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A Domain of the Gene 4 Helicase/Primase of Bacteriophage T7 Required for the Formation of an Active Hexamer*

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Stephen M. Notarnicola‡, Kyusung Park§¶, Jack D. Griffith§¶, and Charles C. Richardson‡‖

From the ‡Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and the §Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

The bacteriophage T7 gene 4 protein, like a number of helicases, is believed to function as a hexamer. The amino acid sequence of the T7 gene 4 protein from residue 475 to 491 is conserved in the homologous proteins of the related phages T3 and SP6. In addition, part of this region is conserved in DNA helicases such as *Escherichia coli* DnaB protein and phage T4 gp41. Mutations within this region of the T7 gene 4 protein can reduce the ability of the protein to form hexamers. The $His^{475} \rightarrow$ Ala and $Asp^{485} \rightarrow$ Gly mutant proteins show decreases in nucleotide hydrolysis, single-stranded DNA binding, double-stranded DNA unwinding, and primer synthesis in proportion to their ability to form hexamers. The mutation $Arg^{487} \rightarrow$ Ala has little effect on oligomerization, but nucleotide hydrolysis by this mutant protein is inhibited by single-stranded DNA, and it has a higher affinity for dTTP, suggesting that this protein is defective in the protein-protein interactions required for efficient nucleotide hydrolysis and translocation on single-stranded DNA. Gene 4 protein can form hexamers in the absence of a nucleotide, but dTTP increases hexamer formation, as does dTDP, to a lesser extent, demonstrating that the protein self-association affinity is influenced by the nucleotide bound. Together, the data demonstrate that this region of the gene 4 protein is important for the protein-protein contacts necessary for both hexamer formation and the interactions between the subunits of the hexamer required for coordinated nucleotide hydrolysis, translocation on single-stranded DNA, and unwinding of double-stranded DNA. The fact that the gene 4 proteins form dimers, but not monomers, even while hexamer formation is severely diminished by some of the mutations, suggests that the proteins associate in a manner with two separate and distinct protein-protein interfaces.

Gene 4 of bacteriophage T7 encodes two proteins that provide helicase and primase activities required for replication (1–6). Along with the T7 DNA polymerase, a 1:1 complex of T7 gene 5 protein, and *Escherichia coli* thioredoxin, these proteins catalyze the reactions required for leading and lagging strand synthesis during phage DNA replication (7, 8). The product of gene 2.5, a ssDNA[1]-binding protein, is required for DNA replication although its specific role is not fully understood (9).

An internal translation initiation sequence in the gene 4 transcript results in expression of the encoded protein as two colinear forms: the 63-kDa gene 4A protein and the 56-kDa gene 4B protein (1). The 63-kDa gene 4 protein exhibits helicase activity and, by virtue of an amino-terminal zinc binding motif, catalyzes the template-directed synthesis of tetraribonucleotides that are used as primers by the T7 DNA polymerase (10, 11). The 56-kDa protein lacks the 63 amino-terminal residues that form the zinc binding motif and consequently catalyzes only helicase activity (12). Since the 63-kDa protein (primase) can provide both primase and helicase activities, it is both necessary and sufficient for productive infection by T7 phage (13, 14).

While the gene 4 primase is sufficient for T7 DNA replication, there is considerable evidence that the helicase and primase proteins interact cooperatively. For example, the 56-kDa helicase stimulates template-dependent tetraribonucleotide synthesis by the 63-kDa primase and enhances DNA replication *in vivo* (14, 15). Further evidence of this interaction was derived from studies (16) with a gene 4 protein containing a defective nucleotide binding site (NBS). The NBS mutant protein inhibits nucleotide hydrolysis by wild-type gene 4 proteins through direct protein-protein interactions, demonstrating the importance of cooperation between the gene 4 proteins in order to translocate on ssDNA and to catalyze the unwinding of double-stranded DNA. Also, the low level of primer synthesis catalyzed by the NBS mutant primase is increased by wild-type helicase, suggesting that the wild-type protein forms a complex with the mutant primase enabling it to translocate along the template DNA to a primase recognition site (17).

The precise mechanism of strand separation by a DNA helicase is not known. However, all helicases examined thus far function as multimeric proteins and use the energy of nucleotide hydrolysis to unwind dsDNA (18–20). Two forms of helicase multimers have been identified: dimer and hexamer. The *E. coli* Rep protein is the best characterized example of a dimeric helicase (21). The reported group of helicases that form hexamers currently includes proteins such as the T7 gene 4 protein (22), *E. coli* proteins DnaB (23), Rho (24), and RuvB (25), SV40 large T antigen (26), and the bacteriophage T4 gp41 (18).

The T7 gene 4 protein is one of a group of bacterial and bacteriophage helicases known as the "DnaB helicase family" that share multiple regions of amino acid sequence similarity (27). Notably, this group includes *E. coli* DnaB, bacteriophage

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‖ To whom correspondence and reprint requests should be addressed. Tel.: 617-432-1864; Fax: 617-432-3362.

[1] The abbreviations used are; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; NBS, nucleotide binding site(s); PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; EM, electron microscopy; PEG, polyethylene glycol.

T4 gp41, and the gene 4 protein of phage T3. Four regions of sequence similarity were identified in the DnaB helicase family; the first two motifs are known to be associated with nucleotide binding, while the roles of the third and fourth motifs are not yet known. We compared the amino acid sequence of the T7 gene 4 protein with its homologs in the closely related phages T3 and SP6. Other than the NBS, the only continuous region of conserved amino acid sequence among these three proteins occurs toward their carboxyl terminus and corresponds to T7 gene 4 residues 475–491 (refer to Table I). This region of the T7 gene 4 protein overlaps the fourth DnaB helicase family motif, corresponding to T7 gene 4 protein residues 481–500. Interestingly, the phage SP6 gene 4 protein has sequence similarity to the fourth motif of the DnaB family of helicases only within the common overlapping region (corresponding to T7 gene 4 protein residues 481–491).

Since the function of this highly conserved carboxyl-terminal region is not known, we have investigated its role in T7 gene 4 protein through site-directed mutagenesis. We show that mutations within this region affect both the formation of gene 4 protein hexamers and cooperative protein-protein interactions within the hexamer that are required for nucleotide hydrolysis and translocation on ssDNA.

EXPERIMENTAL PROCEDURES

Materials

Bacteriophage and Bacteria Strains—E. coli DH5α (Life Technologies, Inc.) was used for cloning and complementation analysis, and *E. coli* HMS 174(DE3) was used for protein production. Bacteriophage T7 wild-type (28) and T7 Δ4-1 (14) have been described.

DNA, Nucleotides, and Enzymes—Single-stranded M13mp6 DNA was purified as described (29). T7 DNA polymerase was provided by S. Tabor (Harvard Medical School). Wild-type gene 4A protein was prepared by B. Beauchamp (Harvard Medical School) using the protocol described under "Methods." Restriction enzymes were obtained from Amersham and New England Biolabs, Inc. DNA sequencing reagents and β,γ-methylene dTTP were obtained from United States Biochemical Corp. "Ultma" DNA polymerase from Perkin Elmer was used for the mutagenic polymerase chain reaction. Oligonucleotides were purchased from Oligos, Etc. Radiolabeled nucleotides were purchased from Amersham and DuPont NEN.

