The K318A Mutant of Bacteriophage T7 DNA Primase–Helicase Protein Is Deficient in Helicase But Not Primase Activity and Inhibits Primase–Helicase Protein Wild-Type Activities by Heterooligomer Formation†

Smita S. Patel,* Manju M. Hingorani, and Winnie M. Ng

Department of Biochemistry, The Ohio State University, 484 West 12th Avenue, Columbus, Ohio 43210

Received January 28, 1994; Revised Manuscript Received April 18, 1994®

ABSTRACT: Lysine 318 in the conserved sequence SXXXGXGKS of bacteriophage T7 gene 4A′ protein was mutated to an alanine to understand the effect of this substitution on the helicase and primase activities. The dTTPase activity of 4A′/K318A mutant protein was much lower than that of 4A′, and both K_m and k_{cat} values were affected. The K_m of the mutant protein was 3–5-fold higher, and the k_{cat} was about 100-fold lower, than that of 4A′. The mutation did not affect the ability of 4A′/K318A to assemble into hexamers or bind DNA in the presence of MgdTTP. Interestingly, the mutant protein does not bind DNA in the presence of MgdTMP-PCP. The reduced dTTPase activity, however, decreased the helicase activity of the mutant protein to an undetectable level, whereas its primase activity was only 1.5–2.5-fold lower. When 4A′/K318A mutant protein was mixed with 4A′, heterooligomers were formed and the helicase and the DNA-dependent dTTPase activities of 4A′ were inhibited, but the DNA-independent activity actually increased. The extent of decrease in activities upon heterooligomer formation depended both on the length of time 4A′ and 4A′/K318A proteins were incubated and on the concentration of the mutant protein. In addition, the decrease in the dTTPase activity was observed only when the two proteins were incubated in the absence of MgdTTP and DNA, conditions under which both proteins form unstable hexamers. Even though 4A′/K318A does not bind a 30-mer DNA in the presence of MgdTMP-PCP, heterooligomers were capable of binding DNA with the same stoichiometry as 4A′. Protein–DNA cross-linking experiments with $(dT)_{30}$ and poly(5-BrdU) showed that DNA interacts with five and perhaps all six subunits of 4A′. Therefore, unless heterooligomer restores the ability of the mutant protein to bind DNA in the presence of MgdTMP-PCP, these results suggest that the DNA can bind 4A′ by interacting with a few subunits. However, a fully active hexamer is required for both the helicase and the single-stranded M13 DNA-dependent dTTPase activities.

Bacteriophage T7 gene 4A and 4B proteins provide the helicase and primase functions for T7 DNA replication. The helicase activity unwinds duplex DNA ahead of the DNA polymerase during leading strand DNA synthesis, and the primase synthesizes small sequence-specific RNA primers that initiate lagging strand DNA synthesis. 4A and 4B proteins are both products of T7 gene 4 translated in the same reading frame (Dunn & Studier, 1983, 1984). 4A is the full-length product, and it contains both helicase and primase activities (Bernstein & Richardson, 1988a, 1989). The smaller 4B protein is made from an internal initiation site in 4A, and it contains only the helicase activity. Thus, 4B is identical to 4A except for the 63 amino acids that are missing from the N-terminus. Because of their similar natures, 4A and 4B proteins copurify from recombinant *Escherichia coli* cells that express both proteins from cloned gene 4 DNA. To study the properties of the individual proteins, pure preparations of 4A and 4B proteins have been obtained from clones that express each protein separately. Expression of 4A alone was achieved by mutating either the 4B initiation codon (Rosenberg *et al.*, 1992) or the 4B ribosome binding site (Mendelman & Richardson, 1991). Mutation of the 4B initiation codon abolishes 4B synthesis, but it results in a necessary substitution of methionine 64. The M64L mutant protein, 4A′, was shown to have primase and helicase activities that were comparable to those of the wild-type mixture of gene 4 proteins (Rosenberg *et al.*, 1992; Patel *et al.*, 1992). Therefore, most of our studies have been performed with the 4A′ protein.

Recent studies have shown that both 4A′ and 4B proteins self-assemble into hexamers in the presence of MgdTMP-PCP[1], the nonhydrolyzable analog of dTTP (Patel & Hingorani, 1993). Hexamer formation is required for DNA binding, as oligomers smaller than hexamers are incapable of binding DNA (Hingorani & Patel, 1993). 4A′ and 4B hexamers protect about 25–30 bases of single-stranded DNA, and the proteins have a tendency to stack on long single-stranded DNA. Quantitative DNA binding studies indicated that the gene 4 proteins bind single-stranded DNA preferentially over double-stranded DNA, and DNA binding was observed only in the presence of MgdTMP-PCP and not in the presence of Mg^{2+} alone or MgdTDP (Hingorani & Patel, 1993).

Most if not all helicases bind and hydrolyze NTP, and the NTPase activity is essential for DNA unwinding. The gene 4 proteins hydrolyze NTPs with a broad substrate specificity (Patel *et al.*, 1992). Therefore, nucleotides such as dTTP, dATP, dGTP, and ATP are used as substrates and are hydrolyzed by the gene 4 proteins with varying k_{cat} and K_m values. The preferred nucleotide for the helicase and primase

† This research was supported in part by grants from the American Cancer Society (NP-832 and IRG16-32).

* To whom correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* June 1, 1994.

[1] Abbreviations: dTMP-PCP, deoxythymidine [β,γ-methylene]triphosphate; HPLC, high-pressure liquid chromatography; BSA, bovine serum albumin; IPTG, isopropyl β-D-thiogalactopyranoside.

0006-2960/94/0433-7857$04.50/0

activities is, however, dTTP, which also has the lowest K_m. High concentrations of other NTPs do support helicase activity, yet for some unknown reason the primase activity specifically requires dTTP. Interestingly, Matson and Richardson (1983a) reported that dTMP-PCP supported qualitatively the same amount of primer synthesis as dTTP, suggesting that dTTP hydrolysis may not be required for primer synthesis. However, a recent report by Mendelman and Richardson (1991) shows that dTMP-PCP supports less than 1% primer synthesis relative to dTTP. Consequently, the role of dTTP in RNA primer synthesis still remains unclear.

Numerous nucleotide binding proteins including helicases contain a glycine-rich sequence, G/AXXGXGKT/S, that may be involved in nucleotide binding (Walker *et al.*, 1982). Such a glycine-rich sequence is also present in the gene 4 proteins located approximately in the middle of the 4A protein, and the sequence is conserved among helicases that share regions of amino acid sequence homology with the gene 4 proteins (Ilyina *et al.*, 1992). High-resolution structures of a few nucleotide binding proteins such as adenylate kinase (Muller & Schulz, 1992), elongation factor EF-Tu (Jurnak, 1985; La Cour *et al.*, 1985; Kjeldgaard & Nyborg, 1992), *ras* p21 protein (Tong *et al.*, 1991), and rec A protein (Story & Steitz, 1992) show that the conserved sequence forms a flexible loop that lies between a β-strand and an α-helix. The amino acids in the loop interact with the phosphate residues of the nucleotide. The lysine in this loop, for instance, interacts with the β,γ-phosphates of GTP analog in *ras* p21 protein (Bourne *et al.*, 1991) and the β-phosphate of GDP in elongation factor EF-Tu (Kjeldgaard & Nyborg, 1992). Mutation of an analogous lysine in RAD3 helicase abolished its ATPase and helicase activities (Sung *et al.*, 1988), and recently, a double mutant of gene 4 protein, G317V–K318M, was shown to lack dTTPase and helicase activities (Notarnicola & Richardson, 1993).

We have mutated the lysine residue in the putative nucleotide binding site at position 318 in 4A′ to an alanine to investigate the role of lysine and the effect of its substitution on the dTTPase, primase, and helicase activities of 4A′. The 4A′/K318A mutant protein was purified to homogeneity, and its biochemical properties have been studied in detail. The various activities such as dTTPase, primase, and helicase as well as the ability of the protein to bind DNA and form oligomers have been compared to those of 4A′. The mutant protein was also mixed with 4A′ to investigate heterooligomer formation between the two proteins and to study the effect of the mutant subunits on the wild-type activities of 4A′ subunits.

EXPERIMENTAL PROCEDURES

Nucleotides and Other Reagents. All dNTPs, rNTPs, and 5-BrdUTP were purchased from Sigma, and dTMP-PCP was purchased from United States Biochemical Corp. The nucleotides were used without further purification. Nucleotide stock solutions were prepared by dissolving the solids in 50 mM Tris-HCl buffer and adjusting the pH to 7.5 with NaOH before storing the solutions in small aliquots at −70 °C. Radiolabeled nucleotides [α-^{32}P]dTTP (3000 Ci/mmol), [γ-^{32}P]ATP (4000 Ci/mmol), [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]CTP (3000 Ci/mmol) were purchased from ICN Biochemicals. The fluorescent cross-linker SAED (sulfo-*N*-succinimidyl [[2-(7-azido-4-methylcoumarin-3-acetamido)ethyl]dithio]propionate) was purchased from Pierce. The Immobilon-P transfer membrane was purchased from Millipore.

Proteins. 4A′ and 4B proteins were purified as described (Patel *et al.*, 1992). T7 gene 5 exo$^-$ protein and thioredoxin were purified as described (Patel *et al.*, 1991). SDS protein markers were purchased from Bio-Rad, and chicken egg albumin and β-galactosidase were purchased from Sigma.

Preparation of 4A′/K318A Mutant. Kunkel's method of site-directed mutagenesis was used to prepare the K318A mutant of 4A′ (Kunkel *et al.*, 1991). The lysine codon, AAG, at amino acid position 318 in 4A′ was changed to GCG, which codes for an alanine, using the oligodeoxynucleotide 5′-GAA CGT TGA CGC ACC CAT ACC. Mutagenesis was performed on the 4A′ gene (11565–13263 fragment of T7 DNA that contains the M64L mutation) subcloned into a phagemid vector, pMa (Stanssens *et al.*, 1989). Six positive clones were identified by oligonucleotide hybridization and confirmed by the dideoxy method of double-stranded DNA sequencing. The 4A′/K318A expression clone was prepared by subcloning the *Sna*BI–*Xba*I fragment from the phagemid into pAR5018. The construction of the pAR5018 overexpression clone of 4A′ has been described (Rosenberg *et al.*, 1992). The 4A′/K318A gene in pAR5018 is under the control of a T7 *lac* promoter that allows protein expression in HMS174(DE3) cells after induction with IPTG.

