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Adenovirus DNA binding protein: helix destabilising properties

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ABSTRACT

Adenovirus DNA binding protein is a multifunctional protein essential for viral DNA replication. To investigate the role of the DNA binding protein in this process its interaction with partial DNA duplexes was examined. Duplex regions of DNA, created when a short DNA strand is annealed to its complementary sequence present in the single stranded form of M13 phage DNA, were efficiently unwound by DNA binding protein in a reaction that required neither ATP nor $MgCl_2$. The unwinding activity of DNA binding protein was reduced by conditions which increased the stability of DNA duplexes. DNA unwinding by DNA binding protein was highly co-operative and required the single stranded DNA to be completely coated with the protein. Completely double stranded DNA could also be unwound by DNA binding protein but this reaction was sensitive to the G + C content of the DNA and could only be observed with relatively short DNA duplexes up to 45 base pairs in length. When these short double stranded DNA molecules contained binding sites for the transcription factors NFI and NFIII addition of the cognate factor blocked DNA binding protein mediated unwinding of that particular DNA duplex. Cleavage of DNA binding protein with chymotrypsin and isolation of the 39, 000 molecular weight C-terminal fragment indicated that the unwinding activity was located in this domain of the protein. In support of this contention a monoclonal antibody, which had previously been mapped to this region, specifically inhibited the DNA unwinding activity. These activities of DNA binding protein are likely to be involved in DNA replication, where the destabilisation of DNA duplexes could be important both during initiation and elongation.

INTRODUCTION

Synthesis of adenovirus DNA initiates by protein priming at the termini of the genome and proceeds via a strand displacement mechanism. The viral genome can be replicated *in vitro* by the action of five proteins of which three are viral and two are cellular. Determination of the mechanism of adenovirus DNA replication has been facilitated by cloning and expression of each of these genes in a variety of heterogeneous systems, from which the proteins can be obtained in a highly purified form. The adenovirus coded DNA polymerase (140 kDa, pol), precursor to the terminal protein (80 kDa, pTP) and the DNA binding protein (59 kDa, DBP) are each essential for viral DNA replication but this process is stimulated greatly by the additional presence of two cellular proteins nuclear factor I (NFI) and nuclear factor III (NFIII), (reviewed in references 1–4). Both of these proteins are transcription factors, NFI being equivalent to CAAT transcription factor (5), while NFIII is the homeodomain containing protein oct-1 (6). Over recent years a model has been formulated for the formation of a preinitiation complex, in which each of these proteins is incorporated into a large nucleoprotein complex at the viral origin of DNA replication. The highly abundant DBP first coats the linear, protein-linked genome then NFI and NFIII are bound at their DNA recognition sites located within the viral origin of DNA replication. A direct protein–protein interaction between NFI and pol (7–9) is then required to direct the pTP-pol heterodimer to its DNA recognition site in the core of the viral replication origin (10). By an as yet undetermined mechanism the single stranded origin template is exposed and pol catalyses the transfer of dCMP onto the β-hydroxyl group of a serine residue in pTP. Once initiated DNA synthesis is continued by pol in a highly processive manner. DBP functions at multiple stages in the replicative process participating directly both in initiation and elongation. During initiation DBP increases the affinity of NFI for its binding site in the origin of DNA replication (11, 12) and decreases the Km for dCTP in the transfer of dCMP onto pTP (13). Once elongation has begun DBP increases the processivity of pol (14, 15) and maintains the displaced single strand in a protected form as a rigid nucleoprotein fibre (16). As the precise action of DBP in these processes has not been determined, we examined the interaction between DBP and a variety of templates containing double stranded DNA. DBP has the properties of a helix destabilising protein and can displace short DNA strands from their complementary sequences in the single stranded form of M13, in a reaction which requires neither ATP nor $MgCl_2$. Unwinding of DNA was highly cooperative and was inhibited by conditions which increased the stability of DNA duplexes. Completely double stranded DNA duplexes could also be unwound, but this activity was inhibited if the DNA fragments were bound by either NFI or NFIII. These activities were localised to the C-terminal domain of DBP that has

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previously been shown to participate in viral DNA replication. The ability of DBP to destabilise DNA duplexes may provide an explanation for the role of this protein at multiple stages in the process of viral DNA replication.

MATERIALS AND METHODS

Construction of a baculovirus containing the gene for the adenovirus type 2 DNA binding protein

The gene for the DBP was prepared in 2 fragments. The fragment containing the start codon and the first 573 bp of the gene was copied from Ad2 DNA by polymerase chain reaction (PCR), using the following oligonucleotide primers synthesised on an Applied Biosystems model 381A:

Primer 1: 5'GCGCGGATCCTATAATATGGCCAGTCGGGAAGAGGAGCAGCGCGA
Primer 2: 5' CATCAACGCGCGCGCAGCCTCCATGCCC

The PCR product was cut with XhoI and the 151 bp fragment corresponding to the 5' end of the DBP gene was gel purified (fragment 1). The second fragment was generated by cutting PUB54 (17) with KpnI and XhoI and gel purifying the fragment corresponding to 22233–23924 bp of the Ad2 genome. The linker oligonucleotide GATCGTAC was ligated to the fragment to convert the KpnI overhang to a BamHI overhang (fragment 2). Fragments 1 and 2 were ligated and the gel purified product digested with BamHI and cloned into the BamHI site of the transfer vector pVL1393 (Invitrogen). Orientation of the insert was determined by EcoRV digestion and plasmids containing the DBP gene in the correct orientation (pVLDBP), were purified from transformed bacteria by isopycnic centrifugation in caesium chloride.

