Interactions of Bacteriophage T7 DNA Primase/Helicase Protein with Single-Stranded and Double-Stranded DNAs†

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ABSTRACT: Protein–DNA interactions of bacteriophage T7 DNA primase/helicase protein 4A′ with small synthetic oligodeoxynucleotides were investigated using a 20-base-paired hairpin duplex, and 10-, 30-, and 60-base-long single-stranded DNA. The effect of nucleotide cofactors on DNA binding was examined using membrane binding assays which showed that 4A′ binds DNA optimally only in the presence of MgdTMP-PCP, the nonhydrolyzable analog of dTTP. About 20% of single-stranded DNA binding was observed in the presence of MgdTDP, but none was detectable in the absence of nucleotides. Native polyacrylamide gel electrophoresis showed that the DNAs bind predominantly to the hexameric form of 4A′. Larger oligomers of 4A′ can bind DNA, but no DNA binding was observed to species smaller than the hexamer. Quantitative equilibrium binding studies at increasing 4A′ concentrations and at increasing DNA concentrations showed tight binding of one 10-mer or 30-mer per hexamer. The 4A′ hexamer can bind a second strand of DNA, but with a 50-fold weaker affinity than the first strand. The 60-mer showed tight binding to two 4A′ hexamers, suggesting that a hexamer may interact with only 30–40 bases of single-stranded DNA. This was corroborated by nuclease protection experiments where the smallest length of DNA protected by 4A′ or 4B protein was found to be about 30 bases. Equilibrium binding studies and competitive DNA binding data are consistent with a weaker affinity of 4A′ for the duplex DNA. Only 20–25% of duplex DNA binding was observed at increasing 4A′ protein in the presence of MgdTMP-PCP. About four duplex DNAs can bind each 4A′ hexamer at increasing DNA concentrations, but their weaker binding was evident from their facile dissociation from 4A′ in the presence of competing single-stranded DNA.

DNA unwinding is catalyzed by a class of enzymes known as DNA helicases (Kornberg & Baker, 1992; Matson & Kaiser-Rogers, 1990). The unwinding process involves disruption of hydrogen bonds between bases of duplex DNA and perhaps stabilization of the resulting single-stranded DNA regions. Even though the enzymatic mechanism of DNA unwinding is not fully understood, it is very likely that protein–DNA interactions play a key role during the enzymatic process. The protein–DNA interactions can be modulated by the NTPase activity present in all helicases. For instance, *Escherichia coli* Rep helicase shows differential binding to single-stranded or double-stranded DNAs in the presence of ADP or AMP-PNP, the nonhydrolyzable analog of ATP (Wong & Lohman, 1992). In addition, the oligomeric structure of helicases may assist the unwinding process by providing multiple DNA binding sites (Lohman, 1992, 1993). In order to understand the enzymatic mechanism of DNA unwinding, a quantitative understanding of the interactions between helicase and DNA is essential. Such detailed DNA (or RNA) binding studies have been carried out only with a few helicases such as the dimeric Rep helicase (Wong *et al.*, 1992) and hexameric *E. coli* Rho protein, which is a transcription terminator and also an RNA–DNA helicase (Geiselmann *et al.*, 1992).

Our studies are focused on the bacteriophage T7 gene 4 proteins which are involved in DNA replication. The helicase activity in T7 is present in two phage-encoded proteins, 4A and 4B (Bernstein & Richardson, 1988, 1989). Both proteins are products of the same gene, and both are synthesized in the same reading frame (Dunn & Studier, 1983). 4A is the full-length product of T7 gene 4 containing helicase and primase activities, and 4B is a smaller protein synthesized from an internal initiation site, and it contains only helicase activity. All the experiments in the present study have been performed using 4A′ protein, which is an M64L mutant of 4A. Methionine-64 in 4A is the initiation codon for 4B, and it was mutated to leucine to allow expression and purification of 4A alone. The primase and helicase activities of 4A′ were shown previously to be unchanged from the wild-type gene 4 proteins (Rosenberg *et al.*, 1992; Patel *et al.*, 1992). It was recently shown that the gene 4 proteins, 4A′ and 4B, self-assemble into stable hexamers in the presence of MgdTMP-PCP[1] and ss-DNA (Patel & Hingorani, 1993). It is not known, however, if it is the hexameric helicase that interacts with the DNA, whether it interacts with both single-stranded and double-stranded DNA, and if the hexameric structure is important for DNA unwinding.

In this paper, we investigate in detail the interactions of 4A′ protein with various small synthetic DNAs, both in the absence and in the presence of nucleotide cofactors. Studies in the literature indicate that gene 4 proteins bind DNA only in the presence of nucleotides, such as dTMP-PCP, dTTP, or dTDP (Matson & Richardson, 1985). The results described in this paper are partly in agreement as we did observe DNA binding in the presence of dTMP-PCP, but not in the presence

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[1] Abbreviations: dTMP-PCP, thymidine 5′-(β,γ-methylenetriphosphate); BSA, bovine serum albumin; DEAE, diethylaminoethyl; PAGE, polyacrylamide gel electrophoresis; ss-DNA, single-stranded DNA; ds-DNA, double-stranded DNA; hp, hairpin.

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of dTDP, as reported previously. Because 4A′ exists in a variety of oligomeric forms, including hexamer (Patel & Hingorani, 1993), native polyacrylamide gel electrophoresis was used to determine which 4A′ oligomeric species bind DNA. Quantitative equilibrium binding and competitive DNA binding studies were carried out to determine the affinity of 4A′ protein for single-stranded and double-stranded DNA. Our results indicate that 4A′ protein binds ss-DNA predominantly in the hexameric form. Each hexamer can bind two strands of ss-DNA, although one DNA strand is bound much more tightly than the second strand. The affinity of 4A′ protein for ds-DNA is much weaker than for ss-DNA; hence, the duplex DNA is easily competed off by ss-DNA.

EXPERIMENTAL PROCEDURES

Enzymes. 4A′ and 4B proteins were purified to homogeneity from the overexpressing clones pAR5018/HMS174(DE3) and pAR3708/HMS174(DE3), respectively (Rosenberg *et al.*, 1992). The purification procedure has been described earlier (Patel et al., 1992). Protein concentrations were determined from multiple absorbance measurements at 280 nm using the calculated extinction coefficients of 4A′ (83 600 M^{-1} cm^{-1}) and 4B (69 000 M^{-1} cm^{-1}), as described (Patel *et al.*, 1992). Marker proteins for native gel electrophoresis were purchased as part of a nondenaturing gel molecular weight determination kit from Sigma Chemicals. T4 polynucleotide kinase (10 units/μL) used to 5′-radiolabel the oligonucleotides and nuclease S7 (micrococcal nuclease) were purchased from Boehringer Mannheim.

Oligodeoxynucleotides. The oligodeoxynucleotides used in the binding assays and nondenaturing gel electrophoresis were synthesized at the Biochemical Instrument Center at The Ohio State University. The DNA sequences are as follows: 60-mer; 5′-AATTC-GTAAT-CATGG-TCATA-GCTGT-TTCCT-CATGA-CGATT-ACCTG-AACCA-TC-CTG-ACTCT; 30-mer, 5′-AGCTT-GCATC-ATAGT-GT-CAC-CTGTT-ACGTT; 10-mer, 5′-ATAGT-GTCAC; hairpin duplex (hp), 5′-GAATT-CGCCA-GTGTC-ATGCG-TTTCG-CATGA-CACTG-GCGAA-TTC. The 60-mer, 30-mer, 10-mer, and hp were purified on 12%, 16%, 18%, and 16% polyacrylamide/7 M urea gels, respectively. The oligodeoxynucleotides were electroeluted from the gels (Elutrap; Schleicher & Schuell), ethanol-precipitated with sodium acetate, desalted using Centricon-3 concentrators (Amicon), and reconstituted in TE buffer. DNA concentrations were determined spectroscopically from absorbance measurements at 260 nm and the calculated molar extinction coefficients of 60-mer (616 890 M^{-1} cm^{-1}), 30-mer (305 010 M^{-1} cm^{-1}), 10-mer (108 920 M^{-1} cm^{-1}), and hp (447 260 M^{-1} cm^{-1}). The absorbances of ss-DNA were measured in TE buffer, and the absorbance of hp DNA was measured in the same buffer that contained in addition 7 M urea.

Nucleotides and Other Materials. The radiolabeled nucleotide [γ-^{32}P]ATP (4000 Ci/mM) was purchased from ICN Radiochemicals. dTMP-PCP was purchased from U.S. Biochemical Corp. and dTDP from Sigma Chemical Co. The nitrocellulose (BA-S) and DEAE (NA-45) membranes used for the equilibrium binding assays were purchased from Schleicher & Schuell. Biogel P-30 resin was purchased from Bio-Rad.