Methods

Mutagenesis—Polymerase chain reaction-mediated oligonucleotide-directed mutagenesis using the megaprimer technique (30) was carried out as described previously (16), except that "Ultma" DNA polymerase was used for increased fidelity. The oligonucleotides used for mutagenesis, mutated residues in bold were: H475A, 5′-CCTTCCTC**AGC**TGCTTTACCTTTGTC-3′; and R487A, 5′-CCAGAACCA**CG**TAGGTCAGTAATAG-3′. The flanking primers were SN101, 5′-CTGGGGTGGTGCTGGTCG-3′, and SN104, 5′-CGTTGTACATACGAACCAGC-3′. Standard techniques were used to clone the final polymerase chain reaction fragments into pGP4-G64$_{S10}$ (16) and to determine the DNA sequence of the resulting clones (31).

Complementation Analysis—E. coli DH5α with plasmids encoding wild-type or mutant gene 4A proteins were used to titer T7 WT and T7 Δ4-1 phage following a standard protocol (32). Plaque size and number were assessed after incubation for 18 h at 37 °C.

Protein Purification—T7 gene 4A overexpression, cell harvest, and cleared lysate production were performed as described previously (16). Gene 4A is overexpressed to levels where the protein production and purification are readily monitored by SDS-PAGE and Coomassie Brilliant Blue staining. The gene 4A protein R487A was precipitated from 47 ml of cleared lysate (3.2 mg/ml total protein, fraction I; Fig. 1, *lane 1*) by adjusting the NaCl concentration to 0.5 M and the polyethylene glycol (PEG4000) concentration to 10%, followed by incubation on ice for 60 min and centrifugation at 12,000 × *g* for 15 min at 4 °C. The gene 4A protein-PEG precipitate was then resuspended in Buffer P (40 mM potassium phosphate, pH 6.8, 5 mM DTT, 5 mM EDTA, 10% glycerol) plus 20 mM KCl (fraction II; Fig. 1, *lane 2*). Fraction II (20 ml at 2.7 mg/ml protein) was loaded onto a phosphocellulose (Whatman P11) column, 2.5 cm^2 × 5 cm, that was equilibrated in Buffer P. The column was then washed with 10 column volumes of Buffer P plus 20 mM KCl, and the gene 4A protein eluted with a 150-ml linear gradient of 20–1000 mM KCl in Buffer P. The fractions containing gene 4A protein were pooled (fraction III; Fig. 1, *lane 3*), and $MgCl_2$ was added to 10 mM. Fraction III (18 ml at 0.9 mg/ml protein) was loaded onto a 2-ml agarose-hexane-ATP type 3 (Pharmacia Biotech Inc.) affinity column equilibrated in Buffer T (Buffer P plus 500 mM KCl and 10 mM $MgCl_2$). In the presence of magnesium, the gene 4A protein is tightly bound to the affinity resin, even at 500 mM KCl, allowing the effective removal of contaminating proteins by thorough washing of the column with 15 ml of Buffer T. The gene 4A protein was then eluted with Buffer P plus 500 mM KCl and 20 mM EDTA. The fractions containing gene 4 protein were pooled (fraction IV; Fig. 1, *lane 4*) and dialyzed extensively at 4 °C against storage buffer (40 mM potassium phosphate, pH 7.0, 20 mM KCl, 5 mM DTT, 5 mM EDTA, 50% glycerol).

Native-PAGE and ssDNA Binding—The electrophoresis samples contained 2 μM gene 4A protein, 40 mM Tris-HCl, pH 7.0, 50 mM NaCl, 10 mM DTT, 10 mM $MgCl_2$, 20% glycerol, and 1 mM nucleotide, when present. The reaction mixtures were incubated for 10 min at room temperature before being applied to the gel. The electrophoresis buffer contained 25 mM Tris, 190 mM glycine, 10 mM magnesium acetate, and 0.1 mM of the same nucleotide present in the reaction mixtures. Nondenaturing electrophoresis was performed using 4–15% linear gradient polyacrylamide gels (Bio-Rad) that were soaked in the electrophoresis buffer for at least 25 min before the samples were loaded. Electrophoresis was at 7.1 V/cm for 30 min and then 14.3 V/cm for 2 h. The gels were then fixed and silver-stained for analysis. The molecular weights of the gene 4 protein oligomers were estimated by comparison with a curve of the R_f *versus* log(M_r) of native PAGE protein standards of 67,000–669,000 (Pharmacia Biotech Inc.). Scanning densitometry of the silver-stained gels was performed using a Personal Densitometer SI (Molecular Dynamics).

DNA binding experiments were performed using the same gel and electrophoresis buffer system described for the native PAGE. The 10-μl binding reactions contained 40 mM Tris-HCl, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, 0.1 μM oligonucleotide, 1 mM β,γ-methylene dTTP, and gene 4A protein at various concentrations. The samples were incubated at room temperature for 5 min, then loaded onto the gel for electrophoresis. The 35-base oligonucleotide used for ssDNA binding experiments had the sequence: 5′-CAGATGCGCGCCTCCTGGCTTATCGGTGTACTTGG-3′ and was end-labeled in a standard reaction with [γ-^{32}P]ATP and T4 polynucleotide kinase; unincorporated label was removed using a spin-column (Microspin S300, Pharmacia Biotech Inc.). After electrophoresis, the gels were fixed, stained with Coomassie Brilliant Blue, and dried. The amount of DNA that co-migrated with the gene 4 protein was determined by PhosphorImager (Molecular Dynamics) analysis.

UV-mediated cross-linking of gene 4 proteins to radiolabeled $(dT)_{20}$ was performed as described previously for UV-cross-linking of gene 4 proteins to dTTP (16). The 10-μl reactions contained 0.2 μM gene 4A protein, 0.01 μM radiolabeled $(dT)_{20}$, 40 mM Tris-HCl, pH 7.0, 10 mM DTT, 100 mM NaCl, 50 μg/ml bovine serum albumin, and 10% glycerol. The $(dT)_{20}$ was 5′-end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. When included in the reactions, $MgCl_2$ was at 10 mM, and nucleotides, dTTP or β,γ-methylene dTTP were at 2 mM. The reactions were incubated for 10 min at 30 °C, then placed on ice and exposed to the UV source (1.0 milliwatts/cm^2) for 15 min. The reaction mixtures were then subjected to SDS-PAGE, and the amount of labeled DNA bound to the gene 4 protein was determined by phosphorimage analysis.

Electron Microscopy—In preparation for electron microscopy, the gene 4A proteins at a concentration of 10 μg/ml (175 nM) were incubated for 10 min at 20 °C in a buffer of 10 mM HEPES, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.1 mM EDTA. When present, the nucleotide concentration was 0.6 mM. The samples were adsorbed onto a carbon film supported by a copper grid and stained with 1% uranyl acetate (33). The photomicrographs were taken on a Philips 400 TLG electron microscope at magnifications of 30,000 and 60,000.