Sf9 cells were maintained as monolayer and suspension cultures at 28°C in TC-100 medium (GIBCO-BRL) supplemented with 10% fetal calf serum and 50μg/ml penicillin and streptomycin. AcRPlacZ DNA linearized with Bsu36I (0.5μg) and the plasmid pVLDBP (2μg) were cotransfected into Sf9 cells using lipofectin (GIBCO-BRL) and the recombinant baculovirus was purified by two rounds of plaque purification (18, 19).

Expression and purification of DBP and 39 kDa fragment

Suspension cultures (500ml) of Sf9 cells were grown to a density of 8×10^5 cells per ml. The cells were collected by centrifugation and infected at a multiplicity of 5 plaque forming units per cell, in 50ml of TC100 for 2 hours at 28°C, before medium containing 5% fetal calf serum was added to 500ml and the infection was allowed to proceed for 72 hours at 28°C. Infected cells were collected by centrifugation at 2500 rpm for 15 minutes and washed in phosphate-buffered saline.

The washed cell pellet from 500ml of Sf9 cells, infected with the DBP recombinant baculovirus, was resuspended in 4ml of ice-cold 50mM Tris/HCl pH8, 5mM KCl, 0.5mM $MgCl_2$, 0.5mM DTT, 1mM PMSF and homogenised with 20 strokes of a Dounce homogeniser using a type B pestle. The nuclei were sedimented at low speed in a microcentrifuge and the supernatant clarified by centrifugation at 60000g for 30 minutes at 4°C. The resulting cytoplasmic extract was applied to a DEAE–sepharose column (10ml) equilibrated with 25mM Tris/HCl pH8, 1mM DTT, 1mM EDTA, 20% glycerol, 1mM PMSF, 50mM NaCl (buffer A). The column was washed with 2 column volumes of buffer A and eluted with the same buffer made 0.2M with respect to NaCl. Fractions containing the DBP were pooled and applied to a 10ml single-stranded DNA–sepharose column equilibrated with the buffer A containing 0.2M NaCl. The column was washed with 2 volumes of equilibration buffer and eluted with a 50ml linear gradient from 0.2–2M NaCl. DBP containing fractions were dialysed against 25mM Tris/HCl pH8, 100mM NaCl, 50% glycerol and stored at −20°C.

Purified baculovirus expressed DBP (10mg) prepared as described above was diluted to 1mg/ml in 25mM Tris/HCl pH8, 1mM DTT, 1mM EDTA, 20% glycerol and incubated with chymotrypsin (10μg/ml) for 30 minutes at room temperature. Digestion to the 39-kDa fragment was monitored by SDS-PAGE, the reaction was stopped by the addition of PMSF to 2mM and sample was dialysed against Buffer A made 0.2M with respect to NaCl. The 39-kDa DNA binding fragment was purified by single-stranded DNA–sepharose chromatography, dialysed and stored as described for the full length DBP above. Sequence analysis of the 39-kDa fragment was carried out by Bryan Dunbar at the SERC Protein Sequencing Facility (University of Aberdeen).

Nuclear factor I (NFI)

Nuclear Factor 1 was purified as described, (7).

Nuclear factor III (NFIII)

NFIII (aa 270–582) fused to glutathione-s-transferase (gift from P.O'Hare) was isolated from *E.coli* by affinity chromatography on glutathione agarose and cleaved with thrombin. The NFIII preparation was dialysed against 10mM sodium phosphate buffer (pH 7.5) containing 0.5M NaCl and glutathione-s-transferase removed by passage over a second glutathione agarose column.

Preparation of substrates to determine strand displacement activity

DNA substrates were prepared by labelling oligonucleotides, (5 pmol) at their 5' termini with [γ ^{32}P] ATP, specfic activity 3000 Ci/mmol, using T4 polynucleotide kinase. Labelled oligonucleotides were isolated in an 8% non-denaturing polyacrylamide gel and eluted overnight in 10 mM Tris–HCl (pH8.0), 1 mM EDTA, 100 mM NaCl. DNA hybrids were formed by annealing 5 pmol of 5'-^{32}P-labelled oligonucleotides, with 1 pmol of complementary single stranded M13 mHR1 DNA (20), in 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl. Following three minutes at 100°C, the mixture was slowly cooled to room temperature and ^{32}P-labelled M13 DNA hybrids were separated from unannealed oligonucleotide by gel filtration on a Sepharose CL-4B column equilibrated in 10 mM Tris–HCl (pH8.0), 1 mM EDTA, 100 mM NaCl.

