reaction, no initiation is observed in the absence of DBP. Addition of DBP strongly stimulates pTP-dCMP formation. At higher pTP-pol concentrations, stimulation drops from 10-fold to 3-fold. Deletion of the C-terminal arm only has a small effect on the level of stimulation of initiation. Δ N Δ C-DBP stimulates 8-fold and this level dropped to 3-fold when the pTP-pol concentration was raised. It is unlikely that high concentrations of pTP-pol somehow squelch the activity of DBP, since DBP is always present in large molar excess. Stimulation of initiation by NFI and Oct-1 depends in a similar manner on the pTP-pol concentration (Mul *et al.*, 1990). This is explained by the fact that these proteins interact with pTP-pol (Bosher *et al.*, 1990; Mul and van der Vliet, 1992; Coenjaerts *et al.*, 1994) and recruit the complex to the origin. This suggests that DBP may also interact with pTP-pol. Indirect evidence for a protein-protein interaction between pTP-pol and DBP has been obtained (Lindenbaum *et al.*, 1986), but we and others have not been able to detect a direct interaction (data not shown). Perhaps the

Fig. 7. $\Delta N\Delta C$ -DBP is defective in elongation after formation of the pTP-CAT trinucleotide intermediate. Initiation/partial elongation reactions were performed on TP-DNA using labeled dCTP (lanes 1–10), labeled dATP (lanes 11–20) or labeled dTTP (lanes 21–30). The pTP-pol concentration was 15 nM. The amounts of DBP and $\Delta N\Delta C$ -DBP added were 0, 62, 125, 250 and 500 ng.

Fig. 8. Elongation on a single-stranded template is independent of DBP. Initiation and partial elongation were tested on a 30 nucleotide long single-stranded template strand of the origin (250 ng) with 50 nM pTP-pol. The amounts of DBP, $\Delta N\Delta C$ -DBP and gp32 were 0, 125, 250, 500 and 1000 ng.

interaction is very transient or DNA dependent. A direct protein–protein interaction between pTP-pol and DBP would explain the stimulatory effect of $\Delta N\Delta C$ -DBP during initiation and the fact that other SSB proteins, like gp32, cannot substitute for DBP. After pTP-dCMP formation, a trinucleotide intermediate is formed, pTP-CAT. Both DBP and $\Delta N\Delta C$ -DBP stimulate pTP-CAT formation to an extent similar to pTP-dCMP formation, which shows that addition of the second and third nucleotide does not require DBP. Since deletion of the C-terminal arm results in a 100-fold lower affinity for ssDNA, it is unlikely that DNA binding by DBP is required for stimulation of initiation.

DBP is required to unwind the template during elongation

DBP is required for strand displacement synthesis (Friedfeld *et al.*, 1983; Prelich and Stillman, 1986). In the presence of DBP, the polymerase becomes highly processive (Lindenbaum *et al.*, 1986) and the sensitivity for inhibitors like (S)-HPMPApp is increased (Mul *et al.*, 1989). Based on the fact that no helicase activity or ATP is required during replication and the observation that DBP can unwind double-stranded molecules up to 200 bp, it was suggested that DBP is involved in unwinding of the template during replication (Zijderveld and van der Vliet,

Fig. 9. $\Delta N\Delta C$ -DBP has a low helix-destabilizing capacity. Unwinding of a 11 bp double-stranded probe with 5' extensions of four nucleotides was tested under replication conditions. The highest concentration of DBP and $\Delta N\Delta C$ -DBP was 1200 nM (1700 and 1000 ng respectively). The percentage of DNA that was unwound was calculated and plotted.

1994). We show that DBP is only required for elongation on a double-stranded template. On a single-stranded template elongation is also observed in the absence of DBP, and addition of DBP does not enhance the level of elongation. Together with the observation that $\Delta N\Delta C$ -DBP has a 15-fold lower unwinding activity and does not support elongation on a double-stranded template, this provides strong evidence that the unwinding activity of DBP is required for elongation. $\Delta N\Delta C$ -DBP stimulates origin-specific replication only when a subsaturating amount of intact DBP is present. Based on our results, we conclude that in this mixing experiment $\Delta N\Delta C$ -DBP only functions during initiation while the intact DBP is required for elongation. In the presence of $\Delta N\Delta C$ -DBP, the small amount of DBP required to observe origin-specific replication cannot be replaced by other SSB proteins like gp32 or the phage $\phi 29$ P5 protein (data not shown), which indicates that cooperative and high affinity binding to ssDNA is not sufficient for strand displacement synthesis. This suggests that there is a specific interaction between DBP and the adenovirus DNA polymerase during elongation.