Buffers. 10 × kinase buffer consisted of 0.5 M Tris-HCl, pH 7.6, 0.1 M magnesium chloride, and 1 mM EDTA, pH 8.0; 10 × binding buffer was 500 mM Tris–acetate, pH 7.5, 100 mM magnesium acetate, 50 mM sodium acetate, and 10 mM DTT; membrane wash buffer was 50 mM Tris–acetate, pH 7.5, 10 mM magnesium acetate, and 5 mM sodium acetate; Tris–glycine buffer was 25 mM Tris (base)/250 mM glycine, pH 8.3; TE buffer was 10 mM Tris-HCl, (pH 7.6)/1 mM EDTA, pH 8.0; and gel loading dye was 0.25% bromophenol blue/15% Ficoll (type 400; Pharmacia) in water.

5′-^{32}P Labeling of Oligodeoxynucleotides. Each oligodeoxynucleotide was 5′-labeled with [^{32}P]phosphate using T4 polynucleotide kinase and [γ-^{32}P]ATP. The reaction mixture contained 10 μM DNA, [γ-^{32}P]ATP (10–50 μCi), 1 × kinase buffer, and 1 μL of kinase (10 units) in a total volume of 50 μL. The reactions were incubated at 37 °C for 1 h. Unincorporated [γ-^{32}P]ATP was removed by gel filtration using a 0.5-mL Biogel P-30 column. Whatman DE-81 ion-exchange filters were spotted with aliquots of reactions before and after gel filtration. The filters were washed with 0.3 M ammonium formate buffer, pH 7.8, and radioactivity bound to filters was measured on a scintillation counter. The concentration of radiolabeled oligodeoxynucleotide was determined from the final volume of the DNA solution and the calculated specific activity (counts per mole).

Equilibrium DNA Binding Assays at Constant 4A′ and Constant DNA Concentrations. Nitrocellulose membrane binding assays were performed using a 96-well dot-blot apparatus (BRL) with a modified version of a previously described procedure (Wong *et al.*, 1992). Each well corresponded to one point in the assay. The membrane assembly consisted of Whatman No. 1 filter paper at the bottom overlaid with a DEAE membrane, followed by a nitrocellulose membrane. Before use, the nitrocellulose and DEAE membranes were soaked for 10 min in 0.5 M NaOH, washed extensively with double-distilled water, and finally equilibrated in the membrane wash buffer for at least 1 h at room temperature (~22 °C). Equilibrium binding assays at constant DNA and increasing 4A′ concentrations were conducted under the following conditions: (a) in the absence of any nucleotide; (b) in the presence of 1 mM dTMP-PCP; and (c) in the presence of 5 mM dTDP. The assays contained 1 μM radiolabeled DNA and 0–30 μM 4A′ in a 20-μL total volume of 1 × binding buffer (50 mM Tris–acetate, pH 7.5, 10 mM magnesium acetate, 5 mM sodium acetate, and 1 mM DTT). Binding assays at constant 4A′ and increasing DNA concentrations were performed using 2 μM 4A′ protein and 0–40 μM radiolabeled oligodeoxynucleotides in 1 × binding buffer in a 20-μL sample volume. The mixtures were incubated for 5 min at room temperature before the samples were filtered through the membrane assembly. The membranes were washed before and after application of samples with 100 μL of membrane wash buffer. After the samples were blotted, radioactivity on both nitrocellulose and DEAE filters was quantitated on a Betascope 603 blot analyzer (Betagen, Waltham, MA). Radioactivity on the nitrocellulose membrane gave a measure of the nucleoprotein complex concentration, and that on the DEAE membrane provided the free DNA concentration. These measurements were used to determine the fraction of DNA bound to 4A′ protein. Nonspecific DNA binding was determined for each experiment by filtering samples in which 4A′ was omitted. Nonspecific binding was about 1% of the total DNA in titrations at increasing protein concentrations and ranged from 0.01 to 10% in titrations at increasing DNA. Nonspecific DNA binding has been corrected in all the titrations reported.

Competitive Binding of ss-DNA and ds-DNA at Increasing 4A′ Protein Concentration. Titration of radiolabeled 60-mer (0.2 μM) with increasing 4A′ protein (0–10 μM) was carried out both in the absence and in the presence of a constant

amount of hp duplex DNA (0.2 μM). Similarly, titration of radiolabeleld hp (0.2 μM) with increasing 4A′ protein (0–10 μM) was carried out in the absence and in the presence of 60-mer DNA (0.2 μM). In titrations where both 60-mer and hp DNAs were present, the DNAs were mixed prior to addition of 4A′ protein. DNA binding was measured using the nitrocellulose and DEAE membrane assembly as described above.

Competitive Binding of ss-DNA and ds-DNA at Increasing 60-mer Concentrations. Competitive binding of ss- and ds-DNA was carried out by titrating 4A′ with increasing amounts of radiolabeled 60-mer (0–10 μM) in the presence of unlabeled hp duplex (0.5 μM). The two DNAs were mixed prior to additon of 4A′ (2 μM) and dTMP-PCP (1 mM) in 1 × binding buffer. The samples were spotted on the nitrocellulose–DEAE assembly, and the amount of 60-mer bound to 4A′ was quantitated as described above. The assay was also performed in the absence of hp duplex. A complementary assay was performed with unlabeled 60-mer (0–20 μM) and radiolabeled hp duplex (0.5 μM) to determine the amount of hp DNA bound to 4A′ in the presence of increasing amounts of 60-mer DNA.

Competitive Binding of ss-DNA and ds-DNA at Increasing hp Duplex Concentrations. Titration of 4A′ with increasing concentrations of radiolabeled hp duplex (0–10 μM) in the presence of unlabeled 60-mer (0.5 μM) was carried out by mixing the two DNAs prior to adding 4A′ (2 μM) and dTMP-PCP (1 mM) in 1 × binding buffer. The titration was also performed in the absence of 60-mer. The complementary experiment with unlabeled hp duplex (0–20 μM) and radiolabeled 60-mer (0.5 μM) was performed to quantitate 60-mer binding to 4A′ in the presence of increasing amounts of hp duplex DNA.

Data Analysis. Quantitation of radioactivity bound to nitrocellulose and DEAE membranes provided the molar amounts of 4A′–DNA complex and free DNA, respectively. Equilibrium binding titrations at constant DNA and increasing enzyme concentrations were analyzed by plotting the fraction of total DNA bound versus 4A′ monomer concentration. Titrations at constant 4A′ and increasing DNA concentrations were analyzed by plotting the fraction of DNA bound per 4A′ hexamer versus the total DNA concentration. The error bars represent standard errors of the mean determined from at least three separate binding experiments. The equilibrium dissociation constants were estimated manually by comparing the titration data to simulated curves. The simulated curves were generated using the KaleidaGraph software and the equations derived for a model where each 4A′ hexamer binds two DNAs:

$$E + D \overset{K_1}{\rightleftarrows} ED \quad (1)$$

$$ED + D \overset{K_2}{\rightleftarrows} ED_2 \quad (2)$$

where E and D refer to 4A′ hexamer and ss-DNA, respectively, and K_1 and K_2 are the equilibrium binding constants for the first and the second DNA binding, respectively. The fraction of DNA bound per 4A′ hexamer (D_b/E_t) in the two binding events was derived from eq 1 and 2 as follows:

$$K_1 = ED/E_fD_f$$

$$K_2 = ED_2/(ED)D_f$$

$$E_t = E_f + ED + ED_2$$

$$E_t = E_f + K_1E_fD_f + K_2K_1E_fD_f^2$$

$$D_b = ED + 2ED_2$$

$$D_b = K_1E_fD_f + 2K_1K_2E_fD_f^2$$

$$\frac{D_b}{E_t} = \frac{K_1D_f + 2K_1K_2D_f^2}{1 + K_1D_f + K_1K_2D_f^2}$$

E_t and E_f refer to total and free 4A′ hexamer concentrations, respectively, and D_b and D_f are bound and free DNA concentrations, respectively. Binding curves were simulated by plotting D_b/E_t *versus* total DNA concentrations at various values of K_1 and K_2 which were compared to the 10-mer and 30-mer titration data. The 60-mer titration data were compared to simulation curves obtained by assuming DNA binding to 4A′ dodecameric species rather than the hexameric species.