Preparation of substrate for determination of directionality

8 pmol of the 42mer oligonucleotide (oligo A) was labelled at the 5'-end with T4 polynucleotide kinase and [γ ^{32}P] ATP. The labelled oligonucleotide was annealed with 2 pmol of single-stranded circular M13mp19 DNA, by heating at 100°C for three minutes and slow cooling to room temperature. Annealed DNA was digested with SmaI for three hours at 30°C and the 3'-OH termini of the DNA labelled in the presence of [α-^{32}P] dCTP and five units of DNA polymerase 1 (large fragment). Annealed DNA was separated from unannealed DNA by size exclusion on a 5ml column of Sepharose CL-4B.

Preparation of linear double-stranded DNA templates

DNA templates were prepared by digesting pEXΔ plasmids (21) with EcoR1 and BamH1. DNA fragments released were labelled

at their 3′ termini in the presence of [α ^{32}P] dATP, [α ^{32}P] dCTP and 5 units of DNA polymerase 1 (large fragment), at 23°C for 20 mins. The dATP and dCTP concentrations were increased by the addition of unlabelled nucleotide to 100 μM and the incubation carried out for an additional 20 mins at 23°C. Labelled DNA fragments were isolated from a non-denaturing polyacrylamide gel and eluted overnight in 10 mM Tris−HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl.

DNA unwinding assay

Unless otherwise indicated, reactions (30μl) were performed at 37°C for 30 mins in 40 mM Tris−HCl (pH 8.0), 5 mM DTT, 1 mg/ml bovine serum albumin, containing 10 fmol of DNA substrate and the indicated concentrations of DBP. Reactions were terminated by the addition of 5 μl of 33 mM EDTA (pH 8.0), 6% sodium dodecyl sulfate, 25% glycerol, 0.5% bromophenol blue, 0.5% xylene cyanol and the products separated through an 8% non-denaturing polyacrylamide gel. Following electrophoresis, gels were dried directly onto DE81 paper (Whatman) and exposed to X-ray film in the presence of an intensifying screen at −70°C. Radioactivity in dried gel slices was determined by liquid scintillation counting in Ecosint A.

Gel electrophoresis DNA-binding assay

The DNA binding activity of NFI and NFIII under DNA unwinding assay conditions, was determined by electrophoresis in a non-denaturing polyacrylamide gel. After incubation, 5 μl of 30% glycerol, 0.25% bromophenol blue and 0.25% xylene cyanol was added and applied to a 6% polyacrylamide gel in 50 mM Tris Borate, 0.5 mM EDTA (pH8.3). Gels were processed as above.

Plasmids

Plasmids pEX18Δ, 36Δ, 58Δ contain the terminal 18, 36 and 58 base-pairs of the Ad2 inverted terminal repeat (21), respectively. These were digested with EcoR1 and BamH1 to generate linear double-stranded DNA fragments.

Oligonucleotides

All oligonucleotides were synthesised on an Applied Biosystems 381A. The 39mer oligonucleotide used in the strand displacement assay contained th sequence 5′-ATTGGCTTCAATCCAAAATAAAGTATATTATTGATGATG-3′ and the 42mer oligonucleotide (oligo A), which was complementary to M13mp19 DNA, had the sequence 5′-TCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGG-3′ . Double stranded oligonucleotides containing the binding site for NFI (7) and NF-$\varkappa$B (22) were as described. Double stranded oligonucleotides containing the binding site for NFIII contained the sequence 5′-GATCTGAGTTAATATGCAAATAAGGCGTGAG-3′, (top strand).

RESULTS

Helix destabilisation activity of the adenovirus type 2 DNA binding protein

Although adenovirus type 2 (Ad2) DNA binding protein (DBP) can be purified in high yield from adenovirus infected HeLa cells, it is not straightforward to genetically manipulate DBP in this genetic background. The gene for DBP was therefore inserted into a modified baculovirus genome, which was used to isolate recombinant baculovirus that would express DBP in infected insect cells. DBP was purified from infected insect cells (figure 1), by a simple two column chromatographic procedure, to yield material (10 mg purified DBP per litre of infected insect cells) that was active in DNA binding and adenovirus type 2 DNA replication in vitro (data not shown). Treatment of DBP with chymotrypsin generated a proteolytic fragment that retained DNA binding activity (figure 1) and had the N-terminal sequence Ser−Leu−Pro−Ile−Val, confirming that it was the 39 kDa fragment described previously (23). To further characterise the biochemical properties of the two proteins that might be involved in viral DNA replication, both the full-length DBP and 39 kDa chymotryptic fragment of DBP were examined for their ability to unwind short stretches of double stranded DNA. The substrate utilised to assay for this activity was a ^{32}P- labelled 39 mer oligonucleotide annealed to the single stranded form of M13 DNA. This substrate was therefore incubated with concentrations of both DBP and 39 kDa DBP that were sufficient to completely coat the M13 single stranded DNA, assuming that DBP has a target size of twelve nucleotides (16), and the ^{32}P labelled DNA products of the reaction analysed on a polyacrylamide gel after removal of bound protein by denaturation in the presence of SDS. Both DBP and 39 kDa DBP were capable of releasing the 39 mer oligonucleotide from the M13 DNA substrate, resulting in the appearance of free oligonucleotide (lanes 2 and 5, figure 2). Incubation of the substrate in the absence of DBP did not result in the release of labelled 39 mer oligonucleotide (lanes 3 and 6, figure 2), indicating that the DNA duplex was stable under the conditions employed for the reactions.