Expression and Purification of 4A′/K318A Protein. 4A′/K318A protein was expressed by transforming pAR5018/K318A into HMS174(DE3) *Escherichia coli* cells (Studier *et al.*, 1990). For large-scale protein purification, transformed cells were grown to an absorbance of 0.4 (at 600-nm wavelength) in 10 L of LB broth containing ampicillin (100 μg/mL) and induced with 0.4 mM IPTG. Three hours after induction, cells were collected by centrifugation, and protein expression was checked on an 8% SDS–polyacrylamide gel. The expression levels of 4A′/K318A protein were higher than those of 4A′ protein, and the mutant 4A′/K318A protein was purified by the same procedure used to purify 4A′ (Patel *et al.*, 1992). The behavior of the mutant protein was also comparable to that of 4A′ through the various purification steps. The mutant protein was purified to homogeneity (95% purity) with a yield of about 1 mg/(gram of packed wet cells).

Determination of Protein Concentration. Protein concentrations were determined both spectroscopically from multiple absorbance measurements at 280 nm in TE buffer containing 8 M urea and by the Bradford assay (Bradford, 1976) using BSA as the standard. Both methods provided the same protein concentrations. The extinction coefficient of 4A′ is 76 100 M^{-1} cm^{-1}, and that of 4B is 67 850 M^{-1} cm^{-1}, at 280 nm (Patel *et al.*, 1992). The extinction coefficient of 4A′/K318A protein was assumed to be the same as that of 4A′ protein as no changes in the tryptophan or tyrosine residues were made.

Synthetic Oligonucleotides and Single-Stranded M13 DNA. The oligodeoxynucleotides were synthesized at the Biochemical Instrument Center of the Ohio State University. The sequence of 60-mer DNA used to measure the primase and the helicase activities is 5′-CCAAC CTGCT CGTAA GCAAA CGAGG GTCCG ACCCT AACTT CATCC ATGTG GTCAG CAAAT, and the sequence of 30-mer DNA is 5′-AGCTT GCATC ATAGT GTCAC CTGTT ACGTT. The 60-mer was purified on a 12% polyacrylamide/7 M urea gel, and the 30-mer, on a 16% polyacrylamide/7 M urea gel. The DNAs were recovered from the gel by electroelution (Elutrap, Schleicher & Schuell) and desalted using Centricon-3 (Amicon). DNA concentrations were determined spectroscopically from absorbance measurements at 260 nm using the calculated extinction coefficients of 60-mer (637 270 M^{-1} cm^{-1}) and 30-mer (305 010 M^{-1} cm^{-1}). Single-stranded M13mp18 DNA was purified as described (Miller, 1987). The concentration of single-stranded M13 DNA was calcu-

lated spectroscopically by absorbance measurements at 260 nm and by assuming 1 absorbance unit to represent 33 μg/mL DNA concentration.

Measurement of the dTTPase Activity. The DNA-dependent dTTPase activity of both 4A′ and 4A′/K318A proteins was measured at 22 and 37 °C by monitoring the hydrolysis of [α-^{32}P]dTTP to [α-^{32}P]dTDP + P_i. The hydrolysis buffer contained 50 mM Tris–acetate, pH 7.5, 50 mM sodium acetate, 1 mM DTT, 0.1 mg/mL BSA, and 10 mM magnesium acetate. The gene 4 proteins (0.5–5 μM) were preincubated for 5–10 min at 22 °C with 100 μM dTTP and single-stranded M13 DNA (50 nM). Reactions were initiated with various concentrations of dTTP (100 μM to 8.0 mM) + [α-^{32}P]dTTP. Aliquots were removed after intervals of 1–5 min (for 4A′ protein) or 2–60 min (for 4A′/K318A protein) and subsequently quenched with 0.2 M EDTA.

The quenched solutions were analyzed by spotting aliquots on a polyethyleneimine–cellulose thin-layer chromatography plate (Whatman), which was developed in 0.3 M potassium phosphate buffer, pH 3.4. Both [α-^{32}P]dTTP and [α-^{32}P]-dTDP were quantitated with a Betagen Betascope 603 instrument. The dTTPase rate constants were calculated from the initial velocities of dTDP formation and protein concentrations. dTTPase rates were plotted against dTTP concentrations, and the steady-state k_{cat} and K_m values were obtained from a hyperbolic fit of the data. The method of nonlinear least squares and the Kaleidograph software were used to fit the data. The errors are reported as standard errors of the mean. The dTTPase activity at increasing M13 DNA concentration (25–200 nM) and constant dTTP concentration (6 mM) was measured as described above.

Effect of Varying Incubation Time on dTTPase Activity of 4A′/K318A and 4A′ Protein Mixture. The effect of mixing time on the dTTPase activity of 4A′ and 4A′/K318A was investigated (a) in the absence of any ligands, (b) after preincubation with MgdTTP, and (c) after preincubation with MgdTTP and single-stranded M13 DNA. The experiment was performed at 22 °C by preincubating 4A′ and 4A′/K318A protein (4 μM each) separately in Tris–acetate buffer without Mg^{2+}, in buffer with 10 mM magnesium acetate and 80 μM dTTP, and in buffer containing both MgdTTP (80 μM) and single-stranded M13 DNA (50 nM). The two protein solutions were mixed in equal volumes and incubated for increasing periods ranging from 0.5 to 30 min. The dTTPase activity of the incubated proteins was assayed at each time interval by adding an equal volume of a mixture containing dTTP (500 μM), [α-^{32}P]dTTP (10 μCi per assay), and single-stranded M13 DNA (50 nM). The reactions were continued for 4 min, and the reactions were quenched and quantitated as described above. The dTTPase activities at various mixing times were normalized to the activity of 4A′ in the absence of the mutant protein.

dTTPase Activity of 4A′ in the Presence of Increasing Concentrations of 4A′/K318A. 4A′ and 4A′/K318A were mixed in the absence of any ligands, and the dTTPase activity of the mixture was measured (a) in the absence of DNA, (b) in the presence of single-stranded M13 DNA, and (c) in the presence of 30-mer single-stranded DNA. 4A′ (1 μM) was incubated with 4A′/K318A (0–10 μM) in Tris hydrolysis buffer (see above) without Mg^{2+} for 15 min at 22 °C. A mixture of magnesium acetate (10 mM) and dTTP (80 μM) was added either without DNA, with single-stranded M13 DNA (50 nM), or with 30-mer DNA (10 μM) in separate reactions. After incubating for an additional period of 10 min, the dTTPase reactions were initiated by adding 5 mM dTTP + [α-^{32}P]dTTP (10 μCi). Aliquots were removed and quenched with EDTA (0.2 M) after time intervals ranging from 2 to 60 min (in the absence of DNA), from 2 to 8 min (in the presence of single-stranded M13 DNA), and from 2 to 10 min (in the presence of 30-mer DNA). The reactions were quantitated as described above.

A control assay was performed in which 4A′ (1 μM) and increasing amounts of 4A′/K318A (0–2 μM) were preincubated separately with MgdTTP (80 μM) and single-stranded M13 DNA (50 nM) to allow stable hexamer formation prior to mixing. The two proteins were then mixed and incubated for an additional 5–7 min, and the dTTPase activity was measured as described above.

Equilibrium DNA Binding. DNA binding was measured at 22 °C using nitrocellulose binding assays as described (Wong & Lohman, 1993; Hingorani & Patel, 1993). The binding buffer contained 50 mM Tris–acetate, pH 7.5, 5 mM sodium acetate, 1 mM DTT, and 10 mM magnesium acetate. Increasing concentrations of 4A′ or 4A′/K318A (0–30 μM) were incubated with [5′-^{32}P]30-mer (1 μM) in the presence of 1 mM dTMP-PCP or 5 mM dTTP. The DEAE and nitrocellulose membranes (Schleicher & Schuell) were treated for 10 min with 0.5 M NaOH, washed extensively with double-distilled water, and equilibrated in the binding buffer prior to use. Samples (20 μL) were filtered through a layer of nitrocellulose and DEAE membranes under vacuum (water aspirator). When samples contained dTTP, filtration was performed within 1–2 min after addition of dTTP. Radioactivity bound to each membrane was quantitated with the Betascope instrument. Molar amount of protein–DNA complex was determined from the fraction of DNA bound to nitrocellulose and the total DNA concentration in the assay.

Equilibrium DNA Binding of 4A′ and 4A′/K318A Mixtures. 30-mer DNA binding to various ratios of 4A′ and 4A′/K318A protein mixtures was assayed at constant DNA and increasing protein concentrations. 4A′ and 4A′/K318A proteins were mixed in 1:1, 1:2, and 1:3 ratios in binding buffer without Mg^{2+} (50 mM Tris–acetate, pH 7.5, 5 mM sodium acetate, and 1 mM DTT) and preincubated for at least 10 min prior to use. A constant amount of [5′-^{32}P]30-mer DNA (1 μM) was mixed with increasing concentrations of the various mixes of protein (0–30 μM) in the presence of 1 mM dTMP-PCP and 10 mM magnesium acetate. The reactions were assayed for DNA binding using the nitrocellulose–DEAE double-membrane assay as described above.

Protein Oligomerization. Oligomerization of 4A′/K318A protein was studied by HPLC gel filtration and chemical cross-linking experiments similar to those described for the 4A′ protein (Patel & Hingorani, 1993). Gel-filtration studies in the presence of nucleotide were performed by adding 200 μM dTTP and 10 mM $MgCl_2$ to the gel-filtration buffer. 4A′/K318A mutant protein was also studied by native polyacrylamide gel analysis as described for 4A′ (Hingorani & Patel, 1993).

RNA Primer Synthesis. The primase activity of 4A′ and 4A′/K318A proteins was assayed by measuring the kinetics of RNA primer synthesis. The primase buffer consisted of 50 mM Tris–acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, and 0.1 mg/mL BSA. 4A′ or 4A′/K318A protein (1 μM) was incubated for less than 2 min at 22 °C with 500 μM ATP, 500 μM CTP, [α-^{32}P]CTP (10 μCi), and 6 mM dTTP in the above buffer. Primer synthesis was initiated by adding single-stranded M13 DNA (50 nM). The reactions were incubated at 22 °C, and aliquots were removed after intervals ranging from 0 to 30 min and quenched with a mixture of EDTA (200 mM) and denaturing

dye solution (95% formamide and 0.05% bromophenol blue). Samples were applied to a 25% polyacrylamide/3 M urea sequencing gel (Bio-Rad, 35 × 43 cm, 0.2-mm thickness), and electrophoresis was performed at 100 W. Electrophoresis was stopped after the bromophenol blue dye had migrated half-way through the gel. Unreacted CTP and the various RNA products were quantitated with the Betascope instrument.