Nondenaturing Polyacrylamide Gel Electrophoresis. Native polyacrylamide gel electrophoresis (PAGE) was performed to investigate the oligomeric state of 4A′ protein both in the absence and in the presence of increasing amounts of DNA, and to determine which 4A′ oligomeric species bound DNA. The 6% polyacrylamide gel was prepared in buffer containing 375 mM Tris-HCl, pH 8.8, 100 μM dTMP-PCP, and 10 mM magnesium acetate, and the 3% polyacrylamide stacking gel was prepared in buffer containing 125 mM Tris-HCl, pH 6.8, 100 μM dTMP-PCP, and 10 mM magnesium acetate. Samples (10 μL) contained 12 μM 4A′, 1 mM dTMP-PCP, and 0–2 μM 5′-radiolabeled oligodeoxynucleotides in 1 × binding buffer (50 mM Tris–acetate, pH 7.5, 10 mM magnesium acetate, 5 mM sodium acetate, and 1 mM DTT). After addition of 2 μL of the native gel loading dye (0.25% bromophenol blue/15% Ficoll) to the samples, electrophoresis was performed in Tris–glycine buffer (pH 8.3) for 3 h at constant current (18 mA). Proteins were visualized by Coomassie Blue staining, and the 5′-^{32}P-radiolabeled DNAs were visualized by autoradiography.

The size of the protein species that predominantly bound DNA was determined from a Ferguson analysis (Ferguson, 1964; Hedrick & Smith, 1968). Standard proteins including lactalbumin (14 200), BSA (monomer, 66 000; dimer, 132 000), chicken egg albumin (45 000), carbonic anhydrase (29 000), and urease (trimer, 272 000; hexamer, 545 000) were analyzed by a series of native PAGE experiments. The 4A′ protein (in the presence and in the absence of 2 μM DNA) and the markers were run on 5.5%, 6%, 6.5%, and 7% polyacrylamide gels (375 mM Tris, pH 8.8) with 3% polyacrylamide stacking gels (125 mM Tris, pH 6.8). Electrophoresis was carried out at constant current (18 mA) over a period of 2–3 h using Tris–glycine buffer (pH 8.3). The relative mobilities of standard proteins and the 4A′ species that bound DNA were measured on each gel and plotted against gel concentration. The negative slope of the line obtained for each marker was plotted against the logarithm of its molecular mass to obtain the standard curve. The molecular mass of the 4A′ oligomer was determined from interpolation of the standard curve.

Nuclease Protection. Nuclease protection of a uniformly radiolabeled poly(dT) DNA by 4A′ and 4B proteins was carried out as described previously (Arai & Kornberg, 1981; Patel & Hingorani, 1993). [α-^{32}P]Poly(dT) (0.2 μM DNA concentration) was incubated with 4A′ (10–40 μM) and 4B (5–30 μM) in the presence of dTMP-PCP (1 mM) at 22 °C in nuclease buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM DTT, and 50 mM NaCl). Reactions were initiated upon addition of nuclease S7 (1.6 μg/mL) and quenched after 30 s with SDS (1%). The DNA products

after nuclease digestion were resolved on a 20% polyacrylamide/7 M urea sequencing gel. 5′-Radiolabeled 10-mer, 30-mer, 40-mer, 60-mer, and DNA V markers (Boehringer Mannheim) were used as size markers. After electrophoresis, the DNA markers and the nuclease-protected products were visualized by autoradiography, and the sizes of the protected bands were determined from interpolation of the standard plot of DNA markers.

RESULTS

Effect of MgdTDP and MgdTMP-PCP on the Equilibrium Binding of DNA to 4A′ Protein. Membrane binding assays were performed to investigate the effect of nucleotide cofactors on the equilibrium binding of DNA to 4A′ protein. In this assay, nucleoprotein complexes are retained by the nitrocellulose membrane and free DNA by the DEAE membrane (Wong *et al.*, 1992). Quantitation of the radioactivity bound to each membrane provided the fraction of total DNA bound to 4A′, which was plotted against 4A′ monomer concentration. DNA binding to 4A′ was examined both in the absence of any nucleotide cofactors and in the presence of dTDP and dTMP-PCP. The assays were performed with a 30-nucleotide-long ss-DNA and a 20-base-paired hairpin (hp) duplex DNA. The hairpin DNA was used to avoid possible ss-DNA contamination from duplexes prepared using two complementary ss-DNAs. The results of the binding assays with the 30-mer are shown in Figure 1A. It is clear that 4A′ protein does not bind DNA in the absence of nucleotide cofactors and only 20% of DNA binds 4A′ in the presence of MgdTDP. On the other hand, in the presence of MgdTMP-PCP, >95% of 30-mer is bound by 4A′ protein. Furthermore, the binding curve appears to be stoichiometric, suggesting that DNA binding is tight in the presence of MgdTMP-PCP. Binding of hp duplex to 4A′ is shown in Figure 1B. The hp duplex, similar to ss-DNA, does not bind 4A′ in the absence of nucleotides or in the presence of MgdTDP. However, in contrast to ss-DNA, a maximum of 20–25% of hp DNA binding occurs in the presence of MgdTMP-PCP. These experiments show that 4A′ protein requires MgdTMP-PCP to bind either ss- or ds-DNA and the protein does not bind a significant amount of DNA in the presence of MgdTDP.

Stoichiometry of the 4A′–DNA Complex. As 4A′ binds DNA optimally only in the presence of MgdTMP-PCP, all binding experiments reported here have been carried out in the presence of the analog. The equilibrium binding of oligodeoxynucleotides of various lengths to 4A′ was investigated using membrane binding assays similar to those described above. Binding assays were performed with 10-mer, 30-mer, and 60-mer ss-DNAs. Titrations were conducted at increasing 4A′ protein concentrations in the presence of 1 mM dTMP-PCP as the nucleotide ligand. The binding of 10-mer is shown in Figure 2A. The 10-mer binding isotherm appears to be stoichiometric, and 1 μM 10-mer requires 6–8 μM 4A′ monomers for saturation. The same ratio of DNA binding is also observed for the 30-mer ss-DNA as shown in Figure 1A. Titration of the longer 60-mer DNA, on the other hand, requires 2-fold higher 4A′ protein (12–13 μM) for saturation (Figure 2B). Because all the titration curves are stoichiometric, the data do not allow accurate estimation of the equilibrium dissociation constants, but the isotherms can be used to determine the stoichiometries of 4A′–DNA binding. With 10-mer and 30-mer DNA, the stoichiometry appears to be 1 DNA per 6–8 protein monomers, and the 60-mer titration indicates a stoichiometry of 1 DNA per 12–13 protein monomers. We know from our previous gel filtration

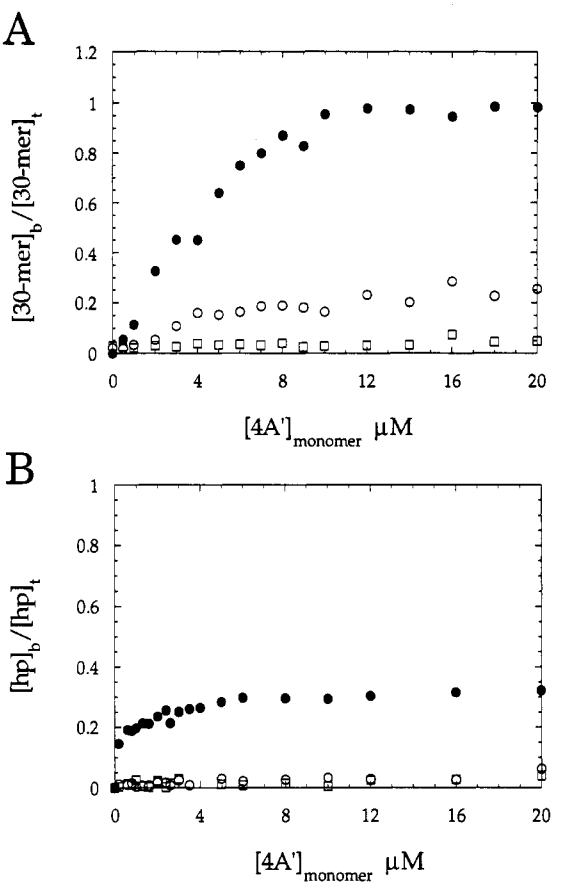

FIGURE 1: Effect of nucleotide cofactors on the equilibrium binding of DNA to 4A′ protein. The interaction of 4A′ with ss- and ds-DNA in the presence and in the absence of nucleotide ligands was investigated by equilibrium binding assays that were conducted at 22 °C in 1 × binding buffer as described under Experimental Procedures. Panel A shows the equilibrium binding of 5′-^{32}P-radiolabeled 30-mer (1 μM) to increasing concentrations of 4A′ protein (0–20 μM) in the presence of 1 mM dTMP-PCP (●) and 5 mM dTDP (○) and in the presence of Mg alone (□). Panel B shows the equilibrium binding of radiolabeled hp DNA (1 μM) to increasing 4A′ protein concentrations (0–20 μM) in the presence of 1 mM dTMP-PCP (●) and 5 mM dTDP (○) and in the presence of Mg alone (□). In each case, the fraction of total DNA bound is plotted against the 4A′ monomer concentration.