Characterisation of the DNA unwinding activity of DBP

One of the surprising properties of the DNA unwinding activity described above was that it appeared to take place in the absence of any added energy source. To investigate this further reactions were carried out in the presence of a variety of nucleotide triphosphates and analogues, and DBP catalysed DNA unwinding

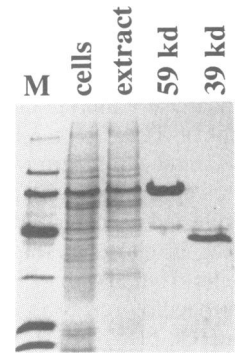

Figure 1. Purification of DBP and 39 kDa DBP. *Spodoptera frugiperda* cells were infected with a recombinant baculovirus containing the gene for DBP, (cells). At 72 hours post infection, cell extracts (extract) were prepared and DBP purified by column chromatography, on DEAE Sepharose and denatured DNA Sepharose, (59 kDa). The 39 kDa DBP was generated by digestion of the 59 kDa DBP with chymotrypsin and repurifying on the denatured DNA−sepharose column, (39 kDa). Fractions from each stage of the purification were analysed on an 8% SDS polyacrylamide gel and proteins stained with Coomassie brilliant blue for comparison with molecular weight markers (M, 200 kDa, 92 kDa, 68 kDa, 43 kDa, 29 kDa, 18 kDa and 14 kDa).

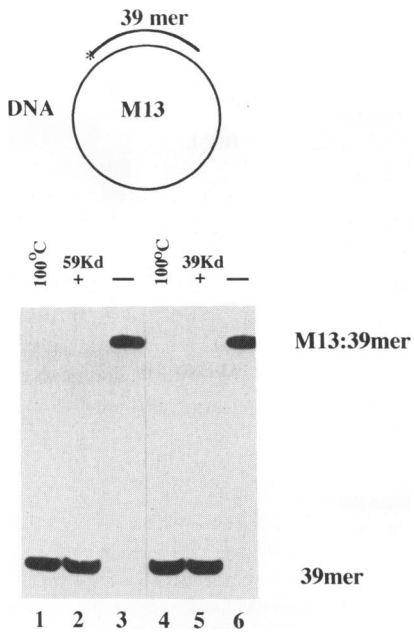

Figure 2. DNA unwinding by DBP. The assay was performed in the presence of 10 fmol M13−39mer DNA substrate and 34 pmol of either full length DBP (59 kDa) or the 39 kDa chymotryptic fragment (39 kDa) as described in Material and Methods. The substrate for the assay is shown in the upper part of the figure and consists of a 5′-^{32} P labelled 39 mer oligonucleotide, annealed to the single stranded form of phage M13 DNA. Lanes 1 and 4 are the heat-denatured substrate and lanes 3 and 6 are the native substrate incubated in the absence of DBP. Lane 2 is the reaction with DBP (59 kDa) and lane 5 the reaction with the 39 kDa chymotryptic fragment of DBP (39 kDa).