Nucleotide Hydrolysis Assay—The assay for examining nucleotide hydrolysis by gene 4 proteins was performed essentially as described previously (16, 34). The reactions were carried out in a volume of 20 μl at 30 °C for 20 min and analyzed by thin layer chromatography on polyethyleneimine-cellulose followed by scintillation counting of the isolated dTDP spot. The reaction buffer (dTTPase buffer) contained 40 mM Tris-HCl, pH 7.0, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM DTT, and 50 μg/ml bovine serum albumin and varying concentrations of [^{3}H]dTTP. Gene 4A proteins were at a concentration of 200 nM, and M13mp6 ssDNA, when present, was used at 50 μM (expressed as nucleotide equivalents). To ensure an adequate magnesium concentration in the

TABLE I

Alignment of the conserved carboxyl-terminal domain of the gene 4 proteins of bacteriophage T7, T3, and SP6

The corresponding amino acid sequences of the T3 and SP6 gene 4 proteins and the *E. coli* DnaB protein are aligned with T7 gene 4 protein amino acids 475 to 500. The T7 gene 4 protein residues mutated for this study are in **bold**. The region of sequence similarity among T7, T3, and SP6 gene 4 proteins is indicated by the upper bracket. The region of sequence similarity among bacteriophage T7, T3 and the fourth motif of the *E. coli* DnaB protein helicase identified by Ilyina *et al.* (27) is indicated by the lower bracket. The dots within the brackets above and below the amino acid sequences indicate residues common to each group, and the numbering corresponds to the T7 gene 4 protein amino acid sequence.

	T7-T3-SP6																			
	475																			491
T7[a]	**H**	E	E	G	R	P	V	S	I	T	**D**	L	**R**	G	S	G	A	L	R	Q	L	S	D	T	I	I
T3[b]	H	E	E	G	R	A	V	S	I	T	D	L	R	G	S	G	A	L	R	Q	L	S	D	T	I	I
SP6[c]	H	E	E	G	G	E	V	I	L	S	D	F	R	G	S	G	A	I	G	F	W	A	S	Y	A	L
DnaB[d]	R	A	D	K	R	-	P	V	N	S	D	L	R	E	S	G	S	I	E	Q	D	A	D	L	I	M
	481																			500
	T7-T3-*dnaB*																			

[a] T7 gene 4 protein amino acids 475 to 500 (1).
[b] T3 gene 4 protein amino acids 474 to 499 (36).
[c] SP6 gene 4 protein amino acids 553 to 578 (J. Rush and C. C. Richardson, unpublished data).
[d] *E. coli* DnaB protein amino acids 389–413 aligned with T7 gene 4 protein sequence as in Ilyina *et al.* (27).

reactions, the magnesium acetate concentration of the [^{3}H]dTTP solutions was equal to the nucleotide concentration. The kinetic constants were derived with the aide of the *Enzyme Kinetics* program for the Macintosh computer (Trinity Software).

Helicase Assay—The helicase substrate consisted of a 75-base oligonucleotide with a partially complementary radiolabeled 25-base oligonucleotide annealed to its 3′-end. The 25-mer (10 pmol) was 5′-end labeled with [γ-^{32}P]ATP and diluted with unlabeled 25-mer (120 pmol) for the annealing reaction. The 75- and 25-base oligonucleotides, 4.5 μM final concentration each, were annealed in a 27-μl reaction containing 40 mM Tris-HCl, pH 7.0, 50 mM NaCl, and 10 mM $MgCl_2$. The annealing mixture was incubated at 70 °C for 2 min and cooled slowly to 30 °C, 5 μl loading buffer was added, and the reaction mixture was loaded onto a 3% agarose gel (MetaPhor Agarose, FMC BioProducts) in TAE buffer and electrophoresed at 14 V/cm for 1 h. The gel was briefly exposed to film, and the autoradiograph was used to locate and excise the labeled substrate band. The helicase substrate was eluted from the agarose slice by freezing the slice at −80 °C, thawing at 37 °C, and then filtering the slice through a microcentrifuge filter device (Bio-Rad). The gel slice was rewet with TE buffer and incubated at room temperature for 30 min, filtered, and combined with the first filtrate. The concentration of the helicase substrate was determined spectroscopically by measuring the absorbance at 260 nm.

The helicase assay measures the ability of the gene 4 protein to separate the partially complementary oligonucleotides of the helicase substrate. The helicase reactions (50 μl) contained dTTPase buffer, 2 mM dTTP, 44 nM helicase substrate, and 20 nM gene 4A protein. The reaction mixtures were assembled on ice, and the reaction was started by the addition of gene 4 protein and incubation at 30 °C. At timed intervals, 7.5-μl samples were removed from the reaction and added to 7.5 μl of stop buffer containing 40 mM EDTA and 25% glycerol. The reaction samples were analyzed by nondenaturing 20% PAGE and phosphorimage analysis. The decrease with time in the amount of radiolabeled 25-mer migrating as duplex DNA with the 75-mer was measured, and the percent oligonucleotide displaced was calculated as described previously (35).

RNA-primed DNA Synthesis—Analysis of gene 4 protein stimulation of DNA synthesis on ssDNA by T7 DNA polymerase was performed as described previously (15) with the following specific changes: the 20-μl reactions contained dTTPase buffer plus 50 mM potassium glutamate, 0.3 mM NTP, 0.3 mM d(G,A,C)TP, and 2.0 mM [α-^{32}P]dTTP. The reactions were incubated at 30 °C for 20 min, and the amount of radioactivity incorporated into DNA was determined by scintillation counting. The specific activity of the [α-^{32}P]dTTP solutions varied from 4 to 11 cpm/pmol.

RESULTS

Comparison of Conserved Helicase Motifs

Ilyina *et al.* (27) observed that the T7 gene 4 protein shares four conserved amino acid sequence motifs with a number of procaryotic DNA helicases. This group of helicases includes the *E. coli* DnaB protein and was termed the DnaB helicase family (27). Among the other helicases in this group are phage T4 gp41 and the T3 gene 4 protein. The T7 and T3 gene 4 proteins share 90% sequence identity and have the lowest overall similarity to the other members of the DnaB helicase family. We performed a pairwise comparison of the gene 4 protein amino acid sequences of the closely related phages T7 (1), T3 (36), and SP6.[2] Of the four DnaB helicase motifs only motif 1, the NBS (T7 gene 4 protein residues 313–319) and the region just preceding and overlapping the fourth helicase motif (T7 gene 4 protein residues 475 to 481, refer to Table I) were conserved among the three gene 4 protein homologs. The fourth and most carboxyl-terminal DnaB helicase motif of T7 gene 4 protein has 94% and 65% identity with the same region of the T3 and SP6 gene 4 proteins, respectively. The T7 and T3 gene 4 proteins have 8 of the 11 most highly conserved residues in this helicase motif, whereas SP6 has only 4 of these residues in common, all within the central region of similarity (residues 481 to 491, Table I). Conversely, the amino acid sequence from 475 to 480 that is conserved in the T7, T3, and SP6 gene 4 proteins are not conserved in any of the other DnaB-related helicases. This sequence analysis has further refined a highly conserved helicase motif represented here by T7 gene 4 residues 485 to 491 and demonstrates the uniqueness of the T7-related gene 4 proteins.

Mutagenesis and Complementation Analysis

The observation that similar sequence motifs are shared between the T7, T3, and SP6 gene 4 homologs and other members of the DnaB family of helicases suggests that this domain may play an important role in gene 4 protein functions. Consequently, we examined the effect of mutations within this region on the properties of the gene 4 proteins. The mutations were constructed in a plasmid, pGP4-G64$_{S10}$, which encodes a copy of the T7 gene 4 protein with a mutation of Met^{64} → Gly (M64G) (17). This mutation allows expression of the 63-kDa gene 4 protein and prevents expression of the 56-kDa protein. The M64G mutation has no detectable effect on the helicase or primase activities of the 63-kDa gene 4 protein (14, 17). A similar mutation in the gene 4 protein of M64L was also shown to not change the function of the protein (13). Therefore, in the interest of clarity, in this report we refer to the gene 4A protein with the M64G mutation as "wild-type." The amino acid changes His^{475} → Ala (H475A) and Arg^{487} → Ala (R487A) were

[2] J. Rush and C. C. Richardson, unpublished data.