experiments that 4A′ protein forms stable hexamers (Patel & Hingorani, 1993). The observed stoichiometries of DNA suggest that each 4A′ hexamer binds 1 DNA of length 10–30 nucleotides and the longer 60-mer DNA binds 2 hexamers or perhaps 1 4A′ dodecamer. The stoichiometry of 1 DNA bound per hexamer was confirmed by high-pressure gel filtration experiments. In this experiment, a constant amount of 30-mer (2 μM) was titrated with increasing 4A′ protein. The 4A′ hexamer and free DNA were the only species, and both were well resolved on the gel filtration column. Formation of 4A′–DNA complex was therefore monitored by quantitating the disappearance of free 30-mer. The result of this experiment was in agreement with the nitrocellulose binding assay as 30-mer binding was found to saturate with a stoichiometry of 1 DNA per 4A′ hexamer (data not shown).

Titration of hp DNA with increasing 4A′ protein shows only 20% DNA binding (Figure 1B); therefore, it is difficult to determine the stoichiometry of hp binding. The lower binding of duplex DNA is actually puzzling, because if the duplex DNA binds with a weaker affinity then a gradual saturation of DNA binding with increasing 4A′ concentrations is expected. To determine if the presence of the 3-nucleotide hairpin loop was responsible for the 20% binding, titrations

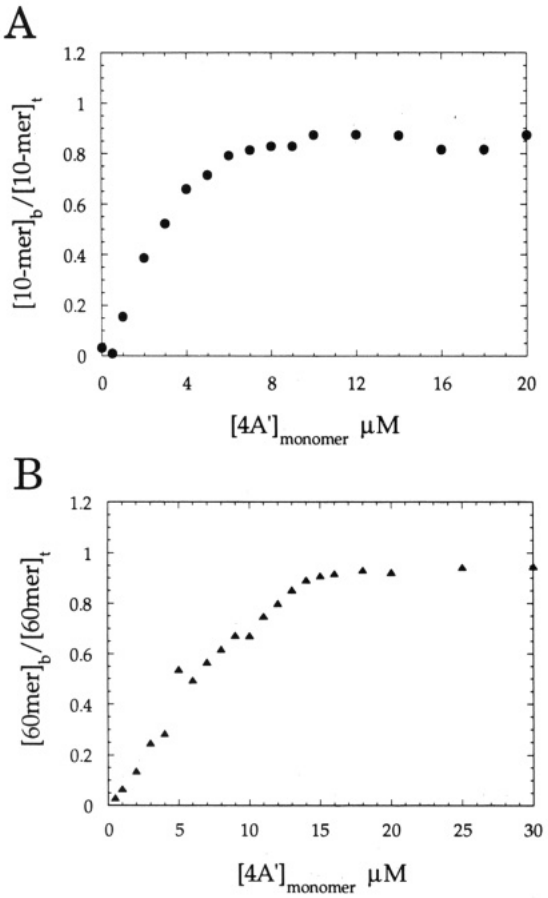

FIGURE 2: Stoichiometry of ss-DNA binding to 4A′ protein. Membrane binding assays with DNAs of various lengths were carried out at constant DNA and increasing 4A′ protein concentrations. Titrations were performed at 22 °C in 1 × binding buffer in the presence of 1 mM dTMP-PCP as described under Experimental Procedures. In each case, the fraction of total DNA bound is plotted againt 4A′ monomer concentrations. Panel A shows titration of 1 μM 10-mer (●) with increasing 4A′ protein concentrations (0–20 μM), and panel B shows titration of 1 μM (60-mer (▲) with increasing 4A′ protein concentrations (0–30 μM). The stoichiometries of DNA binding were determined from the inflection points in the titration curves. The 10-mer and 30-mer (Figure 1A) bind 4A′ with a stoichiometry of 1 DNA per 6–8 4A′ monomers, and 60-mer binds 4A′ with a stoichiometry of 1 DNA per 12–13 4A′ monomers.

were carried out with duplexes of 30- and 40-nucleotide-long complementary ss-DNAs (without the hairpin loop). Between 5 and 20% of DNA binding was observed with these 30- and 40-base-long ds-DNAs (data not shown), indicating that the hairpin loop is not responsible for the observed binding of hp. The hp DNA sample that we have used in these studies actually contained a 1:1 ratio of hairpins to intermolecular hybrids. To determine if 4A′ binds one form of the hp DNA preferentially, we prepared a homogeneous population of hairpins by heating and cooling a low concentration of hp DNA (0.1 μM). Native polyacrylamide gel confirmed that all the intermolecular hybrids were converted to hairpins. Nitrocellulose binding assays showed the same 15–20% binding with the hairpin DNA, indicating that 4A′ does not bind one form of hp DNA preferentially over the other (data not shown). A likely explanation for the lower binding of ds-DNA is perhaps the dissociation of 4A′–ds-DNA complex during the filtration process. Omitting the membrane wash step after filtration or using the nitrocellulose membrane alone did not change the percentage of hp binding (not shown). The weaker binding of hp duplex DNA has been confirmed by competition binding experiments that are described later.

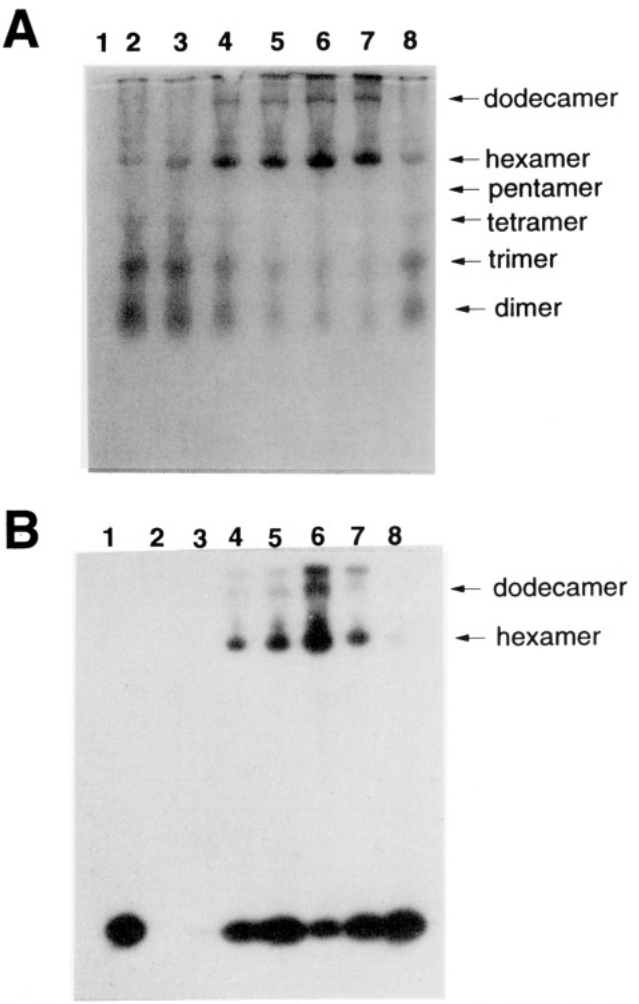

FIGURE 3: Native polyacrylamide gel electrophoresis of 4A′. Panel A shows a Coomassie-stained native polyacrylamide gel of 4A′. The various 4A′ oligomeric species are indicated. Panel B is an autoradiogram of the same gel showing DNA bound to 4A′ oligomers. Free DNA and DNAs bound to 4A′ hexamer and dodecamer species are indicated. The 6% native polyacrylamide gel was prepared in 375 mM Tris-HCl, pH 8.8, and the 3% stacking gel was prepared in 125 mM Tris-HCl, pH 6.8. Both the separating and stacking gels contained 100 μM dTMP-PCP and 10 mM magnesium acetate. All samples were made up in 1 × binding buffer and contained in addition 1 mM dTMP-PCP and native gel loading dye (see Experimental Procedures). The lanes in panels A and B represent the following samples: lane 1, 30-mer ss-DNA (2 μM); lane 2, 4A′ protein (12 μM); lane 3, 4A′ + 10-mer (0.1 μM); lane 4, 4A′ + 10-mer (1 μM); lane 5, 4A′ + 10-mer (2 μM); lane 6, 4A′ + 30-mer (2 μM); lane 7, 4A′ + 60-mer (2 μM); lane 8, 4A′ + hp duplex DNA (2 μM). In lanes 3–8, the 4A′ concentration was 12 μM.