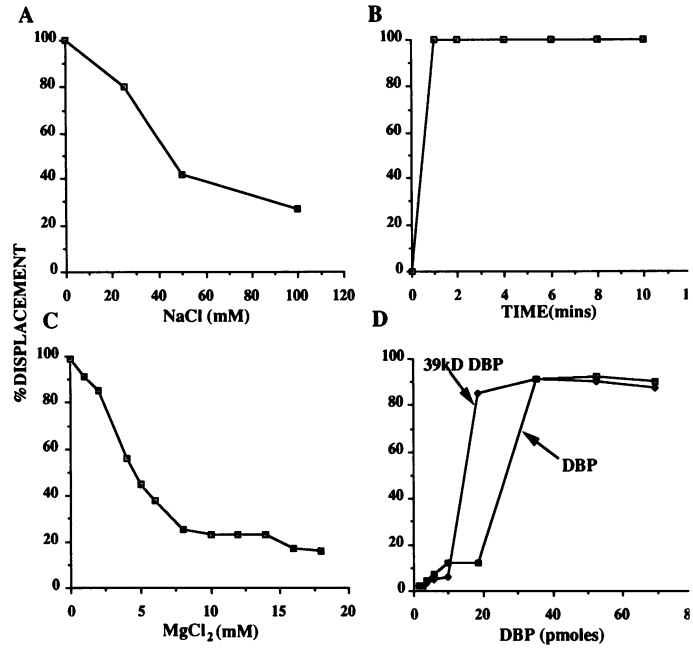

Figure 3. Factors affecting DNA unwinding by DBP. Assays were carried out as described in the legend to figure 2 apart from variation of NaCl concentration (**A**), time of incubation (**B**), $MgCl_2$ concentration (**C**) and amount of DBP added to the assay (**D**). The fraction of the 5′-^{32}P labelled oligonucleotide displaced from its complementary sequence in M13 phage DNA was calculated after separation of the reaction products on a native polyacrylamide gel and determination of the radioactivity in excised bands representing M13:39 mer hybrid and the free 39 mer.

quantitated after electrophoretic separation of the products. DNA unwinding activity was not stimulated by the addition of ATP, CTP, UTP, dATP, dCTP or the presence of an ATP regeneration system and activity was not influenced by the addition of the ATP analogue ATPγS or EDTA. The reaction was not stimulated by the addition of $MgCl_2$ and the data indicate that the DNA unwinding activity of DBP requires neither an energy source nor a divalent cation. In fact addition of $MgCl_2$ has a strong inhibitory effect on the DNA unwinding activity, with 80% inhibition at 8 mM $MgCl_2$ (figure 3C). Inclusion of NaCl in the reaction also reduces DNA unwinding activity of DBP, with less than 30% of the activity remaining at 100 mM NaCl (figure 3A). Titration of increasing amounts of both the DBP and the 39 kDa proteins into the reaction, revealed that strand displacement only took place after the DNA was completely covered with bound protein (figure 3D), although it is not clear why the 39 kDa fragment appears to be fully active at a lower concentration than DBP. At saturating DBP concentrations the unwinding reaction is rapid with all of the 39 mer oligonucleotide released from the M13 DNA within 1 minute (figure 3B).

Involvement of the C-terminal region of DBP in DNA unwinding

To identify which region of the protein was involved in the DNA unwinding activity and to confirm that DNA unwinding was being catalysed by DBP, rather than a copurifying contaminating protein, the DBP specific B6-10 monoclonal antibody (24) was included in the unwinding reactions. While inclusion of purified

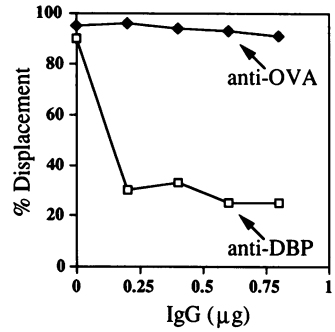

Figure 4. Inhibition of DBP catalysed DNA unwinding by a monoclonal antibody (B6-10) directed against the C-terminal region of DBP. The indicated amount of IgG, purified by elution from protein-G Sepharose, from monoclonal antibodies directed against DBP (anti-DBP) or ovalbumin (anti-OVA) was added to DNA unwinding reactions and the fraction of 39 mer DNA displaced from its complementary sequence in M13 calculated as described in the legend to figure 3.

IgG from a monoclonal antibody raised against ovalbumin had no effect on the unwinding activity of DBP, equivalent amounts of antibody specific for DBP strongly inhibited strand displacement activity (figure 4). The epitope recognised by the B6-10 antibody is present within the C-terminal chymotryptic fragment of DBP (24) and thus confirms that this region of the protein, which retains DNA binding activity, participates in the unwinding activity of DBP.

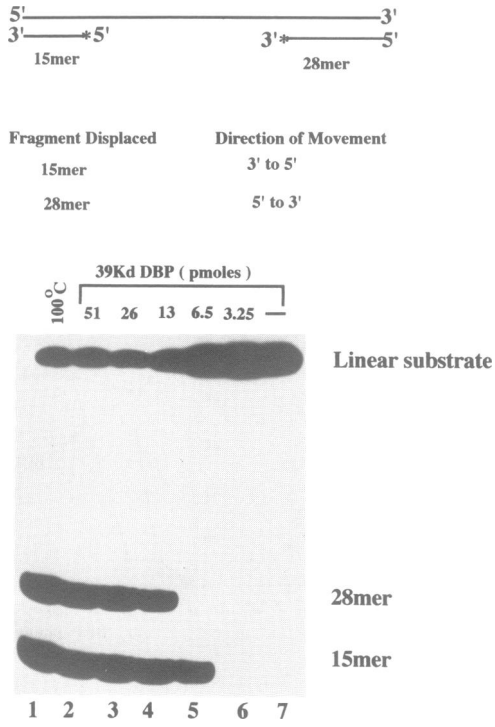

Figure 5. Directionality of DBP catalysed DNA unwinding. The assay was performed in the presence of an increasing concentration of 39 kDa DBP with the template, illustrated at the top of the figure, as described in Material and Methods. Lanes 1 and 7 are the heat-denatured and native substrate, respectively. Lanes 2–6 contain 51, 26, 13, 6.5 and 3.25 pmol of 39 kDa DBP respectively.