Chain formation by DBP is required for DNA unwinding

The exact mechanism of unwinding by DBP is not known. It has been suggested that the formation of a growing DBP protein chain is the driving force in unwinding DNA (Zijderveld and van der Vliet, 1994). We show here that deletion of the C-terminal arm abolishes chain formation in solution and results in a reduced unwinding activity. Strong binding of a DBP monomer to a growing DBP chain on the displaced strand may provide the energy needed to open the DNA duplex. Although $\Delta\text{N}\Delta\text{C}$ -DBP is still able to form protein chains on DNA, we think that deletion of the C-terminal arm weakens the protein-protein interactions considerably, which would explain the greatly reduced affinity for ssDNA and the low helix-destabilizing activity. Therefore, binding of a $\Delta\text{N}\Delta\text{C}$ -DBP monomer to the chain is not strong enough to open the duplex. $\Delta\text{N}\Delta\text{C}$ -DBP stimulates initiation, which indicates that it can still interact functionally with the polymerase, but for elongation this is clearly not sufficient. This shows that for strand displacement synthesis multimerization of DBP via the C-terminal arm resulting in a high affinity for ssDNA is required besides a specific functional interaction between DBP and the adenovirus polymerase.

Multimerization and cooperative DNA binding of helix-destabilizing proteins may be a general mechanism to unwind DNA

Cooperative binding to ssDNA is a common feature of helix-destabilizing proteins. Cooperativity is the result of protein-protein interactions between monomers. This cooperative binding is required to fully saturate the ssDNA and to protect it against nuclease activities. gp32 resembles DBP in many aspects. It forms oligomers in solution (Carroll *et al.*, 1975) and forms protein chains on ssDNA (Kowalczykowski *et al.*, 1981). Efficient unwinding of DNA requires removal of the C-terminus (Hosoda *et al.*, 1974), which does not affect cooperative DNA binding. ICP8, the SSB of herpes simplex virus, forms long filaments in solution (O'Donnell *et al.*, 1987). It binds ssDNA in a highly cooperative fashion and unwinds duplex DNA (Boehmer and Lehman, 1993). Not all SSB proteins form oligomers in the absence of DNA. The SSB of bacteriophage $\phi 29$, P5, and the eukaryotic heterotrimeric SSB RP-A are monomeric in solution. DNA binding is cooperative, however, and continuous arrays of proteins are formed on the ssDNA (Alani *et al.*, 1992; Soengas *et al.*, 1994; Kim and Wold, 1995). These proteins unwind DNA (Georgaki *et al.*, 1992; Soengas *et al.*, 1995; Treuner *et al.*, 1996), and in the case of P5 it was suggested that this was driven by the cooperativity. Therefore, other helix-destabilizing proteins may employ a similar mechanism to that of DBP by which the energy required to unwind DNA is provided by strong protein-protein interactions instead of ATP hydrolysis as in the case of true DNA helicases.

Materials and methods

Construction of DBP mutants

The intact DNA-binding domain of Ad5 DBP (ΔN -DBP: amino acids 174–529) was expressed using a baculovirus expression system (Kanellopoulos *et al.*, 1995). The DNA-binding domain lacking the

C-terminal arm ($\Delta\text{N}\Delta\text{C}$ -DBP: amino acids 174–512) was constructed by means of PCR using the coding sequence of the intact DNA-binding domain in the pVL1392 baculovirus vector (Luckow and Summers, 1988; O'Reilly *et al.*, 1992) as a template. The oligonucleotide primers contained *Bgl*III and *Eco*RI restriction sites (underlined): 5' TTTAGATCTTCATGAGTGTGCCGATCGTGTCTGC and 5' TTTGAATCTCATGTTGCGATACTGGTGTTTAG. The PCR fragment was cloned between the *Bgl*III and *Eco*RI sites of the pVL1392 baculovirus transfer vector and checked by DNA sequencing.