TABLE II
Complementation analysis of the T7 gene 4 wild-type and mutant proteins

The efficiency of plating of wild-type and mutant T7 Δ4–1 bacteriophage on *E. coli* DH5α carrying recombinant plasmids encoding gene 4A proteins with the indicated amino acid substitutions was determined as described under "Experimental Procedures."

T7 gene 4A plasmids[a]	T7 phage[b]		Plaque-forming units on T7 Δ4–1[c]
	WT	Δ4	
Wild-type	+	+	8.3×10^9
H475A	+	+	4.0×10^8
D485G	+	+	2.5×10^9
R487A	+	−	$<10^{-8}$

[a] Plasmid-encoded wild-type and mutant gene 4A proteins.
[b] The T7 bacteriophage used were: WT, wild-type; Δ4, T7 Δ4–1, gene 4 deleted.
[c] T7 Δ4–1 plaque-forming units on *E. coli* strains with plasmids encoding the indicated gene 4 protein.

constructed by oligonucleotide-directed mutagenesis. In the process of constructing the mutants for this and other gene 4 protein studies, we discovered that the original gene 4 clone, pGP4-6, carried a mutation of A to G at phage T7 nucleotide 13,018, changing gene 4 protein amino acid residue 485 from Asp to Gly. This mutant protein D485G has been incorporated into this study, and the gene 4 plasmids were reconstructed with the correct sequence.

The effect these mutations have on the ability of gene 4A protein to support T7 bacteriophage reproduction *in vivo* was examined by complementation analysis. Gene 4-deleted T7 phage (T7 Δ4-1) will not lyse *E. coli* unless a functional copy of gene 4 is provided in *trans*. Previous studies have shown that the 63-kDa form of gene 4 protein is sufficient for T7 phage replication (13, 14). *E. coli* DH5α-carrying plasmids encoding the wild-type and mutant gene 4A proteins were infected with wild-type T7 or T7 Δ4-1 phage, and the number and size of the plaques produced were determined. When infected with T7 Δ4-1, the strains carrying the mutations H475A and D485G produced 20.8- and 3.3-fold fewer plaques, respectively, than wild-type gene 4A (Table II). Also, both of these mutations resulted in plaques that were on average smaller in diameter (pinpoint to 2.5 mm) than those produced by wild-type gene 4 (2.5 to 5 mm) under the same conditions. No plaques were produced by phage T7 Δ4-1 when plated on cells containing the plasmid with gene 4A mutation R487A.

Gene 4 Protein Oligomerization

To determine the basis of the defects, the mutant gene 4 proteins were purified to homogeneity for biochemical and biophysical analysis. We chose to purify the 63-kDa version of the gene 4 protein since it possesses both helicase and primase activities.

Protein Purification—We have improved and streamlined our earlier procedures for the purification of the gene 4 protein (11, 12, 16, 37) by combining an effective precipitation and enrichment step with a very efficient affinity chromatography resin (see "Methods" for details). The gene 4 protein is selectively precipitated from the cleared lysate with polyethylene glycol 4000 (PEG) and NaCl (Fig. 1, *lane 2*). This step provides an enrichment of the gene 4 protein, and the PEG does not interfere with subsequent procedures. The resuspended gene 4 protein-PEG pellet was loaded onto a phosphocellulose column and eluted with a KCl gradient; the gene 4 protein eluted between 300 and 400 mM KCl. Magnesium was added to 10 mM, and the pooled fractions were loaded onto an agarose-hexane-ATP column. In the presence of magnesium, the gene 4 protein binds tightly to the affinity resin at KCl concentrations of at least 500 mM. After the column was washed, the gene 4 protein was eluted with buffer containing EDTA (Fig. 1, *lane 4*). Based on analysis of silver-stained gels (not shown), we estimate that the gene 4 proteins purified following this protocol are greater than 99.8% pure with a yield for the R487A protein of 3.4 mg from 152 mg of total cell protein. The yield and purity of the wild-type protein was similar, 10.3 mg from 390 mg of total cell protein.

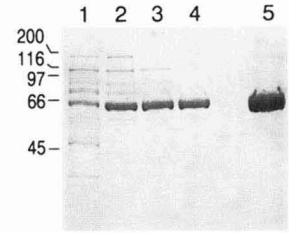

FIG. 1. **SDS-PAGE analysis of the purification of the T7 gene 4A mutant protein R487A.** Protein samples were separated on a 10% SDS-polyacrylamide gel by electrophoresis at 20 V/cm for approximately 50 min and stained with Coomassie Brilliant Blue. *Lanes: 1*, fraction I, cleared lysate from induced culture of *E. coli* HMS 174 (DE3)/pGP4-G64$_{S10}$R487A; *2*, fraction II, resuspended polyethylene glycol precipitate; *3*, fraction III, phosphocellulose chromatography pool; *4*, purified gene 4A R487A protein, fraction IV, agarose-hexane-ATP column pool; *5*, purified R487A protein overloaded to demonstrate purity. *Lanes 1–4* contain approximately 1.2 μg of total protein/lane, and *lane 5* contains approximately 5 μg of protein. The positions of the molecular mass standards (kDa) are indicated to the *left* of the figure.

Native PAGE Analysis—Recently, the gene 4 protein was shown to form hexamers (22, 38), confirming studies indicating that the gene 4 protein was active as a multimer (16, 37, 39). We examined the effect of the mutations within the conserved domain on the oligomerization of gene 4 proteins using native PAGE. The apparent molecular weights of the bands formed by the gene 4 proteins were estimated by comparison with known standards. The gene 4 proteins migrate to positions that correspond to the following forms with estimated molecular mass, from the top to the bottom of the gel: hexamer, 408-kDa (Fig. 2, *open arrows*); pentamer, 322-kDa; tetramer, 263-kDa; trimer, 215-kDa; and dimer, 121-kDa (Fig. 2, *closed arrows*). In each case, the estimated molecular mass is very close to that of the calculated mass for that form of gene 4 63-kDa protein oligomer. No gene 4 protein was detected at the position in the gels that would correspond to a monomer (63-kDa). The migration pattern of the proteins was not affected by the presence (Fig. 2*A*) or absence of magnesium (not shown). In the absence of a nucleotide, the proteins formed primarily hexamers and dimers with some minor intermediate oligomers. Scanning densitometry of the silver-stained gels showed that wild-type protein migrated predominantly as a hexamer, whereas protein R487A was evenly divided between dimers and hexamers (Table III). The H475A and D485G proteins, however, migrated predominantly as dimers. This direct comparison of the proteins demonstrated that in the absence of a nucleotide ligand each of these mutations weakens the gene 4 protein-protein interactions required for hexamer formation.