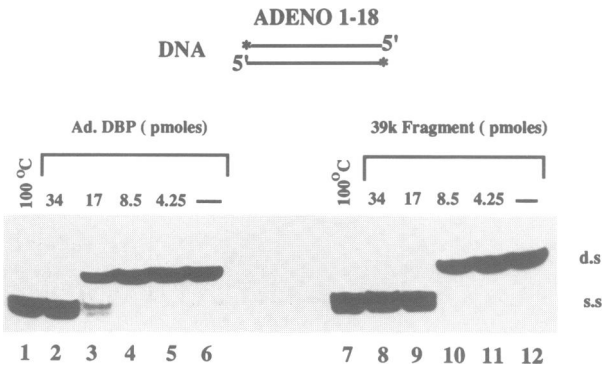

Figure 6. DBP and 39 kDa DBP can unwind completely double stranded DNA. Reactions were performed in the presence of 3 pmol of a 3'-^{32}P labelled 27 base pair DNA duplex containing bases 1–18 from the Ad2 origin of DNA replication and the indicated amounts of either DBP or 39 kDa DBP, as described in Material and Methods. After the reaction duplex DNA (d.s) and single stranded DNA (s.s) were separated on a 16% non-denaturing polyacrylamide gel.

Directionality of DNA unwinding reaction

The circular nature of the partial duplex routinely used as a helicase substrate (figure 2), does not allow the assignment of directionality to the DNA unwinding activity. A linear DNA substrate with short duplex regions at either end and a large internal single stranded region was therefore constructed (figure 5). The duplex regions of the substrate contain ^{32}P-labelled DNA of 28 and 15 nucleotides, such that if DBP bound to the single stranded region and migrated on the DNA in a 5′ to 3′ direction then the 28 nucleotide fragment would be displaced,

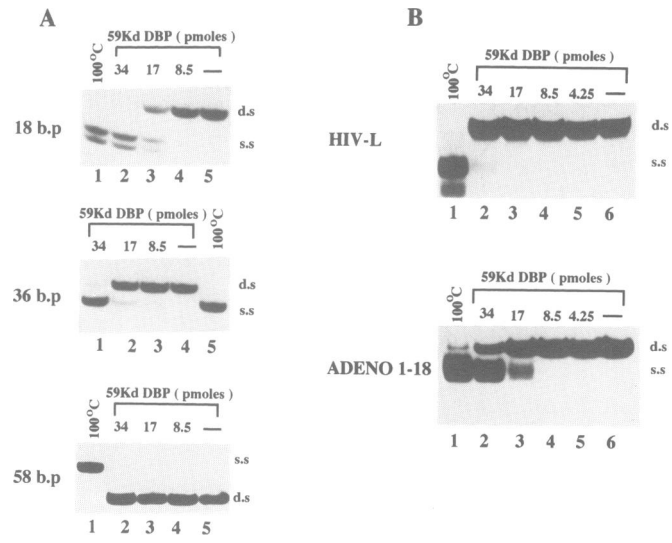

Figure 7. Effect of duplex length and sequence on DNA unwinding catalysed by DBP. Reactions were performed with fully duplex DNA increasing in length (**A**) and two templates with similar lengths but different DNA sequences (**B**), at the indicated amounts of DBP as described in Materials and Methods. Duplex DNA (d.s) and single stranded DNA (s.s) were separated on 16% non-denaturing polyacrylamide gels, apart from the 58 b.p template, which was analysed on a 12% gel. In this case the relative migration of single and double stranded DNA was reversed. The DNA templates used in A were 27 base pairs long containing base pairs 1–18 of the Ad2 origin (18 b.p), 45 base pairs long containing base pairs 1–36 of the Ad2 origin (36 b.p) and 67 base pairs long containing base pairs 1–58 of the Ad2 origin (58 b.p). The DNA templates used in B were a 23 base pair DNA duplex from the NF-κB site in the HIV-1 enhancer (HIV-L) and a 27 base pair DNA duplex containing base pairs 1–18 from the Ad2 origin (ADENO 1–18).

whereas if DBP migrated in a 3′ to 5′ direction on the single stranded DNA then the 15 nucleotide fragment would be released (figure 5). The high affinity of DBP for single stranded DNA and the slow dissociation of the single stranded DNA–DBP complex once formed (16), should ensure that stable binding of DBP takes place on the single stranded region of this substrate. In fact incubation of this substrate with the 39 kDa DBP resulted in both fragments being displaced, although the smaller fragment, being more easily unwound, was detected at a lower concentration of 39 kDa DBP (figure 5). Thus it appears that unlike ATP dependent helicases the strand displacement activity of adenovirus DBP is not strictly directional.