Primer Synthesis in the Presence of MgdTMP-PCP. The primase activity of 4A′ in the presence of dTMP-PCP was measured using a 60-mer DNA as the template that contained both 3′-CTGGG and 3′-CTGGT primer recognition sequences. 4A′ (30 μM) was incubated with 5 mM dTTP or 1 mM dTMP-PCP, 500 μM ATP, CTP, and [α-^{32}P]CTP (10 μCi). Primer synthesis was initiated by adding 60-mer DNA (20 μM). In reactions containing dTMP-PCP, 4A′ was incubated with MgdTMP-PCP for at least 30 min at 22 °C prior to addition of other reagents. Reactions were quenched after time intervals ranging from 0 to 30 min, and RNA products were analyzed as described above.

Helicase Activity. The helicase activity of the mutant protein was measured using the primer-displacement assay as described (Patel *et al.*, 1992). The experiment was performed at 22 °C using 25 nM M13/60-mer DNA complex, 5 mM dTTP, and 0.5 μM 4A′ or 2.5 μM 4A′/K318A protein in separate reactions. Radiolabeled 60-mer was resolved from the complex on a 12% native polyacrylamide gel, which was analyzed on a Betagen Betascope instrument.

Primase and Helicase Activities. Primase and helicase activities of 4A′ and 4A′/K318A mutant were measured simultaneously using the RNA-primed DNA synthesis assay. Reactions were performed in buffer containing 50 mM Tris–acetate, pH 7.5, 50 mM sodium acetate, 10 mM magnesium acetate, 1 mM DTT, and 0.1 mg/mL BSA. The protein, 4A′ (1 μM) or 4A′/K318A (1 μM), was preincubated for 2–4 min at 22 °C with 1 μM T7 DNA polymerase (1:20 ratio of exo$^-$ T7 gene 5 protein and thioredoxin), 1 mM each of dATP, dCTP, dGTP, ATP, and CTP, 6 mM dTTP, and [α-^{32}P]dCTP (10 μCi). Reactions were initiated by adding single-stranded M13 DNA (50 nM) and quenched with 0.2 M EDTA after intervals ranging from 5 to 60 min. A control reaction was performed under the conditions described above except no gene 4 protein was added.

The RNA-primed DNA synthesis products were analyzed both by the DE81 filter binding assays and by denaturing agarose gel electrophoresis. Samples (5 μL) were spotted on individual circles of DE81 paper (Whatman); the filters were dried and then washed four times with 0.3 M ammonium formate buffer, pH 8.0. Radioactivity on both washed and unwashed filters was determined by scintillation counting, and the molar amount of radiolabeled dCMP incorporated in the DNA was calculated. Samples (5 μL) were mixed with the alkaline gel loading dye (50 mM NaOH, 1 mM EDTA, 3% Ficoll 400, and 0.05% bromophenol blue) and applied to a 0.6% alkaline agarose gel (15 × 30 cm). Electrophoresis was carried out at 4 °C for 10–15 h at 400 mA constant current. The DNA products were analyzed on a Betascope instrument.

The mixture of 4A′ and 4A′/K318A mutant protein was also analyzed for primase and helicase activities. A constant amount of 4A′ (0.2 μM) was mixed with increasing concentrations of 4A′/K318A (0–2 μM) in the absence of any ligands and incubated for 10 min at 22 °C. A mixture of magnesium acetate (10 mM), dNTPs, rNTPs, and T7 DNA polymerase was then added, and the reactions were initiated by adding 50 nM single-stranded M13 DNA and analyzed as described above.

Reversible Cross-Linking of Proteins using SAED. All the steps in this experiment were performed in the dark. A stock solution of fluorescent 4A′/K318A mutant protein was prepared by cross-linking 100 μM mutant protein with 100 μM SAED for 1 h at 22 °C in buffer containing 40 mM HEPES, pH 8.0, and 20 mM sodium acetate. Unreacted SAED was removed by passing the reaction mixture through two Sephadex G-25 gel-filtration columns. Twenty micromolar fluorescent 4A′/K318A protein was mixed with 5 or 10 μM 4B, 10 μM chicken egg albumin, and 10 μM β-galactosidase in separate reactions, and the solutions were incubated for 10 min at 22 °C. A mixture of magnesium acetate (10 mM) and dTMP-PCP (1 mM) was added, and the solutions were incubated for 5 min prior to irradiation at 366 nm with a UVGL-25 Mineralight lamp at a distance of 2 cm at 4 °C for 1 h. After photo-cross-linking, the disulfide linkage in the cross-linker was cleaved by incubating the reaction mixtures with 100 mM DTT for 20 min. SDS dye (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) was added, and the samples were applied to a 9% SDS–polyacrylamide gel (Laemmli, 1970). The resolved proteins were blotted on Immobilon-P membrane using a Janssen SemiDry Electroblotter. The membrane was air-dried, and the fluorescent proteins were visualized by excitation with a 366-nm Mineralight lamp and photographed.

Protein–DNA Cross-Linking. Twenty micromolar of 4A′ protein was incubated with 5 μM [5′-^{32}P](dT)$_{30}$ DNA and 10 μM 4A′ was incubated with a uniformly radiolabeled poly(5-BrdU) DNA (0.5 μM) in separate reactions in the presence of 1 mM dTMP-PCP in 50 mM Tris–acetate buffer, pH 7.5, 50 mM sodium acetate, 1 mM DTT, 10 mM magnesium acetate, and 2.5 mM $CaCl_2$. The uniformly radiolabeled poly(5-BrdU) DNA was prepared by elongating a 30-mer DNA (0.1 μM) using 5-BrdUTP (2 mM), [α-^{32}P]dTTP (30 μCi), and terminal transferase enzyme as described previously (Patel & Hingorani, 1993). The reaction mixtures were spotted as 10-μL drops on a Parafilm sheet placed on ice. The solutions were irradiated at 254 nm using a UVGL-25 Mineralight lamp at a distance of 2 cm. Aliquots were removed after 0, 10, 30, and 60 min, and reactions with (dT)$_{30}$ DNA were quenched with an equal volume of SDS gel loading dye (50 mM Tris-HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol). Reaction mixtures containing poly(5-BrdU) DNA were treated with nuclease S7 (1.6 μg/mL) for 5 min to digest free DNA prior to addition of SDS gel loading dye. 4A′ species cross-linked to (dT)$_{30}$ were resolved on a 2–8% gradient SDS–polyacrylamide gel, and those cross-linked to poly(5-BrdU) DNA were resolved on a 3–8% gradient gel. DNA-cross-linked 4A′ protein species were visualized both by autoradiography and by Coomassie Blue staining.

RESULTS

Mutation of Lysine 318 of 4A′ to Alanine. Lysine 318 in 4A protein lies in a putative nucleotide binding motif which is conserved among several helicases that show amino acid sequence homology (Ilyina *et al.*, 1992). To understand the importance of this lysine residue in T7 primase–helicase protein, we have used oligonucleotide site-directed mutagenesis and substituted lysine 318 in 4A′ with alanine. Alanine was chosen because of its smaller size and its neutral side chain. The mutant protein, referred to as 4A′/K318A, was overexpressed using the T7 RNA polymerase expression system

Table 1: Steady-State dTTPase Kinetic Parameters of 4A′ and 4A′/K318A Proteins

protein	temperature (°C)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (mM^{-1} s^{-1})
4A′	37	16.5 ± 1.0	1.7 ± 0.3	9.7
4A′	22	2.8 ± 0.24	0.89 ± 0.2	3.1
4A′/K318A	37	0.19 ± 0.045	4.2 ± 0.8	0.045
4A′/K318A	22	0.043 ± 0.01	4.3 ± 0.03	0.01

(Studier *et al.*, 1990) and purified following the protocol developed for 4A′ protein (Patel *et al.*, 1992). The importance of the lysine residue was investigated by comparing the various properties of the mutant protein to those of 4A′, including the steady-state kinetics of the dTTPase, primase, and helicase activities as well as the ability of the mutant protein to form oligomers and bind DNA.

dTTPase Activity of the 4A′/K318A Protein. The DNA-dependent dTTPase activities of 4A′/K318A mutant and 4A′ proteins were measured at 22 and 37 °C using single-stranded M13 DNA as the effector. In the absence of DNA, the dTTPase activity of the mutant protein was less than 0.001 s^{-1}. In the presence of DNA, the dTTPase activity of 4A′/K318A was detectable but still lower than that of 4A′. Table 1 shows the steady-state k_{cat}, K_m, and k_{cat}/K_m values of the dTTPase activity of 4A′ and 4A′/K318A proteins obtained by measuring the activity over a range of protein concentrations from 0.5 to 5 μM. The mutant protein has a 5-fold higher K_m at 22 °C and a 3-fold higher K_m at 37 °C. The k_{cat} of the mutant protein is about 100-fold lower than that of 4A′ at both 22 and 37 °C. Therefore, the mutation has a larger effect on the k_{cat} than on the K_m of the dTTPase activity. Overall, the k_{cat}/K_m ratio of the mutant protein is 200–300-fold lower than that of 4A′ at both temperatures.

To confirm that the DNA concentration was not limiting in the above experiments, the dTTPase experiments were performed at increasing single-stranded M13 DNA concentrations. The DNA-dependent dTTPase activity increased slightly from 25 to 50 nM DNA but remained constant above 50 nM DNA concentration (data not shown). Therefore, the k_{cat} values shown in Table 1 measured at 50 nM DNA concentration represent the maximum dTTP hydrolysis rate constants of the mutant protein.

Equilibrium DNA Binding Studies of 4A′/K318A Protein. Nitrocellulose DNA binding assays were performed to determine whether the mutation had affected the ability of the protein to bind single-stranded DNA. A 30-nucleotide-long single-stranded DNA was used as the ligand, and experiments were performed at constant DNA and increasing enzyme concentrations as described previously (Hingorani & Patel, 1993). Figure 1 shows the equilibrium binding of 30-mer DNA to 4A′ and 4A′/K318A proteins. In the presence of MgdTMP-PCP, 4A′ protein binds 30-mer DNA tightly with a stoichiometry of one DNA per hexamer. Interestingly, the mutant protein seems to have no affinity for the DNA under these conditions. The mutant protein, however, does bind about 60% of DNA in the presence of MgdTTP, conditions under which 4A′ shows about 30% DNA binding perhaps due to its efficient dTTPase activity. These results indicate that 4A′/K318A protein can bind DNA in the presence of MgdTTP, although its interaction with the DNA may be weaker than that of 4A′ in the presence of MgdTMP-PCP.