The effect of various nucleotides on the association state of the gene 4A proteins can be observed by including the nucleotide in the reaction and electrophoresis buffers. In the presence of dTTP, the preferred nucleotide substrate for T7 gene 4 protein, each of the gene 4A proteins form predominantly hexamers (Fig. 2*D* and Table III). However, a significant portion of the H475A and D485G proteins still migrate as dimers (Fig. 2*D*, *closed arrowhead*). Also, with β,γ-methylene dTTP, the wild-type and mutant R487A proteins form hexamers almost exclusively (Fig. 2, *C* and *D*). In contrast, in the presence of dTDP, none of the proteins formed hexamers as readily as they did with dTTP (Fig. 2*B*). The H475A and D485G proteins are

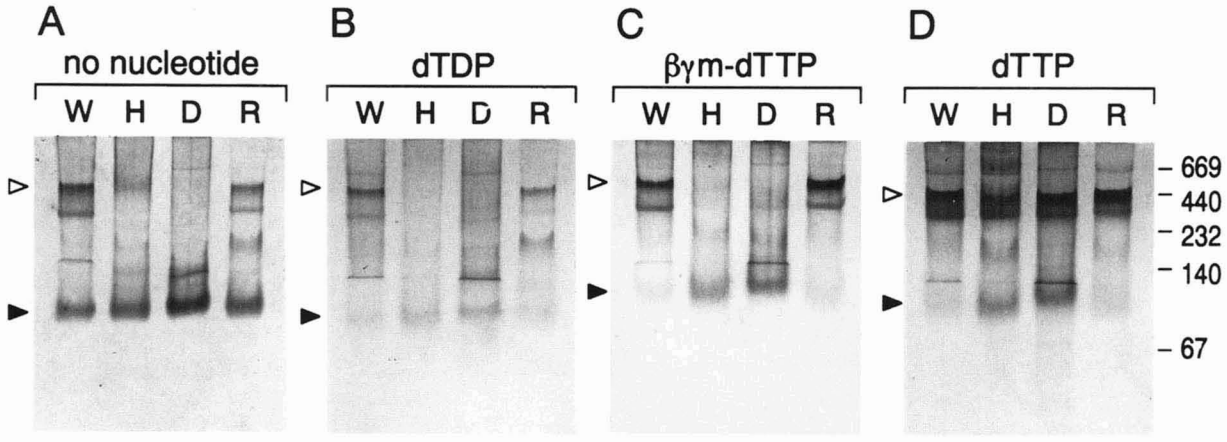

FIG. 2. **Native PAGE analysis of the gene 4A wild-type and mutant proteins.** The purified gene 4A proteins were examined by electrophoresis under nondenaturing conditions using 4–15% polyacrylamide gels that were then silver-stained for analysis. The nucleotides present in the preincubation and electrophoresis buffers for each experiment are indicated at the *top* of each panel: *A*, no nucleotide; *B*, dTDP; *C*, β,γ-methylene dTTP; and *D*, dTTP. The lanes in each panel contained approximately 20 pmol (1.26 μg) of the indicated gene 4A protein: *W*, wild-type; *H*, H475A; *D*, D485G; and *R*, R487A. The positions of the hexamer bands (*open arrows*) and the dimer bands (*closed arrow*) are indicated in each panel. The positions of the native PAGE protein standards and their molecular masses (kDa) are indicated for the gel in *D*.

TABLE III
Scanning densitometry of the native PAGE of the wild-type and mutant T7 gene 4 proteins in the presence and absence of dTTP

The silver-stained gels of the native PAGE of the wild-type and mutant gene 4A proteins with no nucleotide (Fig. 2*A*) or in the presence of dTTP (Fig. 2*D*) were analyzed by scanning densitometry. The peak optical density values corresponding to the hexamer and dimer positions in each gel are given. The background values were subtracted.

Gene 4A protein	No nucleotide		dTTP	
	Dimer	Hexamer	Dimer	Hexamer
Wild-type	0.12	0.38	0	0.95
H475A	0.17	0.06	0.10	0.51
D485G	0.49	0	0.15	0.69
R487A	0.22	0.17	0	1.27

principally dimers in the presence of dTDP and β,γ-methylene dTTP, with few distinct high molecular weight bands visible, indicating either that they do not form discrete complexes or that the complexes are unstable under the conditions used for electrophoresis (Fig. 2, *B* and *C*). In addition to further demonstrating the effect of the mutations in this region on oligomer formation, these results indicate that the gene 4 protein-protein affinity (association affinity) will vary depending on whether a nucleoside di- or triphosphate is bound.

Visualization of the T7 Gene 4 Protein by Electron Microscopy—To assess hexamer formation by an alternative method, the wild-type and mutant gene 4 proteins were examined by EM (Fig. 3). The proteins were incubated with and without nucleotides and then negatively stained with uranyl acetate. In the presence of dTTP, most of the visible complexes formed by the wild-type and mutant proteins were hexamers, in confirmation of the results reported by Egelman *et al.* (38). The percent of the population of each gene 4 protein that formed hexamers in the absence and presence of various nucleotides was determined by counting fields of molecules (Table IV). These estimates are largely consistent with the results of the native PAGE analysis. However, because of the difficulty involved in distinguishing and scoring the smaller multimers, it is possible that the values represent an overestimation of the percent hexamers formed. Alternatively, the time required and the stresses imposed by electrophoresis may have disrupted the weaker protein-protein association of these mutants, thus decreasing our ability to detect hexamers by native PAGE. This is especially evident for the H475A and D485G proteins which show little to no hexamer formation in the absence of dTTP by native PAGE, but, as scored by EM, almost 50% of the complexes were hexamers. Nevertheless, both the results of the native PAGE analysis and the EM show that in the absence of nucleotide the mutant proteins form hexamers less readily than the wild-type protein. Moreover, the enlarged views of individual hexamers (Fig. 3*B*) formed by the mutant gene 4 proteins are indistinguishable from those formed by the wild-type protein, demonstrating that these mutations do not cause any gross morphological defects in the proteins at this level of resolution.

Biochemical Analysis of Wild-type and Mutant Gene 4 Proteins

Earlier studies indicated that the active form of gene 4 protein is oligomeric, and recent evidence has shown that the protein forms hexamers (22), but the dependence of gene 4 protein catalytic activities on hexamer formation has not been examined. To determine if the defects in hexamer formation have an effect on the various enzymatic activities of the gene 4 protein, we compared the biochemical properties of the wild-type and mutant proteins. These activities include nucleotide hydrolysis, helicase activity, and primer synthesis.