DBP promoted unwinding of linear duplex DNA

In the assays described above the substrate used is largely single stranded with only a short stretch of double stranded DNA. Although DBP binds cooperatively and tightly to single stranded DNA it has also been shown that DBP can bind to double stranded DNA (11, 12). It was therefore of interest to determine if DBP could unwind a completely double stranded DNA fragment. A ^{32}P-labelled 27 base pair DNA fragment, which contains base pairs 1–18 of the adenovirus type 2 origin of replication, was incubated with both DBP and the 39 kDa proteolytic fragment and the products separated on a native polyacrylamide gel, which is capable of resolving single and double stranded DNA (figure 6). Both DBP and the 39 kDa proteolytic fragment of DBP were capable of unwinding the short DNA duplex, although again it was noticed that the 39 kDa fragment appeared to more active in the assay than DBP, with maximal activity being acheived at

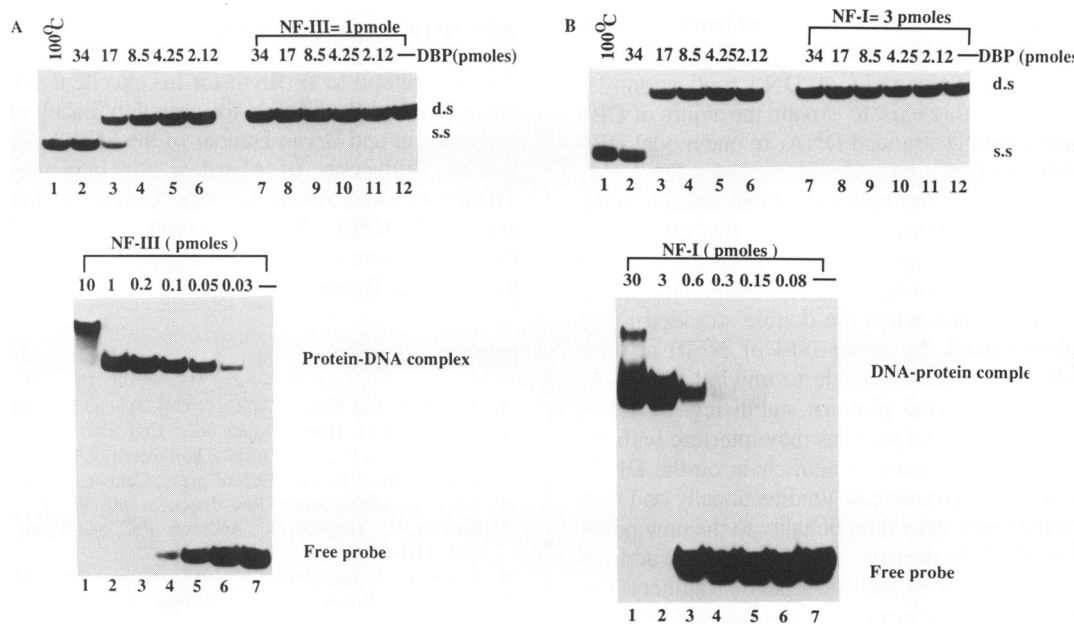

Figure 8. Effect of sequence specific DNA binding proteins on unwinding of duplex DNA by DBP. Duplex DNA containing either the binding site for NFIII (**A**) or NFI (**B**) were incubated with the indicated amounts of DBP, either in the presence or absence of NFI or NFIII. The amount of NFIII (A) or NFI (B) to be used in these assays was determined by gel electrophoresis DNA binding assays (lower panels).

a twofold lower concentration of protein. Under the conditions of the assay the duplex was stable in the absence of DBP (figure 6). To determine the size of double stranded DNA that could be unwound by DBP a series of ^{32}P- labelled double stranded DNA fragments of increasing size were prepared from deleted versions of the adenovirus type 2 origin of DNA replication (21). Three linear templates, figure 7(A), containing 27 (1−18 Ad2 origin), 45 (1−36 Ad2 origin) and 67 (1−58 Ad2 origin) base-pairs of double-stranded DNA were incubated with DBP and the products of the reactions fractionated on native polyacrylamide gels. DBP unwound the 27 and 45 base-pair templates, but not the 67 base-pair substrate. Larger DNA substrates of 100 and 400 base-pairs, could not be unwound by DBP even at high protein concentrations, although it should be noted that the same DNA fragments could be unwound when they were annealed to a single stranded M13 template (data not shown). To determine if the observed strand displacement activity on completely double stranded DNA fragments was dependent on base composition of the DNA, Ad DBP was incubated with two linear, duplex templates of similar size but different base composition. One DNA fragment of 23 base pairs represented the binding site for the transcription factor NF-$\varkappa$B (HIV-L) and had a G+C content of 57%, while the other fragment of 27 base pairs contained the first 18 base pairs of the adenovirus type 2 origin (ADENO 1−18) and had a G+C content of 30%. While DBP could unwind the strands of the DNA duplex with low G+C content (ADENO 1−18, figure 7B), it was unable to unwind the strands of the DNA duplex with high G+C content (HIV-L , figure 7B). DBP therefore appears to be unable to unwind completely double stranded DNA fragments of high structural stability.