Protein Oligomerization. To determine whether the K318A mutation had affected the ability of 4A′/K318A protein to self-assemble into hexamers, we investigated the oligomerization properties of 4A′/K318A mutant protein using HPLC gel filtration experiments similar to those described for 4A′ protein (Patel & Hingorani, 1993). Because the behavior of the mutant protein was very similar to that of 4A′, the gel-filtration profiles of the mutant protein are not shown here. Gel-filtration experiments were performed at increasing 4A′/K318A protein (a) in the absence of nucleotides and DNA, (b) in the presence of 200 μM dTTP and 10 mM $MgCl_2$, and (c) in the presence of MgdTTP and 60-mer DNA. The oligomerization properties of the mutant protein in the absence of ligands were comparable to those of 4A′ protein. At lower protein concentrations (about 5 μM) 4A′/K318A formed dimers, and as protein concentration was increased, dimers plus higher oligomers eluted as a broad peak. In the presence of MgdTTP, 4A′/K318A mutant formed mixtures of stable dimers and hexamers similar to the behavior of 4A′ in the presence of MgdTMP-PCP. The only difference was that 4A′/K318A mutant required 8–10-fold higher protein concentrations to form stable hexamers. The requirement for higher mutant protein concentration may be due to the subsaturating levels of MgdTTP (less than its K_m of 6 mM) used in the gel-filtration experiments. We did not repeat the experiment at higher dTTP concentrations because millimolar amounts of dTTP were required in the gel-filtration buffer to saturate the binding interactions of the mutant protein. However, native polyacrylamide gel analysis (Hingorani & Patel, 1993) conducted at higher MgdTTP concentrations showed that the mutant protein can form stable hexamers in amounts comparable to those formed by 4A′ (data not shown). Gel-filtration experiments in the presence of MgdTTP indicated that the mutant protein hexamers were moderately stabilized by the presence of 60-mer DNA. Chemical cross-linking experiments similar to those described for the 4A′ protein (Patel & Hingorani, 1993) also resulted in cross-linked species of 4A′/K318A mutant protein ranging from dimers to hexamers (data not shown).

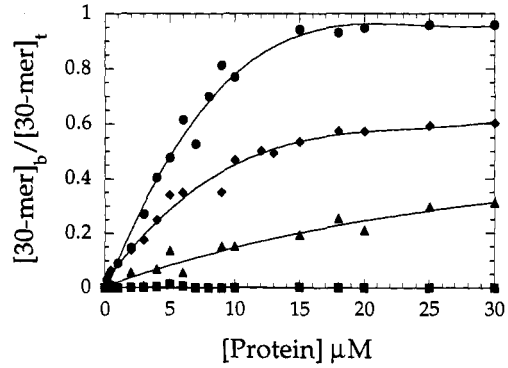

FIGURE 1: Equilibrium binding of 4A′ and 4A′/K318A proteins to 30-mer DNA. A constant amount of [5′-^{32}P]30-mer (1 μM) was titrated with increasing concentrations of 4A′ in the presence of 1 mM dTMP-PCP (●) or 5 mM dTTP (▲). Similar titrations were conducted with increasing concentrations of 4A′/K318A in the presence of 1 mM dTMP-PCP (■) or 5 mM dTTP (◆). The DNA binding experiments were performed at 22 °C using nitrocellulose–DEAE membranes as described in the Experimental Procedures. The 4A′ protein binds nearly 100% of 30-mer in the presence of MgdTMP-PCP with a stoichiometry of 1 DNA per hexamer but binds only 30% of DNA in the presence of MgdTTP. The mutant protein shows almost no DNA binding in the presence of dTMP-PCP but binds about 60% of DNA in the presence of MgdTTP.

Primase Activity. It has been shown that 4A′ synthesizes RNA primers only in the presence of dTTP with sequences 5′-pppACCC, 5′-pppACCA, and 5′-pppACAC at complementary sites on single-stranded DNA such as M13 DNA (Nakai & Richardson, 1988). However, it is not clear whether dTTP hydrolysis is necessary for primer synthesis. Here we

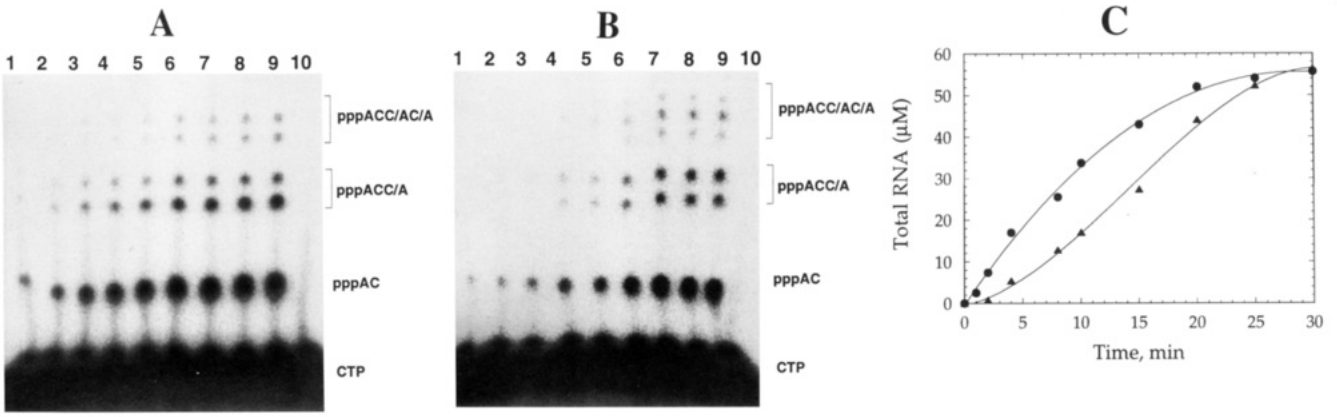

FIGURE 2: Steady-state kinetics of RNA primer synthesis by 4A′ and 4A′/K318A proteins. Panels A and B show various RNA products synthesized by 4A′ and 4A′/K318A proteins, respectively, that were resolved on 25% polyacrylamide/3 M urea gels. Primer synthesis was measured at 22 °C as described in Experimental Procedures. Lanes 1–10 in each gel represent 1, 2, 4, 8, 10, 15, 20, 25, 30, and 0 min of reaction. The lower intense band on each gel is unreacted [α-^{32}P]CTP, and the bands migrating above CTP have been assigned as dimer, trimer, and tetramer RNA products. Panel C shows quantitation of total RNA (dimers to tetramers) synthesis by 4A′ (●) and 4A′/K318A (▲) proteins. The linear rate constants of RNA synthesis were calculated as 0.055 s^{-1} for 4A′ and 0.037 s^{-1} for 4A′/K318A.

had an opportunity to examine the role of dTTP hydrolysis in primase function by studying the primase activity of 4A′/K318A mutant protein which is defective in dTTPase activity. The steady-state kinetics of primer synthesis was measured using single-stranded M13 DNA as the template and by following the incorporation of radiolabeled CMP in the RNA primers. The RNA products were resolved from one another and from the unreacted CTP on a 25% polyacrylamide/urea gel.

Panels A and B of Figure 2 show the kinetics of RNA primer synthesis by 4A′ and 4A′/K318A proteins, respectively. It is clear that the mutant protein can synthesize RNA primers. The distribution and the processivity of RNA synthesis by both proteins is also similar, except for some minor differences. The dimer, pppAC, is the major RNA product in both 4A′ and 4A′/K318A reactions. The trimer products, pppACC and pppACA, are made in equal amounts by the mutant protein, but 4A′ makes one trimer in excess. Similarly, three tetramer products, pppACCC, pppACCA, and pppACAC, are made by the mutant protein, but only two are detectable in the 4A′ reaction. Figure 2C shows quantitation of the RNA products. The initial rate constant of total RNA synthesis by 4A′ is 0.055 s^{-1}, and the rate constant of the mutant protein is 0.037 s^{-1}, only 1.5-fold lower than that of 4A′. The kinetics of RNA synthesis in the 4A′ reaction is also linear up to 10 min, after which RNA synthesis starts to level off. This is most likely due to the depletion of dTTP in the reaction as a result of the higher dTTPase activity of 4A′. The kinetics of primer synthesis by 4A′/K318A, on the other hand, shows a lag of about 2–3 min, but RNA synthesis continues to increase linearly even after 20 min. Therefore, at longer times the amount of RNA products in the mutant protein reaction mixture reaches close to the levels of RNA in the 4A′ reaction. Similar experiments were performed over a range of protein concentrations from 0.01 to 1 μM. Even at the lower protein concentrations the primase activity of the mutant protein was only about 2-fold less than the primase activity of 4A′ (data not shown). These experiments therefore suggest that dTTP hydrolysis may not be necessary for primase activity, and this is consistent with the experiments in the presence of dTMP-PCP that are described below.

Primase and Helicase Activities. Because the mutant protein has a normal primase activity, its helicase activity can be measured using the RNA-primed DNA synthesis assay. In this assay, the primase synthesizes small RNA primers on single-stranded M13 DNA that are used by T7 DNA polymerase to copy the circular M13 DNA. In the absence of helicase activity, the longest DNA product made by the DNA polymerase is around 7 kb in size. In the presence of helicase activity, longer rolling circle DNA products form as the helicase unwinds the newly synthesized duplex DNA. The RNA-primed DNA synthesis reactions with 4A′ and 4A′/K318A were performed at high concentrations of dTTP (6 mM) to facilitate optimum binding of 4A′/K318A protein to the DNA template. The dNTP concentrations were also saturating for the DNA polymerase. Figure 3A shows an alkaline agarose gel used to resolve the RNA-primed DNA products synthesized by 1 μM 4A′ and 4A′/K318A proteins and a control reaction lacking gene 4 protein. DNA products 7 kb and shorter in size facilitated by the primase activity are formed in both the 4A′ and the 4A′/K318A reactions. However, DNA products above 7 kb and greater than 20 kb facilitated by the helicase activity are present only in the 4A′ reaction, and these longer DNA products are absent in reactions containing the mutant protein. DNA products 7 kb in size and a small amout of products 14 kb in size are also present in the control reaction. The 7-kb product most likely results from contaminating primers in the single-stranded M13 DNA preparation, and the DNA products twice that size may result from replication of linear single-stranded M13 DNA primed either by hairpin formation or by a second molecule of linear DNA. The absence of DNA products >20 kb in size in the mutant reaction indicates that the mutant protein does not have helicase activity.