Nucleotide Hydrolysis—The enzymatic activities of T7 gene 4 protein require a hydrolyzable NTP, with the preferred substrate *in vitro* being dTTP; moreover, nucleotide hydrolysis is stimulated greatly by ssDNA (34). Accordingly, we assayed the conversion of dTTP to dTDP by the wild-type and mutant proteins in the presence and absence of ssDNA (Fig. 4). For ease of comparison, the K_m and V_{max} for each protein were determined from the data shown in Fig. 4 and are given in Table V. The wild-type, H475A, and D485G gene 4 proteins hydrolyze dTTP in a reaction that is stimulated by ssDNA. The activity of the R487A protein, however, is inhibited by ssDNA, a result that will be discussed below. In the absence of ssDNA, the V_{max} of the three gene 4A proteins, wild-type, H475A, and D485G are similar. The addition of ssDNA stimulates the activity of the wild-type protein approximately 25-fold whereas the H475A and D485G proteins are stimulated only 7.6- and 18-fold, respectively. Furthermore, the K_m values for dTTP of the H475A and D485G proteins are close to that of the wild-type protein and do not change significantly between reactions with and without ssDNA. The similarity in the values for the K_m and the V_{max} in the absence of ssDNA, together with the lower V_{max} in the presence of ssDNA, indicate that these two

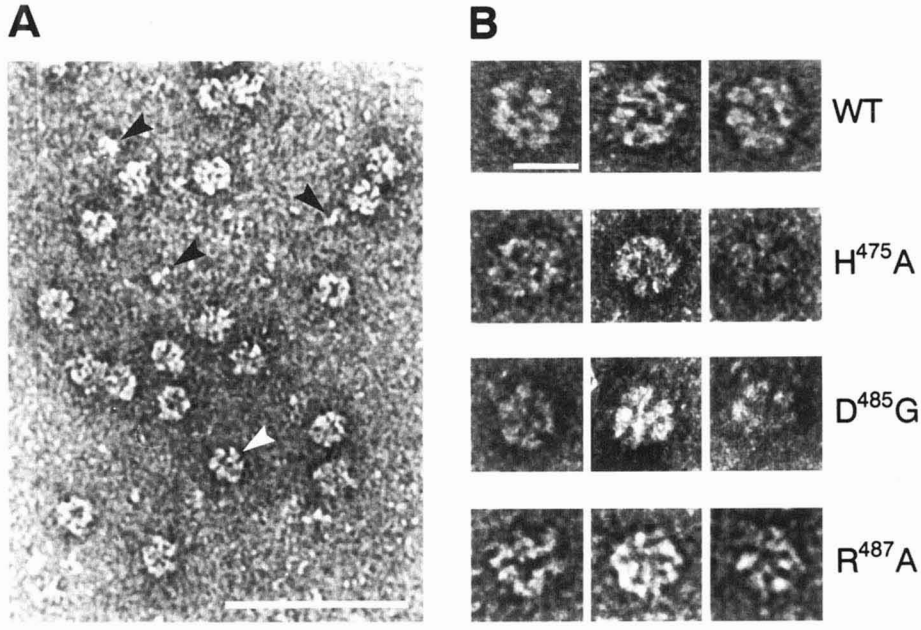

FIG. 3. **Visualization of wild-type and mutant T7 gene 4A proteins.** T7 gene proteins were prepared for EM by negative staining of unfixed samples with 1% uranyl acetate and mounting on thin carbon foils. *A*, a field of wild-type gene 4A protein complexes, largely hexamers, in the presence of 0.6 mM dTTP. The *open arrowhead* indicates a hexamer, and the *closed arrowheads* indicate smaller oligomers. *B*, images of individual hexamers formed by the wild-type and mutant gene 4A proteins in the presence of 0.6 mM dTTP. The identity of the gene 4A protein in each panel of images is indicated on the *right*. The *white scale bars* equal 50 nm (*A*) and 10 nm (*B*).

TABLE IV
Percent of T7 gene 4A wild-type and mutant proteins as hexamers versus smaller oligonucleotides as determined by EM

The wild-type and mutant 63-kDa gene 4 proteins were prepared for EM as described under "Experimental Procedures." The percent of the negatively stained proteins forming hexamers was determined by counting the number of hexamers *versus* smaller multimers; approximately 300 protein complexes were counted for each set (refer to Fig. 3). The procedure was repeated at least two times, and the standard error of the mean is given in parentheses.

Gene 4A protein[a]	Nucleotide[b]			
	dTTP	β,γ-Methylene dTTP	dTDP	None
Wild-type	90 (1)	84 (1)	76 (1)	69 (6)
H475A	89 (1)	71 (6)	70 (3)	51 (14)
D485G	86 (1)	68 (3)	75 (2)	57 (5)
R487A	90 (3)	87 (2)	86 (2)	84 (1)

[a] The proteins were at 10 μg/ml (175 nM).
[b] Each nucleotide was present at 0.6 mM.

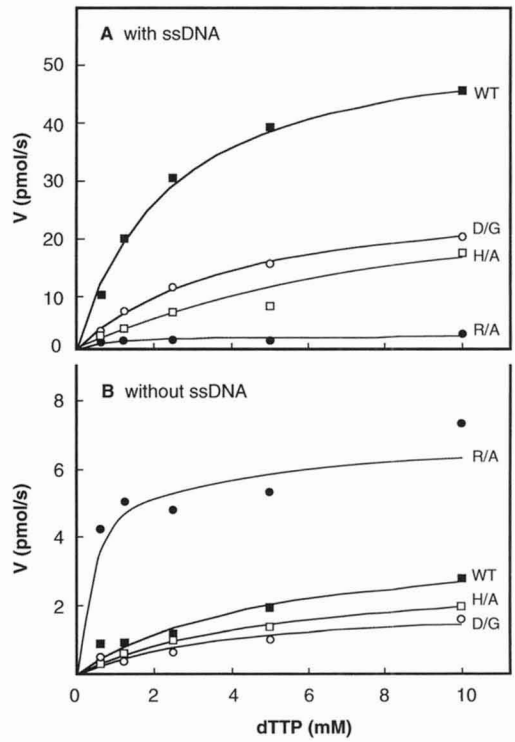

FIG. 4. **Effect of increasing substrate concentration on nucleotide hydrolysis by the wild-type and mutant T7 gene 4A proteins in the presence and absence of ssDNA.** The reactions were performed as described under "Experimental Procedures." All reactions contained gene 4A protein at 200 nM, and the nucleotide concentrations were as indicated. *A*, the nucleotide hydrolysis reactions contained 50 μM M13mp6 ssDNA (nucleotide equivalents). *B*, reactions were performed as in *A* but in the absence of ssDNA. The curves are labeled: *WT*, wild-type; *H/A*, H475A; *D/G*, D485G; and *R/A*, R487A. Each curve represents the average of two experiments, and each experiment was performed in duplicate.

mutations, H475A and D485G, do not directly affect the ability of the gene 4 proteins to bind and hydrolyze dTTP. Rather, considering the effect these mutations have on oligomerization, they suggest that the reduced hydrolysis activity in the presence of ssDNA is the result of fewer active hexamers.

The velocity of the dTTP hydrolysis reaction catalyzed by the R487A protein decreases in the presence of ssDNA, the opposite of the reaction observed with wild-type protein. This apparent inhibition of activity by ssDNA was confirmed in experiments where nucleotide hydrolysis was measured before and after the addition of ssDNA to reactions with the R487A protein. The rate of hydrolysis by the R487A protein decreased immediately after the addition of ssDNA to the reaction mixtures (data not shown). In addition, this mutant protein has an almost 5-fold higher affinity for dTTP than the wild-type protein and forms hexamers as well as the wild-type protein. The results show that the ability of the mutant protein to hydrolyze NTP is intact, but the R487A mutation interferes with the mechanism by which the gene 4 proteins within the hexamer interact in the presence of ssDNA to coordinately hydrolyze nucleotides.

ssDNA Binding—DNA binding by the gene 4 protein is dependent on the ability of the protein to bind nucleotides (40) and form hexamers (41). We used a gel-shift assay with ^{32}P-labeled oligonucleotides to measure ssDNA binding because it also reveals the oligomeric nature of the protein bound to the DNA. In the presence of β,γ-methylene dTTP, the highest concentration of wild-type gene 4A protein (2.0 μM) was able to retain all of the labeled 35-mer present in the assay (Fig. 5). However, the highest concentrations of the H475A and D485G proteins (also 2.0 μM) bound only 30 and 55% of the ssDNA, respectively. Binding of ssDNA by the R487A protein was

TABLE V
K_m and V_{max} for dTTP hydrolysis by the wild-type and mutant T7 gene 4 proteins

The nucleotide hydrolysis activity of wild-type and mutant 63-kDa gene 4 proteins was measured in the presence and absence of ssDNA. The K_m for dTTP and the V_{max} for the conversion of dTTP to dTDP plus P_i were determined from the data shown in Fig. 4.