Native Polyacrylamide Gel Electrophoresis of 4A′. Previous gel filtration experiments had indicated that 4A′ formed stable hexamers in the presence of MgdTMP-PCP and DNA at protein concentrations as low as 0.2 μM (Patel & Hingorani, 1993). We have used native polyacrylamide gel electrophoresis to identify the 4A′ oligomeric species that can bind DNA. Native gel experiments were performed both in the absence and in the presence of ss-DNAs such as 10-mer, 30-mer, 60-mer, and duplex hp DNA. MgdTMP-PCP was added to the polyacrylamide gels to optimize binding of DNA to 4A′. As shown in Figure 3A, in the absence of added DNA (lane 2) various oligomeric species of 4A′, from dimer to hexamer and beyond, are well resolved. When ss-DNA is added in substoichiometric amounts (<1 DNA per hexamer), the hexameric speices becomes more prominent (lanes 3 and 4), and as the ss-DNA concentration is increased to above saturation, all lower oligomeric forms collapse into the hexamer

(lanes 5–7). In addition, oligomeric species beyond hexamers and up to dodecamer become more prominent after addition of DNA. There is a slight increase in these higher species in the presence of 30-mer and 60-mer DNAs relative to the 10-mer DNA.

The hexameric species on the native gel was identified by Ferguson analysis (see Experimental Procedures), which provided a mass of 389 ± 10 kDa, in agreement with the calculated mass of 4A′ hexamer (378 kDa). On the native gel, the presence of only four protein bands below the hexamer indicates that a band from monomer to pentamer is missing. We believe that the 4A′ monomer is likely to be the missing species, because the relative mobilities of bands, from dimer to hexamer, showed a linear relationship when plotted against the log of their masses (not shown). A monomer band was not observed even at protein concentrations as low as 0.1 μM. This is in agreement with the gel filtration results, where the smallest oligomeric species at lower protein concentrations was found also to migrate as a dimer.

The autoradiogram of the native gel (Figure 3B) shows that the DNAs bind predominantly to the hexameric form of 4A′. Species smaller than hexamer do not bind DNA, but oligomers larger than hexamer, such as dodecamer and species between hexamer and dodecamer, do bind DNA to some extent. Despite the saturating protein concentration, a large fraction of 10-mer DNA is observed in the free form (lane 5) relative to the 30-mer DNA (lane 6). Even though the K_d values of the two DNAs are comparable as measured by the membrane binding assays (shown later), it is possible that under the conditions of gel electrophoresis the 10-mer DNA dissociates from 4A′ more easily than the 30-mer DNA. The binding of 60-mer also appears to be lower than the 30-mer (lane 7). This may be due to the weaker binding of 60-mer to 4A′ under the native gel conditions or due to the subsaturating levels of 4A′ in the assay, as 60-mer requires about 2-fold higher 4A′ protein for saturation relative to 10-mer or 30-mer DNAs (see Figures 1 and 2B). The hp duplex, in contrast to ss-DNA, does not appear to induce a significant amount of 4A′ hexamer. It also interacts weakly with the 4A′ hexamer (lane 8), in agreement with the equlibrium DNA binding data shown in Figure 1B. At the same time, the hp duplex does not appear to interact with species other than the hexamer.

Titration of 4A′ with Increasing Concentrations of DNA. Titration of ss-DNA with increasing 4A′ protein indicates that only one DNA binds to either the 4A′ hexamer or the dodecamer. To determine if more than one DNA can interact with the hexamer, titrations were conducted at constant 4A′ protein and increasing 10-mer, 30-mer, and 60-mer DNA concentrations. Figure 4A shows the equilibrium binding of 4A′ to 10-mer DNA. The binding curve is biphasic, and at high DNA concentrations, the stoichiometry appears to be about 2 DNAs per hexamer. The first phase of the curve shows tight binding of close to one DNA per 4A′ hexamer (shown as an inset), and the second phase shows weaker binding of the second DNA. The 30-mer binds in a similar manner, showing tight binding of one strand of DNA per hexamer and weak binding of a second strand (Figure 4B). The titration of 4A′ with 60-mer, however, is different (Figure 4C). One 60-mer binds tightly to two hexamers, which is consistent with the results of 60-mer titration at increasing protein concentrations (Figure 2B). The final stoichiometry of 60-mer binding, however, is the same as that of the 10-mer and 30-mer binding as two 60-mer DNAs bind per hexamer at higher DNA concentrations.

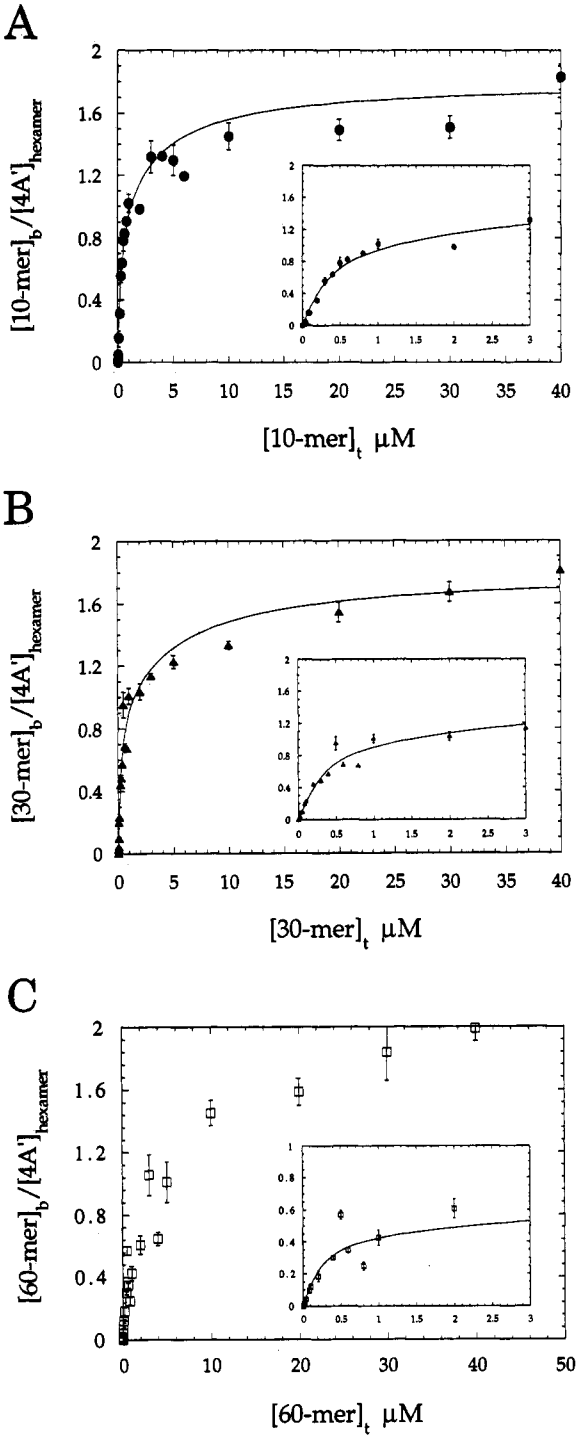

FIGURE 4: Titration of 4A′ with increasing concentrations of ss-DNA. A constant amount of 4A′ protein was titrated with increasing amounts of ss-DNA of various lengths. Titrations were carried out at 22 °C in 1 × binding buffer in the presence of 1 mM dTMP-PCP as described under Experimental Procedures. Panel A shows titration of 4A′ (2 μM monomer) with 0–40 μM 10-mer DNA (●), panel B shows titration of 4A′ with 0–40 μM of 30-mer DNA (▲), and panel C shows titration of 4A′ with 0–40 μM 60-mer DNA (□). In each case, the fraction of DNA bound per 4A′ hexamer is plotted against the total DNA concentration. The titration curves in the insets show binding from 0 to 3 μM DNA. The error bars represent standard errors of the mean determined from at least three independent binding experiments. The stimulated curves shown in solid lines were generated from equations defined for a model in which one 4A′ hexamer binds two 10-mer or 30-mer DNAs, and a 4A′ dodecamer binding 60-mer DNAs (Experimental Procedures). Both 10-mer and 30-mer titrations best-fit to K_d values of 1×10^{-7} and 5×10^{-6} M, and the initial phase of 60-mer binding best-fit to a K_d of 1.4×10^{-7} M.