Quantitation of DNA products in the 4A′ and mutant reactions after correction for the DNA products in the control reaction is shown in Figure 3B. The rate of DNA synthesis in reactions containing the mutant protein is 0.08 s^{-1}, about 2.5-fold lower than the rate of 0.2 s^{-1} in reactions containing 4A′. Similar 2-fold lower RNA-primed DNA synthesis activity of the mutant protein was observed when the above experiments were performed over a range of protein concentrations from 50 nM to 1 μM. These results are consistent with the direct primer synthesis assays that also showed only 1.5–2-fold lower primase activity of the mutant protein. The helicase activity of the mutant protein was also measured using the primer-displacement assay, and the results are shown in Figure 4. Whereas 4A′ efficiently displaced the 60-mer primer annealed to M13 DNA, no detectable displacement of the primer was observed with the mutant protein even at

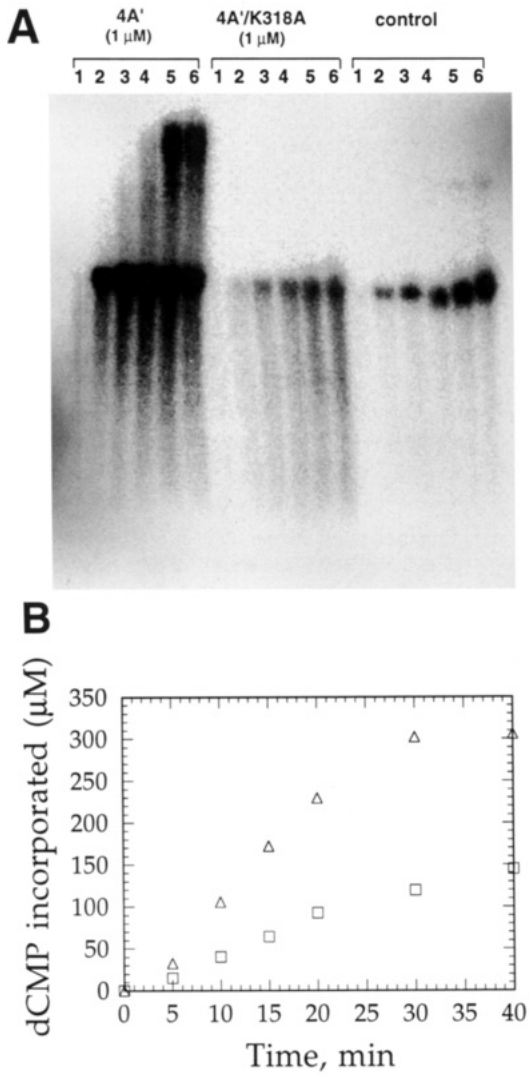

FIGURE 3: RNA-primed DNA synthesis catalyzed by 4A′ and 4A′/K318A proteins. Panel A shows a Betascope image of a 0.6% alkaline agarose gel that was used to resolve the RNA-primed DNA products synthesized by 1 μM 4A′ and 4A′/K318A protein and DNA products synthesized in a control reaction with no gene 4 protein. The assays were performed at 22 °C as described in Experimental Procedures. Lanes 1–6 represent reactions quenched after 5, 10, 15, 20, 30, and 60 min. Panel B shows the kinetics of total DNA synthesis by 1 μM 4A′ (△) and 1 μM 4A′/K318A (□) proteins measured by DE-81 filter binding assays (see Experimental Procedures) after correction for the DNA products in the control reaction. The DNA synthesis rate constants were calculated from the initial slopes. The rate constant for DNA synthesis by 4A′ is 0.2 s^{-1} and by 1 μM 4A′/K318A, 0.08 s^{-1}.

a 5-fold higher mutant protein concentration. Therefore, both the RNA-primed DNA synthesis assay and the primer-displacement assay indicate that the mutant protein lacks helicase activity.

Primase Activity of 4A′ in the Presence of MgdTMP-PCP. Even though 4A′/K318A has a lower dTTPase activity relative to 4A′, it appears to have a normal primase activity. This suggests that dTTP binding may be sufficient for the primase activity. If this were true, then dTMP-PCP (the nonhydrolyzable analog of dTTP) should support primer synthesis by 4A′ protein. We therefore investigated the primase activity of 4A′ in the presence of MgdTMP-PCP using the primer synthesis assay. The assay was performed in the presence of dTTP or dTMP-PCP using a 60-mer DNA as the template (see Experimental Procedures for the DNA sequence). Panels A and B of Figure 5 show that 4A′ can indeed synthesize RNA primers in the presence of MgdTMP-PCP in amounts

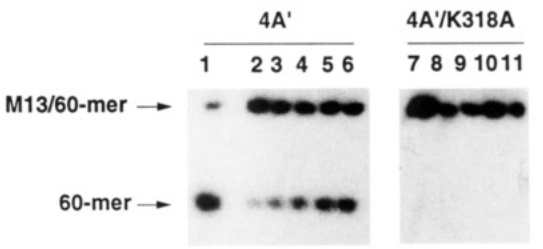

FIGURE 4: Helicase activity of 4A′ and 4A′/K318A mutant protein. Helicase activity was measured by the primer displacement assay at 22 °C as described in Experimental Procedures. The displaced 60-mer was resolved from the M13/60-mer complex on a native 12% acrylamide gel. Lane 1 represents heat-denatured DNA. Lanes 2–6 and 7–11 represent reactions quenched after 5, 10, 15, 20, and 30 min and catalyzed by 0.5 μM 4A′ and 2.5 μM 4A′/K318A, respectively.

comparable to those of RNA products in the presence of MgdTTP. A control experiment containing normal amounts of ATP and CTP but no dTTP or dTMP-PCP showed no RNA primer synthesis (data not shown). Therefore, ATP or CTP does not support primer synthesis. Even though no dTTP contamination in dTMP-PCP was detectable by thin-layer chromatography, 4A′ protein was preincubated with MgdTMP-PCP for at least 30 min to hydrolyze any contaminating dTTP. Quantitation of RNA products shows a 2-fold lower initial rate of primer synthesis in reactions containing MgdTMP-PCP versus MgdTTP (Figure 5C). At longer times the amounts of RNA products synthesized in the two reactions become the same. When the same experiment was performed using lower (1 μM) protein concentrations, the primase activity in the presence of dTMP-PCP was 10-fold lower than the activity of 4A′ in the presence of dTTP (not shown). The same results were also obtained with single-stranded M13 DNA as the template. These results indicate that dTMP-PCP supports primer synthesis to about the same extent as dTTP at higher protein concentrations but not at lower protein concentrations.

Studies described thus far show that 4A′/K318A mutant protein has a reduced dTTPase activity and an undetectable helicase activity. However, the mutant protein appears to have a nearly normal primase activity, and it can bind DNA in the presence of MgdTTP as well as self-assemble into hexamers in the presence of MgdTTP and single-stranded DNA. In the absence of any ligands, both 4A′ and the mutant protein exist in various oligomeric forms ranging from dimers to hexamers. Therefore, it is likely that the two proteins will form heterooligomers when mixed in the absence of ligands, similar to heterodimer formation between 4A′ and 4B proteins (Patel *et al.*, 1992). If heterooligomers are formed, then we can investigate the effect of substituting an active subunit of 4A′ with an inactive subunit. Cross-linking experiments described below were performed to investigate whether the mutant protein forms heterooligomers.

Heterooligomer Formation between 4A′/K318A and 4B Proteins. Because 4A′ and 4A′/K318A proteins have the same molecular mass, we used 4B protein to demonstrate heterooligomer formation. It was technically difficult to resolve homooligomers of either 4B or the mutant protein from the heterooligomers using gel-filtration or chemical cross-linking techniques. Therefore, we have used a reversible cross-linking technique similar to that used by Wong *et al.* (1993) to show heterodimer formation. In this method, 4A′/K318A mutant was first covalently modified with the sulfo-*N*-succinimidyl ester group of SAED fluorescent cross-linker. The modified 4A′/K318A protein was incubated with 4B and photo-cross-linked. Proteins such as chicken egg albumin and

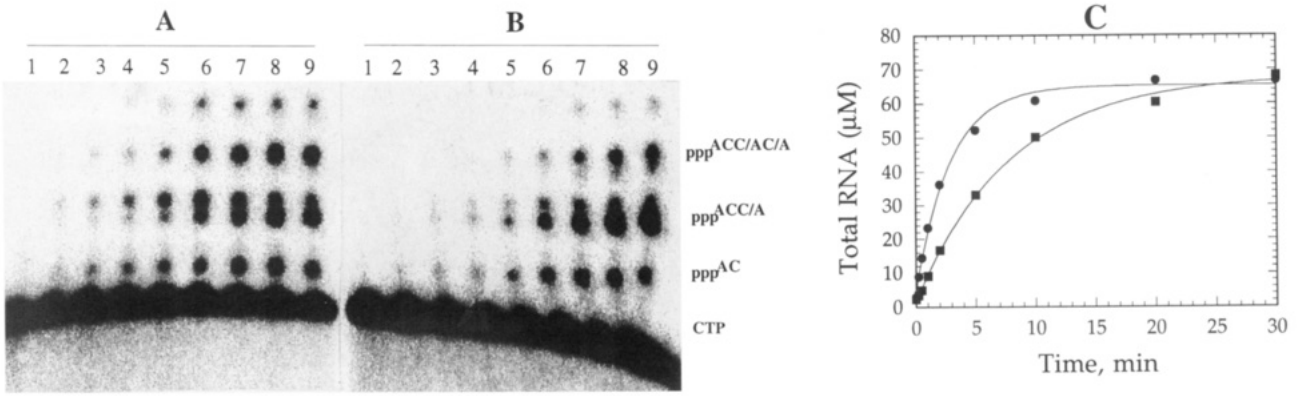

FIGURE 5: RNA primer synthesis by 4A′ in the presence of MgdTMP-PCP. RNA primers synthesized by 4A′ (30 μM) on a 60-mer DNA template (20 μM) in the presence of 5 mM dTTP (A) and 1 mM dTMP-PCP (B) are shown. Primer synthesis reactions were performed at 22 °C as described in Experimental Procedures. Lanes 1–9 in panels A and B represent reactions quenched after 0, 0.25, 0.5, 1, 2, 5, 10, 20, and 30 min. Panel C shows quantitation of RNA products synthesized in the presence of dTTP (●) and dTMP-PCP (■).

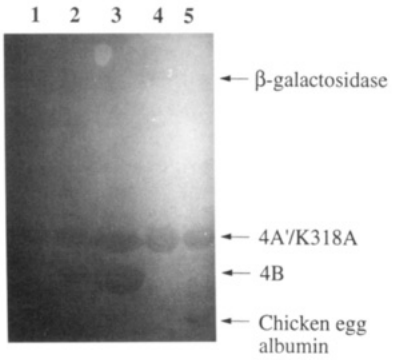

FIGURE 6: Reversible cross-linking of 4A′/K318A mutant protein to 4B using SAED. A photograph of fluorescent proteins resolved on 9% SDS–polyacrylamide gel is shown. 4A′/K318A was chemically cross-linked to SAED, and free probe was removed by gel filtration. The fluorescent 4A′/K318A protein (20 μM) was cross-linked to 4B and control proteins chicken egg albumin and β-galactosidase as described in Experimental Procedures. Lane 1 shows 20 μM fluorescent 4A′/K318A protein photo-cross-linked to itself; lanes 2 and 3 show photo-cross-linking of 20 μM 4A′/K318A to 5 and 10 μM 4B protein, respectively; and lanes 4 and 5 show photo-cross-linking of 4A′/K318A to 10 μM β-galactosidase (MW 116 250) and chicken egg albumin (MW 45 000), respectively.