Purification of ΔN -DBP

Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses at 28°C for 72 h at a multiplicity of 5 p.f.u./cell. Cells were collected by centrifugation, washed in phosphate-buffered saline and resuspended in a buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cells were homogenized using a Dounce homogenizer, and insoluble material was removed by centrifugation at 60 000 g for 30 min at 4°C. The supernatant was loaded on a DEAE-Sephacose column equilibrated in 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, 20% glycerol and 50 mM NaCl. The flow through was applied to a ssDNA-cellulose column equilibrated in 10 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA and 200 mM NaCl. The column was washed with the same buffer containing 500 mM NaCl. ΔN -DBP was eluted with 10 mM Tris-HCl (pH 8.0), 2 M NaCl and was shown to be >90% pure. To remove nuclease activity, ΔN -DBP that eluted from the ssDNA-cellulose column was applied to a heparin cartridge (Bio-Rad) equilibrated in 25 mM HEPES-KOH (pH 8.0), 1 mM DTT, 0.1 mM PMSF, 0.02% NP-40, 20% glycerol and 100 mM NaCl. The column was washed with the same buffer and developed with a linear gradient of 100–500 mM NaCl. ΔN -DBP eluted at ~300 mM NaCl.

Intact DBP was purified from adenovirus-infected HeLa cells as described previously (Dekker *et al.*, 1996).

Purification of $\Delta\text{N}\Delta\text{C}$ -DBP

Monolayer cultures of Sf9 cells were infected with recombinant baculoviruses and soluble extracts were obtained as described for ΔN -DBP. The soluble extract was loaded on a Q-Sepharose column equilibrated in 25 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA, 20% glycerol and 50 mM NaCl. Proteins were eluted with 20 mM Tris-HCl (pH 8.0), 0.5 mM DTT and 200 mM NaCl. The protein solution was applied to a ssDNA-cellulose column equilibrated in 10 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA and 200 mM NaCl. The column was washed with 10 mM Tris-HCl (pH 8.0), 200 mM NaCl and $\Delta\text{N}\Delta\text{C}$ -DBP subsequently was eluted with 10 mM Tris-HCl (pH 8.0), 400 mM NaCl. To remove nucleases, the proteins that eluted from the ssDNA-cellulose column were applied to a heparin cartridge (Bio-Rad) as described above. Under these conditions, $\Delta\text{N}\Delta\text{C}$ -DBP did not bind to the column while the nuclease activity was retained on the column. Subsequently, the $\Delta\text{N}\Delta\text{C}$ -DBP was applied to ssDNA-cellulose via a batch binding procedure in a buffer containing 25 mM HEPES-KOH (pH 8.0), 1 mM DTT, 0.1 mM PMSF, 0.02% NP-40, 20% glycerol and 100 mM NaCl. The resin was then packed into a column and washed with the same buffer. $\Delta\text{N}\Delta\text{C}$ -DBP was eluted with the same buffer containing 400 mM NaCl.

Other proteins

The pTP-pol complex was purified as described (King and van der Vliet, 1994). Terminal protein containing adenovirus DNA was obtained as described (Dekker *et al.*, 1996). gp32 was obtained from Boehringer Mannheim. P5 was a gift from M.S. Soengas and M. Salas.

Gel retardation

A 114 bp *Eco*RI-*Xba*I fragment from pHRI (Hay, 1985) was Klenow end labeled in the presence of [α -³²P]dCTP. DNA was denatured by boiling for 5 min. Binding reactions were carried out on ice for 60 min in a volume of 20 μ l containing 25 mM HEPES-KOH (pH 7.5), 200 mM NaCl, 4 mM MgCl₂, 0.4 mM DTT, 4% Ficoll, 1 μ g bovine serum albumin (BSA), 0.05 ng of denatured DNA and the indicated amounts of proteins. Bound and free DNA were separated on a 6% polyacrylamide gel at 4°C in a running buffer containing 0.5 $\times$ TBE and 0.01% NP-40. Gels were dried and quantified by densitometric analysis using a Phosphorimager.