In order to estimate the equilibrium binding constants of 4A′ and ss-DNAs, we have attempted to fit the binding data to a model where we assumed that two DNAs bind to one 4A′ hexamer (see Experimental Procedures). Simulated curves were compared to the titration data manually, and the best-fit constants for binding were determined. The 10-mer titration data appear to best-fit with dissociation constants $1/K_1$ and $1/K_2$ of 1×10^{-7} and 5×10^{-6} M, respectively. The 30-mer binding data fit to simulated curves with the same values for both dissociation constants. These results indicate that 4A′ hexamer binds one 10- or 30-nucleotide-long ss-DNA with a 50-fold higher affinity than the second DNA strand. The 60-mer binding data were simulated by assuming DNA binding to 4A′ dodecamer rather than the hexamer. The K_d for the first 60-mer was estimated to be 1.4×10^{-7} M. The subsequent K_d values were difficult to determine because of lack of clear transition points in the 60-mer binding isotherm.

Nuclease Protection of ss-DNA. Titrations at constant 4A′ concentration and at constant DNA concentration both indicate that the stoichiometry of DNA binding changes when the length of ss-DNA is increased from 30 to 60 nucleotides. The 30-mer appears to bind one hexamer with a high affinity whereas the 60-mer binds tightly to two hexamers or a dodecamer. These results predict that each 4A′ hexamer must bind 30–40 nucleotides of ss-DNA. The 4A′ DNA binding site was investigated previously using nuclease protection assays (Patel & Hingorani, 1993). A uniformly radiolabeled poly(dT) DNA bound to 4A′ and 4B was subjected to digestion by nuclease S7. The DNA regions protected by 4A′ and 4B proteins were then analyzed on a denaturing polyacrylamide gel. In these experiments, we had observed a ladder of protection bands that increased in increments of about 60 nucleotides. Our interpretation of this result was that protein hexamers were stacked on the long ss-DNA and each hexamer protected about 60 nucleotides of DNA. However, the equilibrium DNA binding results from this study indicate that a 60-nucleotide-long DNA should interact with 2 hexamers. The two results would be consistent if the 60-nucleotide increments observed in the nuclease protection assay were due to protection by stacked dodecamers rather than hexamers. In the previously reported nuclease protection assay, the smallest length of DNA protected by 4A′ and 4B proteins was found to be 36 ± 6 and 55 ± 5 nucleotides, respectively. These small bands ran close to the dye front of the 8% polyacrylamide gel; therefore, we have reexamined the DNAs on a higher percentage gel. Figure 5 shows the nuclease-protected DNAs analyzed on a 20% polyacrylamide gel which resolves DNA down to a few bases. The smallest DNA protected by 4A′ migrates at the 30 ± 5 nucleotide position relative to the markers, and the subsequent band migrates at the 53 ± 5 position (Figure 5A). For the 4B protein, the smallest band is also 30 ± 5 nucleotides (Figure 5B), while the earlier reported smallest band of 55 ± 5 nucleotides is actually the subsequent DNA. Apparently, on the lower percentage gel used previously these small DNAs were stacked at the dye front and hence not resolved. Because a 30-mer DNA binds 4A′ hexamer with a ratio of 1:1, the 30-nucleotide band is most likely the length of DNA protected by 4A′ and 4B hexamers. The subsequent 50–60-nucleotide protection band might result from DNA bound to 2 hexamers or a dodecamer. Similarly, the previously reported 60-nucleotide increments may be due to protection by dodecamers stacked on the long ss-DNA.

Competitive Binding of ss-DNA and ds-DNA at Constant DNA and Increasing 4A′ Concentrations. Equilibrium binding of hp DNA has indicated that 4A′ binds duplex DNA with a much lower affinity than it binds ss-DNA. Competitive DNA binding experiments at constant DNA concentration (shown below) and at constant 4A′ concentration were performed to investigate further the relative affinities of 4A′ protein for single-stranded and duplex DNAs. Figure 6 shows the competitive binding of 60-mer and hp duplex DNA to increasing 4A′ protein concentrations. Both radiolabeled 60-mer and radiolabeled hp DNA were used in separate experiments to monitor the 4A′–60-mer or 4A′–hp complex, respectively. Radiolabeled 60-mer was titrated with increasing 4A′ protein in the absence and in the presence of an equimolar amount of unlabeled hp DNA. The two titration curves are indistinguishable, indicating that the hp duplex does not compete with the 60-mer for binding 4A′. Similarly, radiolabeled hp duplex was titrated with increasing 4A′ protein both in the absence and in the presence of an equimolar amount of unlabeled 60-mer DNA. In the absence of 60-mer, 4A′ binds only 20–25% of hp duplex, as shown in Figure 1B. In the presence of 60-mer, however, titration of hp shows a sigmoidal binding. When 60-mer and hp titration curves are compared, it is clear that 4A′ binds hp DNA only after 60-mer binding is saturated. Thus, under the conditions of the above experiments, the hp duplex does not appear to compete with the 60-mer ss-DNA for binding 4A′.

Competitive Binding of ss-DNA and ds-DNA at Constant 4A′ and Increasing DNA Concentrations. Figure 7A shows titration of 4A′ with increasing concentrations of hp DNA in the presence of a constant amount of 60-mer DNA. The experiment was performed both with radiolabeled hp to monitor the 4A′–hp complex and with radiolabeled 60-mer to monitor the 4A′–60-mer complex. Titration of 4A′ with increasing radiolabeled hp DNA shows up to four hp DNAs bound per hexamer. When titration was carried out in the presence of a constant amount of unlabeled 60-mer, a distinct lag is observed in the binding of hp to 4A′, which is not present in the absence of 60-mer. Similarly, when titration was carried out using radiolabeled 60-mer, the 60-mer DNA remains bound to 4A′ with a high affinity even in the presence of 20-fold higher hp DNA concentrations. When similar competition experiments were carried out with 30-mer ss-DNA, it also competed effectively with hp DNA, and it remained bound to 4A′ in the presence of excess hp (not shown). The only difference was in the stoichiometry of 30-mer binding which was 1 DNA per hexamer instead of 1 per dodecamer as observed with the 60-mer.

Competitive binding experiments at constant 4A′ and increasing 60-mer DNA concentrations in the absence and in the presence of a constant amount of hp DNA are shown in Figure 7B. The radiolabeled hp DNA bound to 4A′ is easily competed off by increasing unlabeled 60-mer DNA. At the same time, 60-mer binding to 4A′ is unaffected by the presence of hp DNA. The above experiments suggest that ss-DNA and ds-DNA both probably bind at the same site on the hexamer; 4A′, however, binds ss-DNA with a higher affinity than ds-DNA.

It is curious that 4A′ binds up to four hp DNAs per hexamer at increasing hp DNA concentrations (Figure 7A), when only 20% hp binding was observed at increasing 4A′ protein concentrations (Figure 1B). To determine if any of the four hp DNAs were bound in a specific manner, a competition experiment similar to that shown in Figure 7B was performed at increasing 60-mer concentration and 8 μM radiolabeled hp (concentration where four hp DNAs bind per hexamer). In this experiment, three hp DNAs were competed off by 0.5