β-galactosidase were used as controls. If 4A′/K318A associates with 4B, then the azido group on the fluorescent end of the cross-linker will covalently attach to 4B. Subsequent treatment with high concentrations of DTT reduces the disulfide linkage in the cross-linker and transfers the fluorescent group to the associated protein. Figure 6 shows the resulting fluorescent proteins that were resolved on an SDS–polyacrylamide gel. When 4A′/K318A protein is photo-cross-linked in the absence of added protein, the mutant protein cross-links to itself. Therefore, fluorescent 4A′/K318A is detected in lane 1. When 4A′/K318A is cross-linked in the presence of 4B protein, the fluorescent group is transferred to 4B (lanes 2 and 3). It appears that 4A′/K318A protein prefers to form heterooligomers because the fluorescence of 4B is more intense than that of 4A′/K318A. The fluorescence intensity of control proteins chicken egg albumin and β-galactosidase is relatively weak, indicating that free cross-linker either is absent or is present in very small amounts in the reactions. This experiment demonstrates heterooligomer formation between 4B and the mutant protein and suggests that 4A′ and the mutant protein may also associate in a similar manner.

Effect of 4A′/K318A Mutant Protein on the Helicase Activity of 4A′. To determine the effect of the mutant protein on the helicase activity of 4A′, RNA-primed DNA synthesis assays were performed using a constant amount of 4A′ protein mixed with increasing concentrations of the mutant 4A′/K318A protein. Figure 7 shows an alkaline gel of RNA-primed DNA products in which formation of DNA products larger than 7 kb is facilitated by the helicase activity of 4A′. When increasing concentrations of 4A′/K318A protein were added to 4A′, a substantial decrease in the helicase activity of 4A′ was observed. At a 5–10-fold higher mutant protein concentration, the helicase activity rapidly became undetectable. The concentration of 4A′ in all the reactions was kept constant. Therefore, the observed decrease in the helicase activity must be due to mixed oligomer formation between 4A′ and the mutant protein. Both single-stranded M13 DNA and dTTP were also present in excess of the protein hexamers. Therefore, limiting substrate is not responsible for the reduction in the helicase activity.

Effect of Varying Incubation Time on dTTPase Activity of 4A′/K318A and 4A′ Protein Mixture. The following experiment was designed to determine whether the mutant protein affected the dTTPase activity of 4A′ and whether the effect was time-dependent. Experiments were also performed under various conditions to investigate whether the presence of ligands affected the dTTPase activity. Equal concentrations of 4A′ and 4A′/K318A proteins were incubated for increasing periods in the absence and in the presence of ligands such as MgdTTP and DNA. As shown in Figure 8, when the two proteins were preincubated in the absence of any ligands prior to mixing, there was a time-dependent decrease in the dTTPase activity of 4A′. When 4A′ and 4A′/K318A proteins were incubated with MgdTTP prior to mixing, only a small time-dependent decrease (about 20%) in the dTTPase activity was observed. However, when 4A′ and 4A′/K318A proteins were treated with both MgdTTP and single-stranded M13 DNA prior to mixing, no time-dependent loss in the dTTPase activity was observed even after 30 min of mixing.

Gel-filtration and native PAGE experiments have shown that both 4A′ and 4A′/K318A proteins form unstable hexamers in the absence of MgdTTP and thus both proteins exist in various oligomeric forms under these conditions. Therefore, the rapid decrease ($t_{1/2}$ of 20 s) in the dTTPase activity of 4A′ in the absence of ligands must be due to heterooligomer formation as well as the lower activity of the heterooligomers. Furthermore, both proteins form stable hexamers in the presence of MgdTTP and DNA. Therefore, the relatively small decrease in the dTTPase activity, when the two proteins were preincubated with MgdTTP or with MgdTTP and DNA prior to mixing, indicates that protein subunits in stable hexamers exchange very slowly.

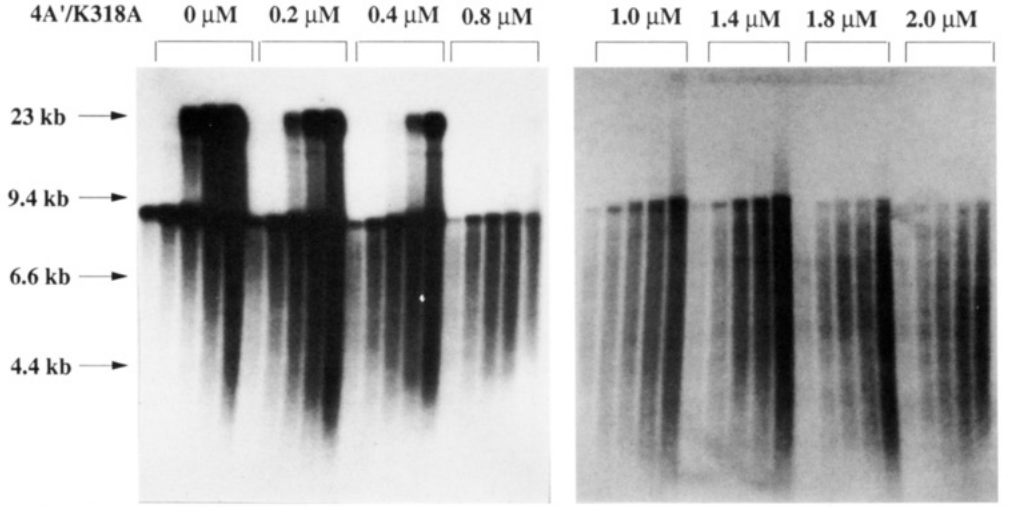

FIGURE 7: RNA-primed DNA synthesis activity of 4A′ in the presence of increasing concentrations of 4A′/K318A. Autoradiograms of 0.6% alkaline agarose gels that were used to resolve the RNA-primed DNA synthesis products of 4A′ (0.2 μM) in the presence of increasing 4A′/K318A protein (0–2 μM) are shown. The concentrations of 4A′/K318A are shown across the top. The two proteins were mixed in the absence of any ligands at 22 °C and assayed as described in Experimental Procedures. The five lanes in each experiment correspond to reactions quenched after 5, 10, 20, 30, and 60 min.

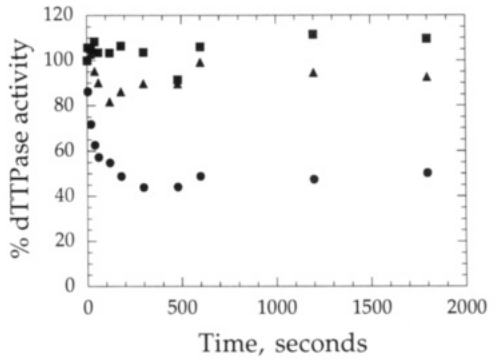

FIGURE 8: Time-dependent decrease in the dTTPase activity of 4A′ mixed with 4A′/K318A. 4A′ and 4A′/K318A (1 μM) were each preincubated under different conditions, and the DNA-dependent dTTPase activity of the mixed proteins was assayed as described in Experimental Procedures. The dTTPase activities of the mixture of 4A′ + 4A′/K318A proteins preincubated in the absence of any ligands before mixing (●), in the presence of MgdTTP before mixing (▲), and in the presence of both MgdTTP and single-stranded M13 DNA before mixing (■) are shown. The activities have been normalized to the DNA-dependent dTTPase activity of 4A′ in the absence of the mutant protein.

dTTPase Activity of Heterooligomers in the Presence of Single-Stranded M13 DNA. The ability of 4A′/K318A to inhibit the dTTPase activity of 4A′ was further investigated by titrating a constant amount of 4A′ with increasing concentrations of the 4A′/K318A mutant protein. 4A′ hydrolyzes dTTP with a steady-state rate constant between 2 and 3 s^{-1} in the presence of single-stranded M13 DNA at 22 °C (Figure 9A). When increasing concentrations of 4A′/K318A protein are added to 4A′, the dTTPase activity of 4A′ drops sharply. At a 1:1 ratio of the two proteins, the activity of the mixture is 5-fold lower than the activity of 4A′. Beyond a 1:1 ratio of the two proteins only a slight loss in activity is observed. Therefore, at a 10-fold higher 4A′/K318A concentration, a constant low level of dTTP hydrolysis at 0.1 s^{-1} is still detectable. This residual activity is not contributed by the mutant protein because the mutant protein has a much lower activity (0.012 s^{-1}). The residual activity, therefore, either represents the activity of a small percentage of 4A′ hexamers that did not exchange or may be the intrinsic activity of the heterooligomers. The dTTPase activity of 4B protein also decreased in a similar manner upon addition of increasing amounts of 4A′/K318A mutant protein. We have confirmed that the decrease in activity is not due to limiting DNA concentrations because the same decrease in activity was observed with 50, 100, and 200 nM single-stranded M13 DNA. In addition, when 4A′ and 4A′/K318A proteins were incubated with MgdTTP and single-stranded M13 DNA prior to mixing, no decrease in the dTTPase activity was observed (inset in Figure 9A).

dTTPase Activity of Heterooligomers in the Presence of 30-mer Oligodeoxynucleotide. The effect of 4A′/K318A protein on the 30-mer DNA-dependent dTTPase activity of 4A′ was investigated at increasing mutant protein concentrations. As shown in Figure 9B, the steady-state dTTPase rate constant of 4A′ in the presence of 30-mer DNA is close to 0.25 s^{-1}. When increasing 4A′/K318A protein is added, initially there is a small reduction in the 30-mer DNA-dependent dTTPase activity. As addition of 4A′/K318A is continued, the activity remains constant at 0.1–0.2 s^{-1} even in the presence of a 10-fold higher concentration of the mutant protein. This experiment indicates that the 30-mer DNA-dependent dTTPase activity of heterooligomers is not very different from the activity of 4A′.

dTTPase Activity of Heterooligomers in the Absence of DNA. Next, we investigated the dTTPase activity of heterooligomers in the absence of DNA. As shown in Figure 9C, the dTTPase activity of 4A′ in the absence of DNA is very low, between 0.01 and 0.013 s^{-1}. When increasing amounts of 4A′/K318A protein were added to 4A′, the dTTPase activity actually increased. At an approximate 1:2 ratio of 4A′ to mutant protein, the hydrolysis rate increases from 0.012 s^{-1} to a maximum of about 0.04 s^{-1}. This increase in the dTTPase activity cannot be due to the activity of added mutant protein because the activity of 4A′/K318A is <0.001 s^{-1}. Therefore, the above results indicate that the DNA-independent dTTPase activity of heterooligomers is comparable to that of 4A′ and higher than the activity of the mutant protein.