DNA unwinding assay

DNA unwinding was studied using a double-stranded oligonucleotide containing 11 bp and a four nucleotide single-stranded extension at each

5' end. Two oligonucleotides with the sequences: 5'AATTCTACCGCCT-CC3' and 5'AATTGGAGGCGGTAG3' were hybridized and the double-stranded oligonucleotide was labeled at the 5' end by T4 polynucleotide kinase in the presence of [γ - 32 P]ATP. DNA (0.05 ng) and various amounts of DBP and Δ NAC-DBP were incubated in 25 mM HEPES-KOH (pH 8.0), 1 mM DTT, 1.5 mM MgCl₂, 1 μ g of BSA and 50 mM NaCl in a total volume of 25 μ l for 1 h at 20°C. Reactions were stopped by addition of 2 μ l of stop mix (40% sucrose, 1% SDS, 0.1% bromophenol blue, 0.1% xylene cyanol) and products were analyzed on an SDS-15% polyacrylamide gel followed by autoradiography. Quantifications were performed using a Phosphorimager.

DNA replication

Adenovirus DNA replication was performed in a volume of 25 μ l in the presence of 25 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, 500 nM [α - 32 P]dCTP, 40 μ M of dATP, dTTP, dGTP and 50 ng of Ad5 terminal protein containing DNA (TP-DNA) cut with *Xho*I. pTP-pol was added to a final concentration of 5 nM. The amounts of DBP or Δ NAC-DBP added are indicated in the figure legends. After incubation for 45 min at 37°C, reactions were stopped by addition of 2.5 μ l of 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 partly dehydrated followed by autoradiography. Replication products were quantified by densitometric analysis using a Phosphorimager.

Initiation and partial elongation

Initiation of replication on TP-DNA was performed in a reaction volume of 25 μ l containing 25 mM HEPES-KOH (pH 7.5), 50 mM NaCl, 1.5 mM MgCl₂, 1 mM DTT, 50 nM [α - 32 P]dCTP and 100 ng of Ad5 TP-DNA. pTP-pol, DBP and Δ NAC-DBP concentrations were as described in the figure legends. When partial elongation on a double-stranded template was tested, the concentrations of nucleotides were: 1 μ M dCTP, 1 μ M dATP, 1 μ M dTTP and 5 μ M ddGTP and 26 nM of [α - 32 P]dCTP, [α - 32 P]dATP or [α - 32 P]dTTP (as indicated in the figure legends). Partial elongation on a single-stranded template was performed in the presence of 1 μ M dATP, 1 μ M dTTP, 0.1 μ M dCTP, 5 μ M ddGTP and 26 nM [α - 32 P]dCTP. As a single-stranded template, 230 ng of an oligonucleotide containing the first 30 nucleotides of the bottom strand of the Ad5 origin (King and van der Vliet, 1994) was used. pTP-pol, DBP and Δ NAC-DBP concentrations were as indicated in the figure legends. Reactions were performed at 37°C for 30 min when TP-DNA was used as template and at 30°C for 60 min when a single-stranded template was used. Reactions were stopped by addition of 80 mM EDTA. The samples were precipitated with 20% trichloroacetic acid (TCA). When a single-stranded template was used, 10 μ g of BSA was added. Precipitates were washed with 5% TCA, resolved in sample buffer and analyzed on an SDS-7.5% polyacrylamide gel and autoradiographed. Replication products were quantified by densitometric analysis using a Phosphorimager.

Electron microscopy and negative staining

Samples of DBP, Δ N-DBP and Δ NAC-DBP were examined for their state of polymerization in the absence of DNA by negative staining. Proteins (0.5 μ l; 10 mg/ml) were diluted with 11 μ l of 20 mM Tris-HCl (pH 8.0), 1 mM EDTA and put on ice for 5 min. Of this protein solution, 2 μ l was applied on a 400 mesh Formvar-coated grid and the excess was removed. The preparation was stained with a 1% uranyl acetate solution and was set to dry after removal of the excess staining solution. The grids were examined in a Philips 400 electron microscope.