Unwinding of DNA duplexes by DBP is inhibited by bound proteins

Prior to initiation of adenovirus DNA replication the origin is bound by the sequence specific DNA binding proteins NFI and NFIII. It was therefore of interest to determine if DBP could unwind DNA duplexes which were already bound by these proteins. DBP was incubated with double stranded oligonucleotides containing the binding sites for NFI and NFIII, either in the absence or the presence of the cognate bound protein. To ensure that the specific binding sites were fully occupied by either NFI or NFIII increasing quantities of the two proteins were incubated with the ^{32}P- labelled double stranded oligonucleotide and the formation of DNA−protein complexes determined in a gel electrophoresis DNA binding assay. Amounts of NFI (1 pmol) and NFIII (3 pmol) were chosen that resulted in full site occupancy but higher order complexes, indicative of non-specific binding, were not detected (figure 8, A and B lower panels). DBP was incubated either with the double stranded oligonucleotides or with the double stranded oligonucleotides already bound to NFI or NFIII. While DBP can unwind the DNA duplexes it is unable to do so when either NFI or NFIII are bound to their cognate sites (figure 8 A and B, upper panels). Thus it seems unlikely that DBP alone is responsible for helix opening at the origin prior to initiation of DNA replication.

DISCUSSION

The adenovirus DBP has been shown to possess the properties of a helix destabilising protein. When templates contain a large amount of single stranded DNA the double stranded portion is efficiently unwound in a highly cooperative reaction. Completely double stranded templates are also unwound but this reaction is restricted by the length and G+C content of the DNA fragment. Like other helix destabilising proteins, DBP promoted unwinding requires neither ATP nor $MgCl_2$. In fact the latter is inhibitory to the process as are other agents, such as NaCl, which increase the stability of duplex DNA molecules. Very similar properties

have recently been ascribed to both the HeLa and herpes simplex virus coded single strand specific DNA binding proteins, which appear to be involved in cellular and viral DNA replication (25, 26). Two possibilities therefore exist to explain the ability of DBP to unwind completely double stranded DNA. In one model DBP could utilise its ability to first bind to double stranded DNA (11, 12), invade the DNA duplex and bind in a stable fashion to the exposed single strands. An alternative model is that DBP binds to transiently single stranded regions of DNA that are exposed during 'breathing' of short double stranded DNA molecules. What is clear however is that when the double stranded DNA fragments are tightly bound, by either NFI or NFIII at their cognate recognition sites, DBP is unable to unwind the DNA. This could be due to the bound proteins stabilising the DNA duplex or alternatively the bound proteins may interfere with the ability of DBP to form a continuos protein chain on the DNA. Unlike DNA helicases which translocate unidirectionally on DNA there does not appear to be a strict directionality to the unwinding reaction catalysed by DBP. In the experiment designed to address this point (figure 4) it was noted that the smaller fragment was released from the template at lower concentrations of DBP than the larger DNA fragment. While this is interpreted as being a consequence of the lower T_m of the smaller fragment, it cannot be ruled out that initial binding of a single molecule of DBP is followed by unidirectional polymerisation of the protein on the single stranded DNA. Determination of the three dimensional structure of DBP by X-ray crystallography (27) has suggested a mechanism for the cooperative binding of DBP to single stranded DNA. It is thought that an extension at the C-terminus of one DBP molecule 'hooks' into a complementary surface on an adjacent DBP molecule and in doing so initiates formation of a protein chain of DBP molecules. The ability of DBP to impose a rigid structure on DNA has recently been shown to be responsible for the ability of DBP to also promote renaturation of complementary single strands (28). However, whereas the denaturation reaction described here is inhibited by high concentrations of monovalent and divalent cations the renaturation reaction described above is highly resistant to their presence (28).

The ability of DBP to destabilise double stranded DNA duplexes could be utilised at a number of points in the viral replicative cycle. While DBP has been shown to stimulate the initiation of Ad2 DNA replication in vitro by decreasing the Km for transfer of dCMP onto pTP and by increasing the binding of NFI to the replication origin, neither of these reactions are likely to involve the melting of duplex DNA. However DBP may stimulate the initiation of DNA replication by additional means and it has previously been demonstrated that Ad4 DBP dramatically stimulates initiation of DNA replication. In this case the extent of stimulation is independent of the concentrations of NFI and dCTP (29). Although not supported by published evidence, one possibility is that DBP may participate with other replication proteins in the unwinding of the DNA double helix that is expected to take place at the termini of the genome prior to initiation. The requirement for DBP during progress of the replication fork has been well established and it is likely to be a consequence of DBP stabilising displaced single strands and altering the properties of the viral DNA polymerase. In the latter case DBP has been shown to convert pol into a form that is capable of strand displacement and highly processive DNA synthesis (15, 16). Both of these activities may well be a consequence of the ability of DBP to transiently destabilise double stranded DNA at the advancing replication fork.

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