Equilibrium DNA Binding Studies of 4A′ and 4A′/K318A Mixtures. 4A′/K318A mutant protein does not bind DNA in the presence of MgdTMP-PCP. To determine whether heterooligomers of 4A′ and the mutant protein were capable of binding DNA in the presence of MgdTMP-PCP, DNA binding studies were performed at constant 30-mer DNA and increasing concentrations of 1:1, 1:2, and 1:3 mixtures of 4A′

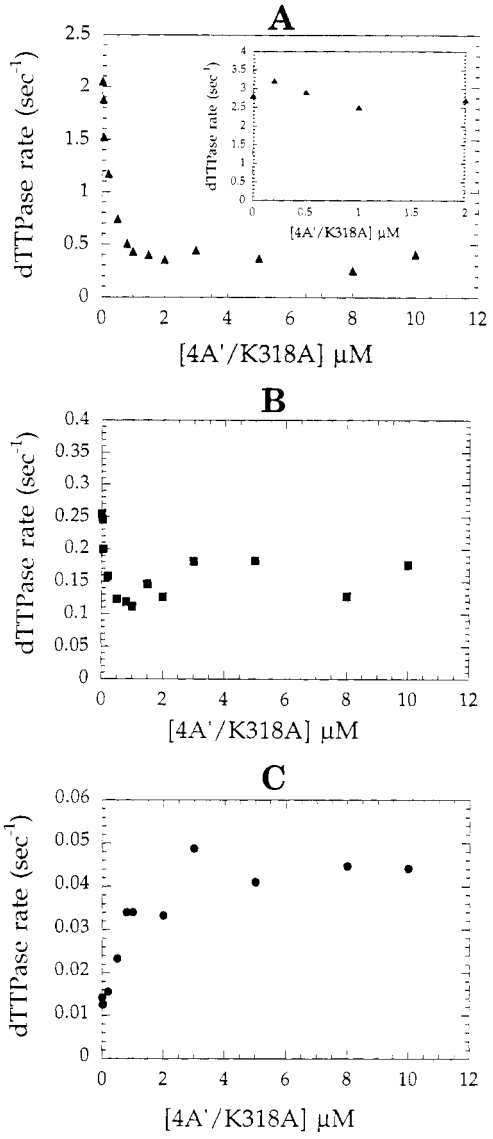

FIGURE 9: dTTPase activity of 4A′ in the presence of increasing 4A′/K318A protein concentrations. A constant amount of 4A′ (1 μM) was mixed with increasing concentrations of 4A′/K318A (0–10 μM) in the absence of any ligands. The mixed proteins were assayed for dTTPase activity under various conditions. Panel A shows the decrease in the dTTPase activity of 4A′ upon addition of increasing amounts of 4A′/K318A protein assayed in the presence of 5 mM MgdTTP and 50 nM single-stranded M13 DNA (▲). The inset shows a control experiment performed similarly except the proteins were incubated with MgdTTP and DNA prior to mixing and measuring the dTTPase activity. Panel B shows the decrease in the dTTPase activity of 4A′ with increasing 4A′/K318A protein assayed in the presence of 5 mM MgdTTP and 10 μM 30-mer DNA (■). Panel C shows the increase in the dTTPase activity of 4A′ with increasing 4A′/K318A protein assayed in the presence of 5 mM MgdTTP (●). The maximum decrease in the activities (with M13 and 30-mer DNA) or the maximum increase in the activity (in the absence of DNA) occurs at an approximate 1:1 to 1:2 ratio of 4A′ to 4A′/K318A proteins.

and 4A′/K318A proteins. Figure 10 shows 30-mer DNA binding to 4A′ with a stoichiometry of about 1 DNA per 8–10 4A′ monomers, and no DNA binding to 4A′/K318A protein. The heterooligomers of 4A′ and 4A′/K318A proteins, however, bind DNA with the same stoichiometry as 4A′ (1 DNA per 8–10 4A′ + 4A′/K318A monomers) but with a weaker affinity. Unless heterooligomer formation somehow restores the DNA binding ability of the mutant subunits in the presence of MgdTMP-PCP, the above results suggest that all the subunits in the hexamer need not be active to bind DNA. A control experiment was also performed with 4A′ and 4A′/K318A

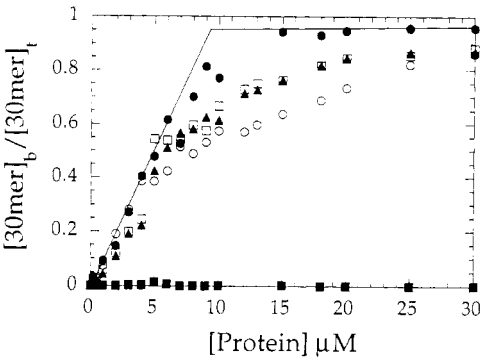

FIGURE 10: Equilibrium DNA binding of 4A′ and 4A′/K318A protein mixtures at constant DNA and increasing protein concentration. 4A′ and 4A′/K318A were mixed in 1:1, 1:2, and 1:3 ratios in the absence of any ligands. A constant amount of [5′-^{32}P]30-mer DNA (1 μM) was titrated with increasing concentrations of 4A′ (●), 4A′/K318A mutant (■), and 1:1 (□), 1:2 (▲), and 1:3 (○) mixtures of 4A′ and 4A′/K318A proteins. Protein concentrations shown on the x-axis represent micromolar amounts of 4A′ alone or 4A′/K318A alone or concentrations of 4A′ + 4A′/K318A mixed in various ratios. DNA binding assays were conducted in the presence of 1 mM dTMP-PCP at 22 °C using nitrocellulose–DEAE membranes as described in Experimental Procedures.

proteins preincubated with MgdTMP-PCP prior to mixing. As expected, the stoichiometry of DNA binding depended only on the concentration of 4A′ and not on the total protein concentration as shown in Figure 10. Thus, 4A′/K318A protein behaved as if it were not present in the binding reactions.

Protein–DNA Cross-Linking. The above DNA binding experiments suggest that the DNA can bind to the hexamer by interacting with only a few subunits. The following protein–DNA cross-linking experiment was performed to determine whether the DNA bound a fully active 4A′ hexamer by interacting with all six subunits or only a few. 4A′ was incubated with [5′-^{32}P](dT)$_{30}$ or a uniformly radiolabeled poly(5-BrdU) DNA in separate reactions in the presence of MgdTMP-PCP and photo-cross-linked for increasing times. Panels A and B of Figure 11 show autoradiograms of SDS–polyacrylamide gels used to resolve the DNA-cross-linked species in poly(5-BrdU) and [5′-^{32}P](dT)$_{30}$ reactions, respectively. Reaction mixtures containing poly(5-BrdU) DNA were treated with nuclease S7 to digest free DNA prior to electrophoresis. In both reactions, DNA cross-linked to 4A′ species ranging from monomers to pentamers. The same protein species were also observed with a Coomassie Blue dye stained gel (not shown). When cross-linked 4A′ species were analyzed on a lower percentage polyacrylamide gel, species as high as octamers were visible (not shown). Control photo-cross-linking experiments in the absence of dTMP-PCP (that promotes stable DNA binding) showed no DNA-cross-linked 4A′ species (data not shown). These experiments therefore indicate that a long single-stranded DNA or a 30-nucleotide DNA may bind 4A′ by interacting with at least five and perhaps all six subunits of the hexamer.

DISCUSSION

Substitution of lysine 318 in 4A′ with alanine reduced the dTTPase activity of the mutant protein relative to that of 4A′. Both the k_{cat} and the K_m values were affected by the mutation. Therefore, the lysine residue is important for both dTTP binding and dTTP hydrolysis. The reduced dTTPase activity of 4A′/K318A is not due to its inability to bind DNA or a defect in hexamer formation. Equilibrium DNA binding experiments showed that the 4A′/K318A protein can bind a

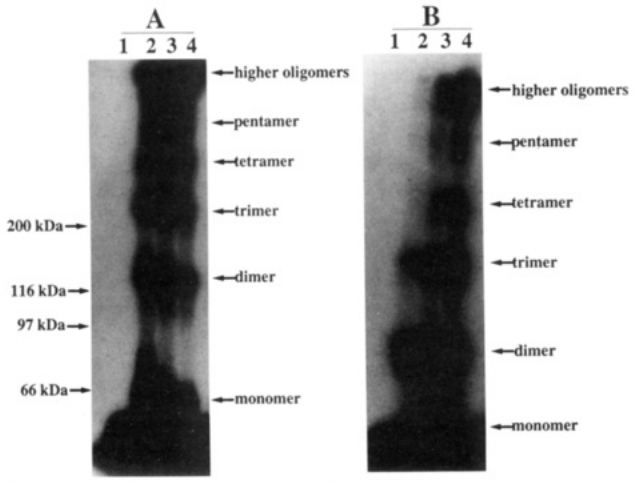

FIGURE 11: Protein–DNA cross-linking. Panels A and B show autoradiograms of 3–8% and 2–8% gradient SDS polyacrylamide gels that were used to resolve 4A′ species cross-linked to poly(5-BrdU) and $(dT)_{30}$ DNA, respectively. 4A′ (10 μM) was cross-linked to [5′-^{32}P]$(dT)_{30}$ DNA (5 μM) and to a uniformly radiolabeled poly-(5-BrdU) DNA (0.5 μM) as described in Experimental Procedures. 4A′ cross-linked to poly(5-BrdU) DNA was treated with nuclease S7 before electrophoresis. Lanes 1–4 in both panels represent DNA-cross-linked 4A′ species formed after 0, 10, 30, and 60 min of irradiation. The positions of protein markers and DNA-cross-linked 4A′ species from monomer to pentamer as well as highly cross-linked species that do not enter the gel are indicated.

30-nucleotide-long single-stranded DNA in the presence of MgdTTP. Interestingly, no DNA binding was observed in the presence of MgdTMP-PCP, probably because the protein may not bind dTMP-PCP. This is surprising because the longer P–C bond length and the shorter P–C–P angle of the analog relative to those of the oxygen-containing nucleotide do not prevent 4A′ protein from binding dTMP-PCP. The failure of K318A mutant protein to bind dTMP-PCP suggests that lysine residue 318 directly or indirectly plays a key role in interactions with dTTP.

Recently, studies of a double mutant, G317V–K318M, of gene 4 proteins (Notarnicola & Richardson, 1993; Mendelman *et al.*, 1993) and an ATPase-deficient mutant of DnaB helicase protein (Shrimankar *et al.*, 1992) have been reported. Both mutant proteins were deficient in NTPase and helicase activities, and unlike 4A′/K318A, the double mutant of 4A showed a highly reduced primase activity. It was interesting that although the double mutant of gene 4 protein was capable of binding dTTP, it did not bind single-stranded DNA in the presence of either MgdTMP-PCP or MgdTTP. Perhaps mutation of both G and K adversely affects a conformational change that is required for DNA binding. Alternatively, the double mutant may be defective in forming hexamers, the active oligomeric form that binds DNA (Hingorani & Patel, 1993). Oligomerization of the double mutant was, however, not reported.