Dynamic light scattering

A concentrated protein solution of DBP, Δ N-DBP and Δ NAC-DBP [10 mg/ml in a buffer containing Tris-HCl (pH 8.0), 1 mM DTT, 1 mM EDTA; the salt concentration was 2 M in the case of DBP and Δ N-DBP and 400 mM in the case of Δ NAC-DBP] was applied to a Dyna Pro-801 Dynamic Light Scattering instrument (Protein Solutions Incorporated). The count of the photons scattered by the molecules in the flow cell was measured and an assessment of the radius of the particles in solution and the molecular weight was made with a program accompanying the instrument.

Acknowledgements

The authors would like to thank Gunter Stier for his advice on the construction of the mutants, Wieke Teertstra and Audrey King for

purified pTP-pol, and Hans van Leeuwen and Marian Walhout for critical reading of the manuscript. This work was supported in part by the Netherlands Foundation for Chemical Research (SON) with financial support from the Netherlands Organization for Scientific Research (NWO).

References

- Alani, E., Thresher, R., Giffith, J.D. and Kolodner, R.D. (1992) Characterization of DNA binding and strand exchange stimulation properties of γ -RPA, a yeast single-strand-DNA-binding protein. *J. Mol. Biol.*, **227**, 54–71.
- Ariga, H., Klein, H., Levine, A.J. and Horwitz, M.S. (1980) A cleavage product of the adenovirus DNA binding protein is active in DNA replication *in vitro*. *Virology*, **101**, 307–310.
- Boehmer, P.E. and Lehman, I.R. (1993) Herpes simplex virus type 1 ICP8: helix-destabilizing properties. *J. Virol.*, **67**, 711–715.
- Bosher, J., Robinson, E.C. and Hay, R.T. (1990) Interactions between the adenovirus type 2 DNA polymerase and the DNA binding domain of nuclear factor I. *New Biol.*, **2**, 1083–1090.
- Carroll, R.B., Neet, K. and Goldthwait, D.A. (1975) Studies of the self-association of bacteriophage T4 gene 32 protein by equilibrium sedimentation. *J. Mol. Biol.*, **91**, 275–291.
- Chase, J.W. and Williams, K.R. (1986) Single-stranded DNA-binding proteins required for DNA replication. *Annu. Rev. Biochem.*, **55**, 103–136.
- Cleat, P.H. and Hay, R.T. (1989) Co-operative interactions between NFI and the adenovirus DNA binding protein at the origin of replication. *EMBO J.*, **8**, 1841–1848.
- Cleghon, V., Voelkerding, K., Morin, N., Delsert, C. and Klessig, D.F. (1989) Isolation and characterization of a viable adenovirus mutant defective in nuclear transport of the DNA-binding protein. *J. Virol.*, **63**, 2289–2299.
- Coenjaerts, F.E.J., van Oosterhout, J.A.W.M. and van der Vliet, P.C. (1994) The Oct-1 POU domain stimulates adenovirus DNA replication by a direct interaction between the viral precursor terminal protein-DNA polymerase complex and the POU homeo domain. *EMBO J.*, **13**, 5401–5409.
- Dekker, J., Coenjaerts, F.E.J. and van der Vliet, P.C. (1996) Reconstitution of adenovirus DNA replication with purified replication proteins. In Adolph, K.W. (ed.), *Viral Genome Methods*. CRC Press, Boca Raton, FL, pp. 269–282.
- Friefeld, B.R., Krevolin, M.D. and Horwitz, M.S. (1983) Effects of the adenovirus H5ts125 and H5ts107 DNA binding proteins on DNA replication *in vitro*. *Virology*, **124**, 380–389.
- Georgaki, A., Strack, B., Podust, V. and Hübscher, U. (1992) DNA unwinding activity of replication protein A. *FEBS Lett.*, **308**, 240–244.
- Ginsberg, H.S., Ensinger, M.J., Kauffman, R.S., Mayer, A.J. and Lundholm, U. (1974) Cell transformation: a study of regulation with type 5 and adenovirus temperature sensitive mutants. *Cold Spring Harbor Symp. Quant. Biol.*, **39**, 419–426.
- Hay, R.T. (1985) The origin of adenovirus DNA replication: minimal DNA sequence requirements *in vivo*. *EMBO J.*, **4**, 421–426.
- Hay, R.T., Freeman, A., Leith, I., Monaghan, A. and Webster, A. (1995) Molecular interactions during adenovirus DNA replication. In Doerfler, W. and Böhm, P. (eds), *The Molecular Repertoire of Adenoviruses II*. Springer Verlag, Berlin, pp. 31–48.
- Hosoda, J., Takacs, B. and Brack, C. (1974) Denaturation of T4 DNA by an *in vitro* processed gene 32 protein. *FEBS Lett.*, **47**, 338–342.
- Kanellopoulos, P.N., van der Zandt, H., Tsernoglou, D., van der Vliet, P.C. and Tucker, P.A. (1995) Crystallization and preliminary X-ray crystallographic studies on the adenovirus ssDNA binding protein in complex with ssDNA. *J. Struct. Biol.*, **115**, 113–116.
- Kanellopoulos, P.N., Tsernoglou, D., van der Vliet, P.C. and Tucker, P.A. (1996) Alternative arrangements of the protein chain are possible for the adenovirus single-stranded DNA binding protein. *J. Mol. Biol.*, **257**, 1–8.
- Kim, C. and Wold, M.S. (1995) Recombinant human replication protein A binds to polynucleotides with low cooperativity. *Biochemistry*, **34**, 2058–2064.
- King, A.J. and van der Vliet, P.C. (1994) A precursor terminal protein-trinucleotide intermediate during initiation of adenovirus DNA replication: regeneration of molecular ends *in vitro* by a jumping back mechanism. *EMBO J.*, **13**, 5786–5792.
- Kowalczykowski, S.C., Lonberg, N., Newport, J.W. and von Hippel, P.H. (1981) Interactions of bacteriophage T4-coded gene 32 protein with nucleic acids. *J. Mol. Biol.*, **145**, 75–104.