Gene 4A protein	+ssDNA[a]		−ssDNA[b]	
	K_m[c]	V_{max}[d]	K_m	V_{max}
Wild-type	2.76	59.36	1.22	2.32
H475A	6.18	21.99	5.26	2.93
D485G	4.39	28.83	1.33	1.60
R487A	0.46	2.22	0.33	6.98

[a] Nucleotide hydrolysis reactions in the presence of 50 μM M13mp6 ssDNA.
[b] Nucleotide hydrolysis reactions in the absence of ssDNA.
[c] dTTP (mM).
[d] Theoretical maximum velocity (dTDP produced, pmol/s).

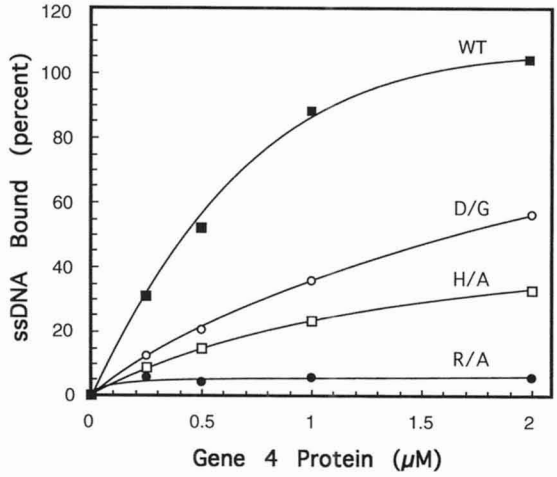

FIG. 5. **ssDNA binding by the wild-type and mutant T7 gene 4A proteins.** Reactions were performed as described under "Experimental Procedures." Increasing concentrations of the gene 4A proteins were incubated with a constant amount of radiolabeled oligonucleotide, and the reactions mixtures were separated by nondenaturing PAGE. The amounts of bound and unbound oligonucleotide were quantified by phosphorimage analysis of the respective bands. Each reaction contained the indicated concentrations of gene 4A protein, 1 mM β,γ-methylene dTTP, and 0.1 μM radiolabeled 35-mer. The curves are labeled: *WT*, wild-type; *H/A*, H475A; *D/G*, D485G; and *R/A*, R487A.

barely detectable. Comparison of the autoradiographs and the Coomassie Blue-stained gels revealed that the radiolabeled ssDNA migrated together with gene 4 protein complexes of a hexamer or greater in size (data not shown). The relative binding ability of the H475A and D485G proteins is proportional to their ability to form hexamers, suggesting that these mutant proteins bind DNA poorly due to defects in protein-protein interaction and not to a reduction in DNA binding affinity.

The fact that the R487A protein bound ssDNA so poorly as measured by the gel-shift assay was puzzling. This mutant protein forms hexamers as well as the wild-type protein and must interact with DNA since its dTTPase activity is inhibited by ssDNA. One possible explanation for the poor binding is that β,γ-methylene dTTP imparts a conformation to this mutant protein that differs somewhat from that imparted by dTTP. However, this gel-shift assay cannot be used with dTTP, because the gene 4 protein translocates off the ssDNA and is then separated from the DNA during electrophoresis. Consequently, to examine DNA binding by the R487A protein with greater sensitivity and determine if there are differences attributable to the nucleotide present, we employed a UV-mediated cross-linking assay (Fig. 6). With this assay we demonstrated that

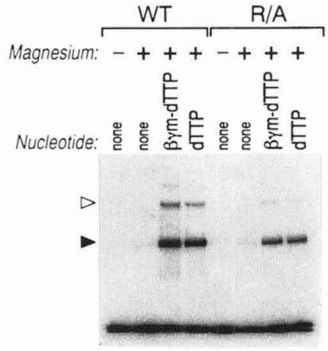

FIG. 6. **UV-mediated cross-linking of gene 4A wild-type and mutant R487A proteins to ssDNA.** The reaction mixtures contained 0.2 μM gene 4A protein, 0.01 μM radiolabeled $(dT)_{20}$, 40 mM Tris-HCl, pH 7.0, 10 mM DTT, 100 mM NaCl, 50 μg/ml bovine serum albumin, and 10% glycerol, plus 10 mM $MgCl_2$ and 2 mM nucleotide as indicated at the *top* of the figure. The reaction mixtures were preincubated for 10 min at 30 °C, placed on ice, and exposed to the UV source for 15 min. The samples were examined by SDS-PAGE and PhosphorImager analysis. The *closed arrow* indicated gene 4A monomer (63-kDa) cross-linked to the labeled oligonucleotide, and the *open arrow* indicates a dimer (120-kDa); higher molecular weight species are also visible. Unbound radiolabeled $(dT)_{20}$ is visible at the *bottom* of the figure.

the R487A protein bound radiolabeled $(dT)_{20}$ approximately 75% as well as the wild-type gene 4 protein (indicated by the *arrowheads* in Fig. 6). Cross-linking occurred only when both nucleotide and magnesium are present in the reaction mixtures, thus demonstrating a specific interaction between the gene 4 protein and the oligonucleotide. The labeled oligonucleotide primarily reacted with a single monomer of gene 4A protein (*closed arrow*, Fig. 6); however, a fraction of the oligonucleotide was cross-linked to multiple gene 4 protein monomers (*open arrow*, Fig. 6). It is unclear if this result was due to multiple proteins cross-linked to a single oligonucleotide or cross-linked proteins bound to a single oligonucleotide. The nucleotide present in the assay has a slight influence on ssDNA binding; both wild-type and R487A proteins were cross-linked to the labeled oligonucleotide only 86% as well with dTTP as with β,γ-methylene dTTP. This result confirms that the R487A protein interacts with ssDNA, but does not reveal the relative strength of the interaction. It may be that this mutant has a tenuous hold on ssDNA that is not sufficiently strong to maintain contact during the gel-shift assay, but is strong enough to inhibit nucleotide hydrolysis.