In vivo complementation assays with 4A′/K318A mutant have shown that the Δ3.8–4A T7 deletion phage defective in primase activity described previously (Rosenberg *et al.*, 1992) makes normal plaques on HMS174 cells that express 4A′/K318A protein under the control of the T7 *lac* promoter (K. Griffin and A. Rosenberg, personal communication). These results suggest that the mutant protein is capable of providing primase activity *in vivo*. However, 4A′/K318A does not support growth of Δ3.8–4.5 T7 deletion phage defective in both primase and helicase activities, suggesting that the mutant protein does not provide helicase activity. Upon IPTG induction, however, 4A′/K318A mutant protein greatly decreased the plaque size of 4A′ T7 phage, indicating that higher mutant protein concentrations inhibit the activities of the wild-type protein most likely by heterooligomer formation.

Our *in vitro* characterizations of the mutant protein described here are in agreement with the above *in vivo* results. Even though 4A′/K318A mutant protein has a greatly reduced dTTPase activity, its primase activity is only 1.5–2.5-fold lower than that of 4A′. The reduced dTTPase activity, however, abolishes the helicase activity. The nearly normal primase activity of 4A′/K318A protein suggested that dTTP hydrolysis may not be necessary for primase function. This was supported by primase assays in the presence of MgdTMP-PCP. The nonhydrolyzable analog supports nearly the same amount of primer synthesis at higher 4A′ protein concentrations. Even though 4A′ can synthesize primers in the presence of dTMP-PCP at lower protein concentrations, it appears that optimum primase activity at lower protein concentrations does require dTTP hydrolysis. This may be because dTTP hydrolysis allows the gene 4 protein to processively translocate on single-stranded DNA to screen for new primase sites (Tabor & Richardson, 1981). At higher protein concentrations, processive translocation may become less important in screening for new primase sites and therefore dTTP hydrolysis may not be required. Another possiblity is that multiple turnovers of primer synthesis in the presence of dTMP-PCP may be limited by the slow rate of DNA dissociation from 4A′, which would also result in the synthesis of lower amounts of RNA primers especially at low protein concentrations.

When 4A′/K318A mutant protein was mixed with 4A′, both the helicase and the DNA-dependent dTTPase activity of 4A′ decreased in a time-dependent and concentration-dependent manner. Similar effects were also reported with the double mutants of the gene 4 proteins (Notarnicola & Richardson, 1993). However, the decrease in the DNA-dependent dTTPase activity was observed only when 4A′ and 4A′/K318A proteins were incubated in the absence of any ligands, indicating that the presence of MgdTTP and single-stranded DNA may inhibit subunit exchange between 4A′ and the mutant protein. This is consistent with our previous observations that 4A′ forms stable hexamers in the presence of nucleotide and DNA (Patel & Hingorani, 1993). In addition, reversible cross-linking experiments with a fluorescent cross-linker showed that 4A′/K318A mutant protein associates with 4B and thus provided direct evidence for heterooligomer formation.

Whereas the DNA-dependent dTTPase activity showed a decrease upon heterooligomer formation, the DNA-independent dTTPase activity of 4A′ increased upon addition of the mutant protein. There are two explanations for the observed higher dTTPase activity of the heterooligomers. First, the increase in total protein concentration upon addition of the mutant protein may drive the equilibrium of 4A′ toward hexamer formation. If hexamers are the active species that hydrolyze dTTP, then an increase in the number of hexamers (either hexamers containing 4A′ alone or heterohexamers) will result in a net increase in the dTTPase activity. In support of this hypothesis, published results show a sigmoidal increase in the dTTPase activity of gene 4 proteins with increasing protein concentration (Bernstein & Richardson, 1988b). Alternatively or in addition to the above explanation, the increased activity may result if all the subunits in 4A′ hexamer either do not bind dTTP or do not hydrolyze dTTP simultaneously. If each heterooligomer contained one or more 4A′ subunits that are as active as in hexamers containing 4A′ alone, then there would be a net increase in the dTTP hydrolysis

activity upon addition of the mutant protein as the number of hexamers increased in the reaction.

The DNA-dependent dTTPase activity of heterooligomers showed a more drastic decrease when the activity was measured in the presence of single-stranded M13 DNA versus 30-mer DNA as the effector. Even the intrinsic dTTPase activity of 4A′ is 8–10-fold higher in the presence of M13 DNA than with 30-mer DNA. The lower activity of 4A′ in the presence of 30-mer DNA may be due to rate-limiting DNA dissociation and rebinding events. In other words, 4A′ bound to small DNAs may have to completely dissociate from the DNA after each dTTP hydrolysis event, whereas 4A′ bound to long and circular M13 DNA need not, if 4A′ can processively translocate on long single-stranded DNA. Thus, DNA binding and release events may not limit the dTTPase activity of 4A′ when measured in the presence of long DNAs as they would with smaller 30-mer DNA.

Therefore, the lower DNA-dependent dTTPase activity of heterooligomers in the presence of single-stranded M13 DNA versus 30-mer DNA can be explained if heterooligomer formation prevents processive translocation of protein on the DNA. When the mutant subunits in a heterooligomer encounter DNA, either the DNA remains tightly bound to the mutant subunits due to the lower dTTPase activity or the DNA may actually dissociate more easily because 4A′/K318A has a higher K_m for dTTP. By either mechanism, the mutant subunits in a heterooligomer can interfere with processive translocation of the protein on longer DNAs. A 30-mer DNA probably has to dissociate and reassociate after each dTTP hydrolysis event when bound even to a fully active 4A′ hexamer. Therefore, we expected no change in the 30-mer DNA-dependent dTTPase activity upon heterooligomer formation. However, formation of heterooligomers did result in a small decrease in the 30-mer DNA-dependent dTTPase activity. This decrease may be accounted for by some percentage of DNA bound to the mutant subunits in the heterooligomers. Because each hexamer prefers to bind one DNA, those oligomers that have DNA bound to the mutant subunits will hydrolyze dTTP at a lower rate which would result in a net decrease in the dTTPase activity.

Previous studies have shown that the hexameric structure is necessary for DNA binding (Hingorani & Patel, 1993). However, it was not known how many subunits in the hexamer interacted with the DNA. The finding that heterooligomers can bind a 30-mer DNA with the same stoichiometry as 4A′ suggested that all the subunits in the hexamer may not interact with the DNA. Yet protein–DNA cross-linking experiments reported here show that the DNA cross-links at least five and perhaps all six subunits of 4A′, indicating that the DNA probably interacts with all six subunits in a fully active 4A′ hexamer. Thus, unless heterooligomer formation somehow restores the ability of the mutant protein to bind DNA, these studies indicate that a single-stranded DNA is capable of binding to the hexamer by interacting with only a few subunits. All subunits must be fully active, however, to unwind DNA or to hydrolyze dTTP in the presence of a long single-stranded DNA.

REFERENCES

Bernstein, J. A., & Richardson, C. C. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85*, 396–400.

Bernstein, J. A., & Richardson, C. C. (1988b) *J. Biol. Chem. 263*, 14891–14899.

Bernstein, J. A., & Richardson, C. C. (1989) *J. Biol. Chem. 264*, 13066–13073.

Bourne, H. R., Sanders, D. A., & McCormick, F. (1991) *Nature 349*, 117–127.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Dunn, J. J., & Studier, F. W. (1983) *J. Mol. Biol. 166*, 477–535; (1984) *J. Mol. Biol. 175*, 111–112 (erratum).

Hingorani, M. M., & Patel, S. S. (1993) *Biochemistry 32*, 12478–12487.

Ilyina, T. V., Gorbalenya, A. E., & Koinin, E. V. (1992) *J. Mol. Evol. 34*, 351–357.

Jurnak, F. (1985) *Science 230*, 32–36.

Kjeldgaard, M., & Nyborg, J. (1992) *J. Mol. Biol. 223*, 721–742.

Kunkel, T. A., Bebenek, K., & McClary, J. (1991) *Methods Enzymol. 204*, 125–139.

La Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 2385–2388.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Matson, S. W., & Richardson, C. C. (1983a) *J. Biol. Chem. 258*, 14009–14016.

Matson, S. W., & Richardson, C. C. (1983b) *J. Biol. Chem. 258*, 14017–14024.

Mendelman, L. V., & Richardson, C. C. (1991) *J. Biol. Chem. 266*, 23240–23250.

Mendelman, L. V., Notarnicola, S. M., & Richardson, C. C. (1993) *J. Biol. Chem. 268*, 27208–27213.

Miller, H. (1987) *Methods Enzymol. 152*, 145–170.

Muller, C. W., & Schulz, G. E. (1992) *J. Mol. Biol. 224*, 159–177.

Nakai, H., & Richardson, C. C. (1988) *J. Biol. Chem. 263*, 9818–9830.

Notarnicola, S. M., & Richardson, C. C. (1993) *J. Biol. Chem. 268*, 27198–27207.

Patel, S. S., & Hingorani, M. M. (1993) *J. Biol. Chem. 268*, 10668–10675.

Patel, S. S., Wong, I., & Johnson, K. A. (1991) *Biochemistry 30*, 511–525.

Patel, S. S., Rosenberg, A. H., Studier, F. W., & Johnson, K. A. (1992) *J. Biol. Chem. 267*, 15013–15021.

Rosenberg, A. H., Patel, S. S., Johnson, K. A., & Studier, F. W. (1992) *J. Biol. Chem. 267*, 15005–15012.

Shrimankar, P., Stordal, L., & Maurer, R. (1992) *J. Bacteriol. 174*, 7689–7696.

Stanssens, P., Opsomer, C., McKeown, Y. M., Kramer, W., Zabeau, M., & Fritz, H.-J. (1989) *Nucleic Acids Res. 17*, 4441–4454.

Story, R. M., & Steitz, T. A. (1992) *Nature 355*, 374–376.

Studier, F. W., Rosenberg, A. H., Dunn, J. J., & Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60–89.

Sung, P., Higgins, D., Prakash, L., & Prakash, S. (1988) *EMBO J. 7*, 3263–3269.

Tabor, S., & Richardson, C. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 205–209.

Tong, L., deVos, A. M., Milburn, M. V., & Kim, S.-H. (1991) *J. Mol. Biol. 221*, 751–754.

Walker, J. E., Saraste, M., Runswick, M. J., & Gay, N. J. (1982) *EMBO J. 1*, 945–951.

Wong, I., & Lohman, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 5428–5432.

Wong, I., Amaratunga, A., & Lohman, T. M. (1993) *J. Biol. Chem. 268*., 20386–20391.