- Lindenbaum, J.O., Field, J. and Hurwitz, J. (1986) The adenovirus DNA binding protein and adenovirus DNA polymerase interact to catalyze elongation of primed DNA templates. *J. Biol. Chem.*, **261**, 10218–10227.
- Luckow, V.A. and Summers, M.D. (1988) Trends in the development of baculovirus expression vectors. *Biotechnology*, **6**, 47–55.
- Monaghan, A., Webster, A. and Hay, T. (1994) Adenovirus DNA binding protein: helix destabilising properties. *Nucleic Acids Res.*, **22**, 742–748.
- Mul, Y.M. and van der Vliet, P.C. (1992) Nuclear factor I enhances adenovirus DNA replication by increasing the stability of a preinitiation complex. *EMBO J.*, **11**, 751–760.
- Mul, Y.M. and van der Vliet, P.C. (1993) The adenovirus DNA binding protein effects the kinetics of DNA replication by a mechanism distinct from NFI or Oct-1. *Nucleic Acids Res.*, **21**, 641–647.
- Mul, Y.M., van Miltenburg, R.T., de Clercq, E. and van der Vliet, P.C. (1989) Mechanism of inhibition of adenovirus DNA replication by the acyclic nucleoside triphosphate analogue (S)-HPMPApp: influence of the adenovirus DNA binding protein. *Nucleic Acids Res.*, **17**, 8777–8789.
- Mul, Y.M., Verrijzer, C.P. and van der Vliet, P.C. (1990) Transcription factors NFI and NFII/Oct-1 function independently, employing different mechanisms to enhance adenovirus DNA replication. *J. Virol.*, **64**, 5510–5518.
- Nagata, K., Guggenheimer, R.A., Enomoto, T., Lichy, J.H. and Hurwitz, J. (1982) Adenovirus DNA replication *in vitro*: identification of a host factor that stimulates synthesis of the preterminal protein-dCMP complex. *Proc. Natl Acad. Sci. USA*, **79**, 6438–6442.
- Nicolas, J., Sarnow, P., Girard, M. and Levine, A.J. (1983) Host range temperature-conditional mutants in the adenovirus DNA-binding protein are defective in the assembly of infectious virus. *J. Virol.*, **47**, 228–239.
- O'Donnell, M.E., Elias, P., Funell, B.E. and Lehman, I.R. (1987) Interaction between the DNA polymerase and single-stranded DNA-binding protein (infected cell protein 8) of herpes simplex virus 1. *J. Biol. Chem.*, **262**, 4260–4266.
- O'Reilly, D.R., Miller, L.K. and Luckow, V.A. (1992) *Baculovirus Expression Vectors, A Laboratory Manual*. Freeman, New York.
- Prelich, G. and Stillman, B.W. (1986) Functional characterization of thermolabile DNA-binding proteins that affect adenovirus DNA replication. *J. Virol.*, **57**, 883–892.
- Pronk, R., van Driel, W. and van der Vliet, P.C. (1994) Replication of adenovirus DNA *in vitro* is ATP-independent. *FEBS Lett.*, **337**, 33–38.