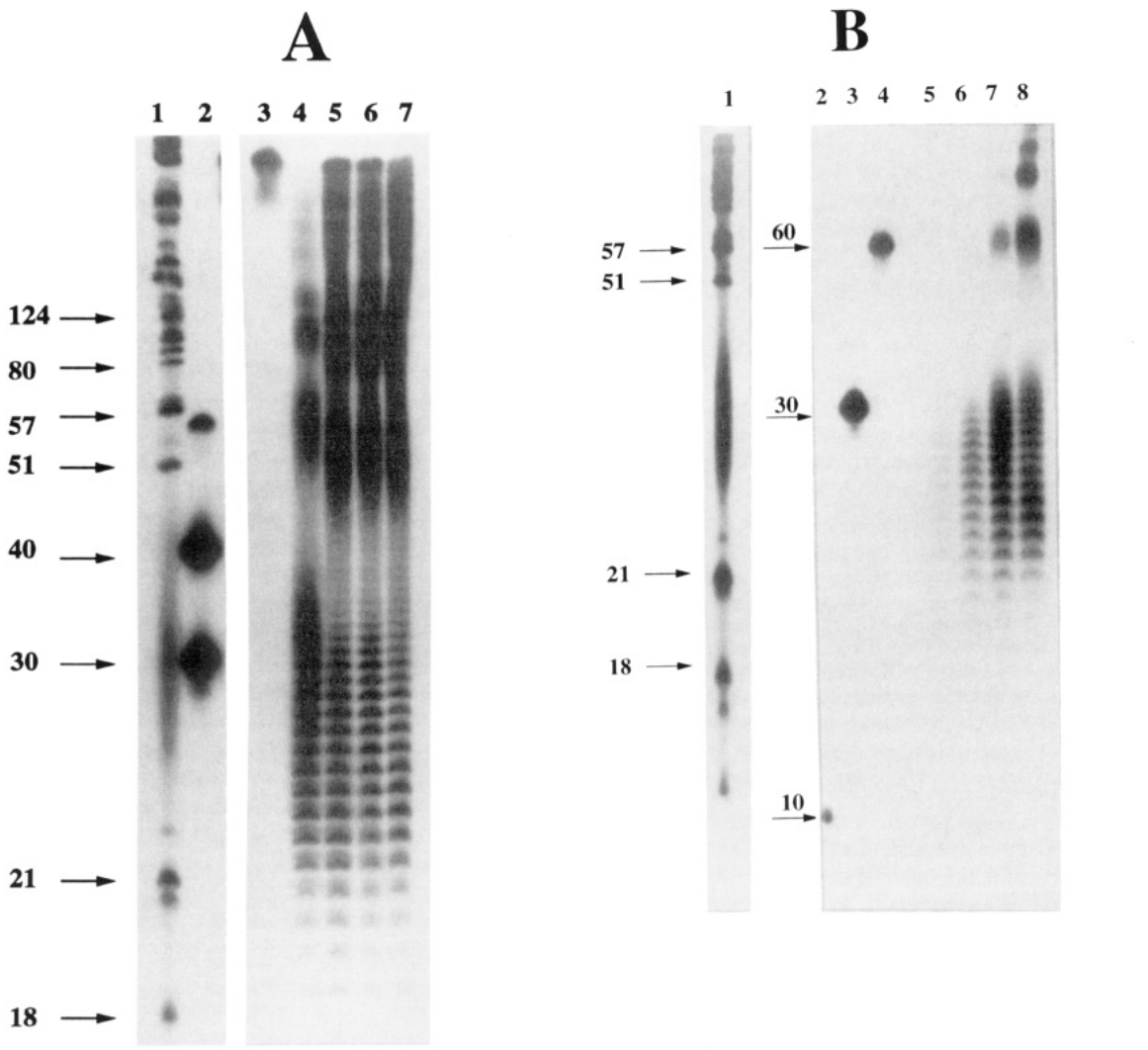

FIGURE 5: Nuclease protection of poly(dT) with 4A′ and 4B proteins. Nuclease protection of a uniformly labeled poly(dT) DNA (0.2 μM) with 4A′ and 4B proteins was carried out at 22 °C in the presence of 1 mM dTMP-PCP using endonuclease S7 as described under Experimental Procedures. Panel A shows the nuclease-protected DNA products of 4A′ protein analyzed on a 20% polyacrylamide/7 M urea gel. The DNAs were visualized by autoradiography, and the lanes correspond to the following samples: lane 1, DNA markers V (lengths indicated on the left); lane 2, DNA markers (30-mer, 40-mer, and 60-mer); lane 3, full-length poly(dT) DNA; lanes 4–7 nuclease-protected DNA products in reactions containing 10, 20, 30, and 40 μM 4A′ protein. Panel B shows the nuclease-protected DNAs of 4B protein. The lanes represent the following samples: lane 1, DNA markers V; lanes 2–4, DNA markers (10-mer, 30-mer, and 60-mer); lanes 5–8, nuclease-protected DNAs in reactions containing 5, 10, 20, and 30 μM 4B protein. The smallest DNA protected by both 4A′ and 4B proteins was calculated to be 30 ± 5 nucleotides. The subsequent length of DNA protected by 4A′ is 53 ± 5 nucleotides and by 4B is 55 ± 5 nucleotides.

μM 60-mer, and the last hp DNA was competed off by 2 μM 60-mer DNA. 4A′ protein, therefore, can bind 60-mer and hp DNA simultaneously (as also seen in Figure 7A), but it is not clear if simultaneous binding of ss-DNA and ds-DNA is any more favorable than binding of two ss-DNAs.

DISCUSSION

We have shown using equilibrium DNA binding experiments that the hexameric 4A′ primase/helicase protein requires MgdTMP-PCP for optimal DNA binding. The protein does not bind DNA in the absence of nucleotide ligands, and only 20% of ss-DNA binding was observed in the presence of MgdTDP. The effect of nucleotide cofactors on ds-DNA binding is similar. 4A′ protein does not bind ds-DNA in the absence of nucleotide ligands or in the presence of MgdTDP. In the presence of MgdTMP-PCP, ds-DNA binding was observed, but it was limited to about 20%. Because 4A′ shows high affinity for DNA in the presence of MgdTMP-PCP, the analog was used in all the binding studies reported here. Whether dTMP-PCP exactly mimics dTTP function in modulating 4A′–DNA interactions cannot be definitively proven at the present time. However, the general behavior of the enzyme in the presence of dTTP and dTMP-PCP appears to be comparable and at the same time different from that in the presence of dTDP. Both dTTP and dTMP-PCP facilitate stable hexamer formation, and both promote DNA bindnig to a greater extent than dTDP (ss-DNA binding in the presence of dTTP are unpublished results). In any case, DNA binding isotherms in the presence of dTTP would be difficult to interpret as dTTP gets hydrolyzed under the conditions of the binding assay.

The 4A′ protein was shown previously by using gel filtration to assemble into stable hexamers in the presence of MgdTMP-PCP (Patel & Hingorani, 1993). Here, we show that the 4A′ oligomers from dimers to hexamers and beyond can be resolved on a native polyacrylamide gel. When 4A′ was analyzed on the native gel in the presence of DNA, the ss-DNAs were found to bind predominantly to the hexameric form of 4A′. The DNAs showed some affinity for the higher oigomeric forms, but no DNA binding was detected to species smaller

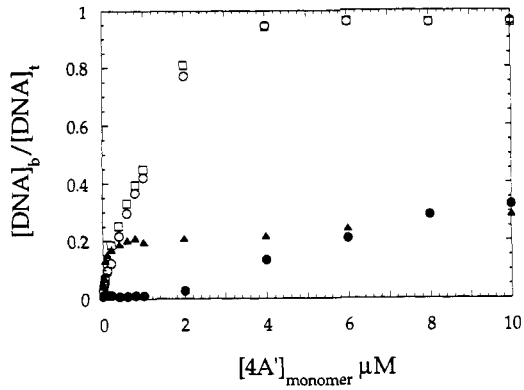

FIGURE 6: Competitive binding of ss-DNA and ds-DNA to increasing concentrations of 4A′ protein. Competitive binding assays at constant DNA and increasing 4A′ protein concentrations were performed at 22 °C in 1 × binding buffer in the presence of 1 mM dTMP-PCP as described under Experimental Procedures. The binding curves, (○) and (▲), show, respectively, titrations of constant amounts of radiolabeled 60-mer (0.2 μM) and hp DNA (0.2 μM) with increasing 4A′ protein concentrations (0–10 μM). Competitive binding of 60-mer and hp DNA was carried out by incubating the two DNAs prior to 4A′ addition. The titration curve (□) shows competitive binding of radiolabeled 60-mer (0.2 μM) to increasing 4A′ concentration in the presence of unlabeled hp DNA (0.2 μM). Similarly, the titration curve (●) shows competitive binding of radiolabeled hp (0.2 μM) to increasing 4A′ protein concentration in the presence of unlabeled 60-mer (0.2 μM).