- Rosenfeld, P.J., O'Neill, E.A., Wides, R.J. and Kelly, T.J. (1987) Sequence-specific interactions between cellular DNA-binding proteins and the adenovirus origin of DNA replication. *Mol. Cell. Biol.*, **7**, 875–886.
- Soengas, M.S., Esteban, J.A., Salas, M. and Gutiérrez, C. (1994) Complex formation between phage phi29 single-stranded DNA binding protein and DNA. *J. Mol. Biol.*, **239**, 213–226.
- Soengas, M.S., Gutiérrez, C. and Salas, M. (1995) Helix-destabilizing activity of phi29 single-stranded DNA binding protein: effect on the elongation rate during strand displacement DNA replication. *J. Mol. Biol.*, **253**, 517–529.
- Stuiver, M.H. and van der Vliet, P.C. (1990) Adenovirus DNA-binding protein forms a multimeric protein complex with double-stranded DNA and enhances binding of nuclear factor I. *J. Virol.*, **64**, 379–386.
- Treuner, K., Ramsperger, U. and Knippers, R. (1996) Replication protein A induces the unwinding of long double-stranded DNA regions. *J. Mol. Biol.*, **259**, 104–112.
- Tsernoglou, D., Tsugita, A., Tucker, A.D. and van der Vliet, P.C. (1985) Characterization of the chymotryptic core of the adenovirus DNA-binding protein. *FEBS Lett.*, **188**, 248–252.
- Tucker, P.A., Tsernoglou, D., Tucker, A.D., Coenjaerts, F.E.J., Leenders, H. and van der Vliet, P.C. (1994) Crystal structure of the adenovirus DNA binding protein reveals a hook-on model for cooperative DNA binding. *EMBO J.*, **13**, 2994–3002.
- van der Vliet, P.C. (1991) The role of cellular transcription factors in the enhancement of adenovirus DNA replication. *Semin. Virol.*, **2**, 271–280.
- van der Vliet, P.C. (1995) Adenovirus DNA replication. In Doerfler, W. and Böhm, P. (eds), *The Molecular Repertoire of Adenoviruses II*. Springer-Verlag, Berlin, pp. 1–30.
- Zijderveld, D.C., d'Adda di Fagagna, F., Giacca, M., Timmers, H.T.M. and van der Vliet, P.C. (1994) Stimulation of the adenovirus major late promoter *in vitro* by transcription factor USF is enhanced by the adenovirus DNA binding protein. *J. Virol.*, **68**, 8288–8295.
- Zijderveld, D.C., Stuiver, M.H. and van der Vliet, P.C. (1993) The adenovirus DNA binding protein enhances intermolecular DNA renaturation but inhibits intramolecular DNA renaturation. *Nucleic Acids Res.*, **21**, 2591–2598.
- Zijderveld, D.C. and van der Vliet, P.C. (1994) Helix-destabilizing properties of the adenovirus DNA-binding protein. *J. Virol.*, **68**, 1158–1164.

Received on October 8, 1996; revised on November 20, 1996