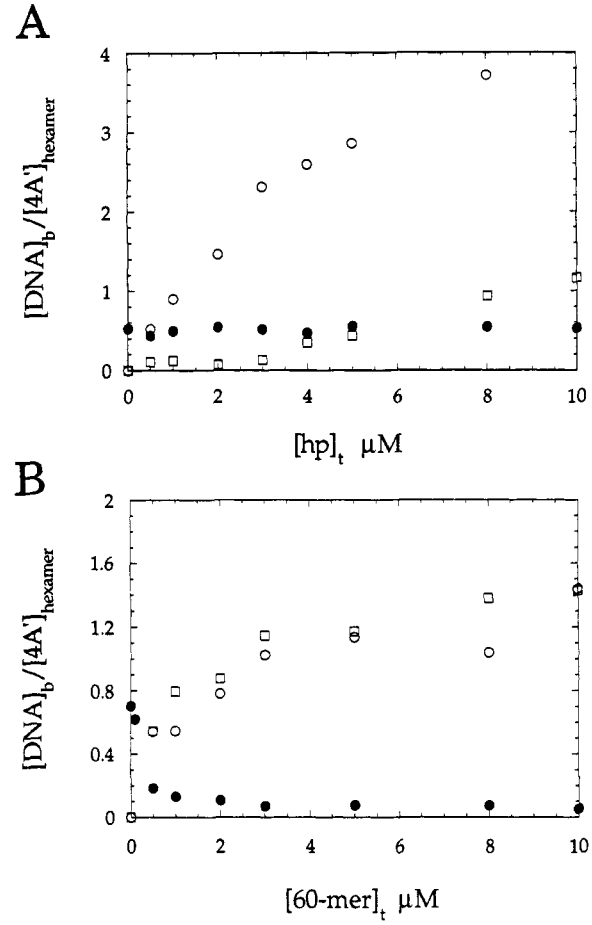

FIGURE 7: Competitive binding of ss-DNA and ds-DNA at constant 4A′ concentrations. Competitive binding assays at constant 4A′ protein and increasing DNA concentrations were conducted in the presence of 1 mM dTMP-PCP at 22 °C as described under Experimental Procedures. Panel A shows titrations of 4A′ (2 μM) with increasing radiolabeled hp DNA concentrations (0–10 μM) in the absence of 60-mer (○) and in the presence of 0.5 μM unlabeled 60-mer (□); and titration of 4A′ with increasing unlabeled hp DNA concentration in the presence of 0.5 μM radiolabeled 60-mer (●). Similarly, panel B shows titrations of 4A′ (2 μM) with increasing radiolabeled 60-mer concentrations (0–10 μM) in the absence of hp DNA (○) and in the presence of 0.5 μM unlabeled hp DNA (□); and titration of 4A′ with increasing unlabeled 60-mer concentrations in the presence of 0.5 μM radiolabeled hp DNA (●). In each case, the fraction of labeled DNA bound per hexamer is plotted against the total hp or the total 60-mer DNA concentration.

than the 4A′ hexamer. A similar observation has been made also for the SV40 T antigen helicase (Sengupta & Borowiec, 1992). Quantitative nitrocellulose binding assays were used to determine the stoichiometry of small oligodeoxynucleotides binding to 4A′. The surprising result from the membrane binding experiments was the preferential binding of 1 strand of ss-DNA to 4A′ protein with high affinity regardless of its length from 10 to 60 nucleotides. Titrations at increasing 4A′ protein concentration showed that a 10-mer or a 30-mer DNA binds 4A′ hexamer with a stoichiometry of 1:1 and the longer 60-mer DNA binds with a stoichiometry of 1:2. Titrations at increasing 10-mer and 30-mer concentrations showed that 4A′ hexamer can bind a second strand of DNA, but only at higher DNA concentrations. The second DNA binds with a 50-fold lower affinity than the first DNA. Similarly, one 60-mer shows tight binding to two hexamers (or a dodecamer), and additional 60-mer DNAs appear to bind with a weaker affinity. The oligodeoxynucleotides used in this study all contain a 5′-GTC primase recognition sequence. However, we would like to point out that this sequence is not necessary for DNA binding as other DNAs ranging from 20 to 60 nucleotides of random sequences show similar tight binding with the same stoichiometries.

In contrast to the stoichiometric binding observed with ss-DNA, only a fraction of ds-DNA binds 4A′ both on the native gel and in the membrane binding assays performed at increasing 4A′ concentrations. The competition assays with ss-DNA and ds-DNA confirmed the weaker binding of ds-DNA. The ds-DNA was easily competed from 4A′ by ss-DNA in these assays, yet under the same conditions, the ss-DNA remained bound to 4A′ in the presence of excess ds-DNA. Titration of 4A′ with increasing ds-DNA in the presence of a constant amount of ss-DNA did show simultaneous binding of ss-DNA and ds-DNA to 4A′. However, it is not clear if the simultaneous binding of ds-DNA and ss-DNA to 4A′ is any more favorable than the binding of two ss-DNAs.

Quantitative DNA binding studies with different lengths of ss-DNAs also provided some information about the size of the DNA binding site on the hexamer. Because a 30-mer binds 1 hexamer and a 60-mer binds 2 hexamers, the maximum length of ss-DNA that can interact with 1 hexamer is inferred to be between 30 and 40 nucleotides. The nuclease protection experiments reported here are in agreement as the smallest length of DNA protected by 4A′ and 4B was found to be 30 ± 5 nucleotides. Assuming that a 30-nucleotide-long DNA interacts with 1 hexamer, then the question is how many subunits of the hexamer are involved in the interaction with DNA. Preliminary electron microscopy studies indicate that the 4A′ subunits are arranged in a ringlike structure and the outer diameter of the ring is between 100 and 120 Å (unpublished results). If we assume that the DNA is wrapped around the periphery of the hexamer making contacts with each subunit, than the total length of ss-DNA that can interact with the hexamer would be 90–100 nucleotides (with a rise of 3.4 Å per nucleotide). According to this calculation, a 30-nucleotide-long DNA cannot wrap completely around the hexamer, but it is only long enough to interact with approximately 2 subunits of the hexamer. This mode of DNA binding

may be useful for a 4A′-like hexameric helicase as discussed below.

DNA helicases show a tendency to self-assemble into oligomers, predominantly as dimers or hexamers [for references, see review by Lohman (1993)]. It is not known whether dimeric and hexameric helicases employ different mechanisms of DNA unwinding, because only a small number of helicases have been studied in any detail at the present time. If we compare the DNA binding properties of hexameric 4A′ helicase to those of a well-studied dimeric helicase Rep, we find significant differences in the two helicases, both in the protein–DNA interactions and in the modulation of these interactions by NTP and NDP. Rep shows favorable binding of two ss-DNAs in the presence of MgADP, yet in the presence of MgAMP-PNP, it prefers to bind one ss-DNA and one ds-DNA simultaneously. In this manner, during the entire cycle of NTP hydrolysis, Rep remains bound to DNA at least via one of its subunits. On the basis of these results, a rolling type of mechanism was proposed for the Rep helicase, in which ATP binding promotes translocation of Rep on the DNA, and ATP hydrolysis facilitates DNA unwinding (Wong & Lohman, 1992). The 4A′ protein, in contrast to Rep, binds ss-DNA optimally in the presence of MgdTTP or MgdTMP-PCP. Under the same conditions, 4A′ binds only a limited amount of ds-DNA which is also competed off effectively by ss-DNA. In the presence of MgdTDP, 4A′ binds a small amount of ss-DNA, but it does not bind any ds-DNA. These results suggest that 4A′ protein may bind DNA upon dTTP binding and dissociate from the DNA upon dTTP hydrolysis. If all the subunits of the hexamer were to bind DNA and hydrolyze dTTP simultaneously, then such dissociation and rebinding events at each catalytic cycle would greatly hinder processive unwinding of DNA. On the other hand, if the hexamer were to interact with the DNA only via one or two of its subunits at any given time, and the remaining subunits were to bind DNA in a sequential manner, then complete dissociation and reassociation of the DNA would not be required. Each cycle of dTTP binding and dTTP hydrolysis would be coupled to consecutive DNA binding and dissociation events that would promote DNA unwinding by a mechanism that is not understood at the present time. The 4A′ helicase using such a mechanism would be able to processively unwind up to 30 nucleotides in a single catalytic cycle.

Various models have been proposed as possible mechanisms of DNA helicases (Lohman, 1992, 1993). The mechanisms have been broadly classified as active or passive modes of action. In the former mechanism, the helicase would contribute directly to unwinding, whereas in the latter mechanism, the helicase would effect unwinding indirectly, perhaps by stabilizing the resulting ss-DNA. To actively unwind ds-DNA, the helicase would bind ds-DNA at the forked-DNA junction and cause unwinding by directly destabilizing the base pairs. Alternatively, the helicase could bind to the single strands at the fork junction, perhaps both 5′ and 3′ strands, and cause distortion of duplex region resulting in DNA unwinding. The NTPase activity here may serve to directly destabilize the duplex structure. In the passive unwinding mechanism, the helicase could interact with ss-DNA, and DNA unwinding could be driven by stabilization of newly unwound ss-DNA. The NTPase activity here would promote, in an unknown manner, unidirectional movement of the helicase on DNA. Even though T7 DNA helicase prefers binding to ss-DNA over ds-DNA, the present studies do not exclude transient interactions of the helicase with ds-DNA regions during DNA unwinding. Additional studies will be required to determine if the hexameric T7 DNA helicase uses the above described active, passive, or some combination of both mechanisms to promote DNA unwinding.

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