Helicase Activity—To determine if helicase activity is intact in the mutant proteins, we used an oligonucleotide substrate to measure dsDNA unwinding. The helicase substrate consists of a 75-base oligonucleotide with a partially complementary radiolabeled 25-base oligonucleotide annealed to its 3′-end (see *inset* to Fig. 7). The 5′-17 bases of the 25-mer base pair with the 3′-17 bases of the 75-mer, leaving the 3′-8 bases of the 25-mer and the 5′-58 bases of the 75-mer as ssDNA. The 25-mer is ^{32}P-labeled at its 5′-end so that its migration in the gel can be detected by autoradiography. Strand separation is determined by measuring the change in the amount of radiolabeled 25-base oligonucleotide that migrates as ssDNA. The relative amount of unwinding activity shown by the wild-type and mutant proteins (Fig. 7) is proportional to the activities observed in the ssDNA binding (Fig. 5) and nucleotide hydrolysis reactions (Fig. 4*A*). The H475A and D485G proteins were not able to separate the oligonucleotides as efficiently as the wild-type protein. This is consistent with the fact that the mutant proteins do not bind ssDNA as well as the wild-type protein. Unwinding activity by the wild-type protein does not reach 100% because the concentration of ssDNA increases as the reaction proceeds and eventually exceeds the concentration of

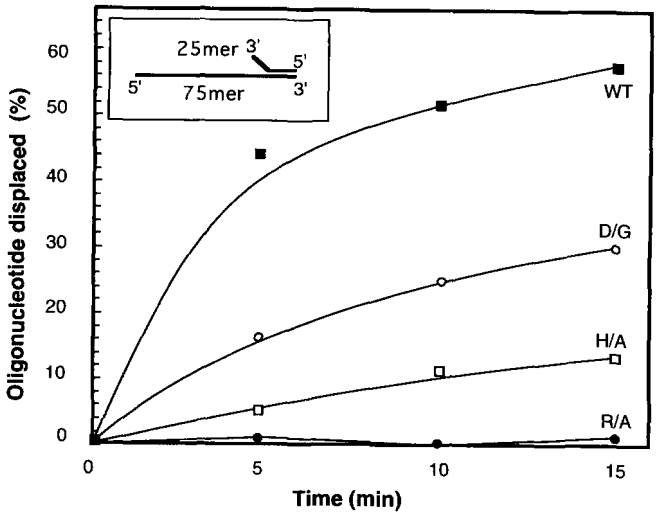

FIG. 7. **Helicase activity of the wild-type and mutant gene 4A proteins.** The helicase substrate consists of a 75-base oligonucleotide with 17 bases of a 25-base oligonucleotide annealed to its 3'-end as diagramed in the *inset*. The 25-mer was 5'-^{32}P-end-labeled so that its position in the gel can be detected and quantified. The reactions mixtures contained 20 nM gene 4A protein, 44 nM helicase substrate, and 2 mM dTTP in a 50-μl volume. Samples were removed at the indicated time, and the reaction was stopped by the addition of buffer with excess EDTA. The reactions were quantified by PhosphorImager analysis of the nondenaturing PAGE. The change in the amount of labeled 25-mer separated from the 75-mer was calculated and expressed as a percent of the total helicase substrate. The curves are labeled: *WT*, wild-type; *H/A*, H475A; *D/G*, D485G; and *R/A*, R487A.

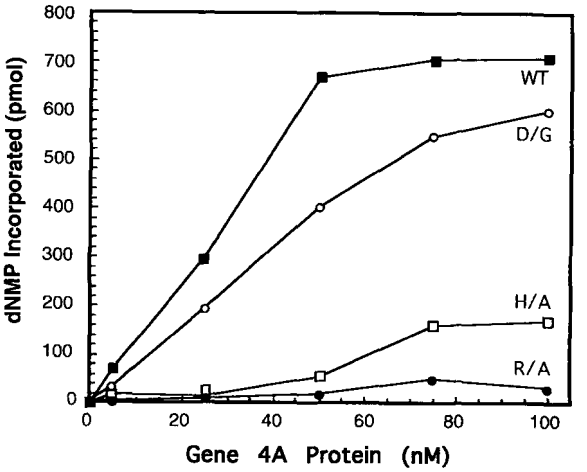

FIG. 8. **The effect of increasing concentrations of T7 gene 4A wild-type and mutant proteins on RNA- primed DNA synthesis by T7 DNA polymerase.** The concentrations of gene 4A proteins are indicated. In addition, the 30-μl reactions contained 40 nM T7 DNA polymerase, 40 μM M13 ssDNA, 0.3 μM NTP, 0.3 μM d(G,A,C)TP, and 1 mM [α-^{32}P]dTTP in dTTPase reaction buffer plus 50 mM potassium glutamate. The incorporation of label into DNA was determined using DEAE-filters (Whatman DE81) and scintillation counting. The curves are labeled as in Fig. 7.

the helicase substrate, effectively competing for the enzyme. The R487A protein cannot hydrolyze dTTP in the presence of ssDNA and, as anticipated, could not separate the strands of the helicase substrate (Fig. 7).

Primase Activity—In addition to the activities examined thus far, the gene 4A protein catalyzes the synthesis of tetraribonucleotide primers essential for the replication of the bacteriophage genome. To assess the effect the mutations described here have on primase activity, we used an assay that couples primer synthesis to DNA synthesis catalyzed by the T7 DNA polymerase. Consequently, this assay measures both the ability of the proteins to synthesize oligonucleotides and to provide functional primers for the DNA polymerase. Primase activity catalyzed by the wild-type protein reaches peak activity at approximately half the maximum protein concentration used in the assay (Fig. 8). None of the mutant proteins stimulated the same level of DNA synthesis even at the highest protein concentrations used in the assay. Each mutant protein, however, is able to prime DNA synthesis indicating that they are all capable of catalyzing the synthesis of tetraribonucleotides that can be extended by T7 DNA polymerase. The level of DNA synthesis measured in reactions with each protein is proportional to the levels of activity observed in each of the biochemical assays previously presented. This result is consistent with the loss of activity as a result of changes in the ability of the mutant proteins to form hexamers and in turn interact with ssDNA.

DISCUSSION

The hexameric nature of the T7 gene 4 protein was previously demonstrated by EM, gel filtration analysis, and chemical cross-linking experiments (22, 38). Studies with a T7 gene 4 protein having mutations in its NBS support this physical evidence (16). In these latter studies, we exploited the ability of the NBS mutant gene 4 protein to inhibit ssDNA-dependent nucleotide hydrolysis by the wild-type protein to investigate the stoichiometry of the gene 4 protein complex on ssDNA. Both this inhibition reaction and the stoichiometry of ssDNA binding indicated that the gene 4 protein was active as a hexamer. The ability of the NBS mutant protein to inhibit completely the activity of the wild-type gene 4 protein also demonstrated the importance of cooperative nucleotide hydrolysis within the hexamer.

In the present study, we have identified mutations in the gene 4 protein that affect its ability to form hexamers and to interfere with coordinated interactions between the subunits of the hexamer. The mutations lie within a conserved domain in the carboxyl-terminal region of the protein. This domain spans amino acid residues 475 to 500 and shares sequence similarities with the gene 4 proteins of phages T3 and SP6, the DnaB proteins of *E. coli* and *Salmonella typhimurium*, the phage T4 gp41 helicase and others (refer to Table I of this report and Ilyina *et al.* (27)). Moreover, the core of this region, T7 gene 4 protein residues 481 to 491, is highly conserved in each protein of the DnaB helicase family, many of which form hexamers. Our finding that this conserved domain is responsible for oligomerization and protein-protein interactions required for nucleotide hydrolysis on ssDNA suggests that this related group of NTP-dependent hexameric helicases may share a common mechanism for translocation on ssDNA and unwinding dsDNA.

Each of the mutations within this domain affected the ability of the gene 4A protein to complement a T7 phage lacking gene 4. The H475A and D485G mutations decreased T7 Δ4-1 plaque size and number, and the R487A mutation prevented growth of this phage. EM analysis revealed that each of the mutant gene 4 proteins form hexagonal rings, and, morphologically, these hexamers were indistinguishable from those formed by the wild-type protein. Further analysis of the mutant proteins revealed that the amino acid substitutions cause two distinct yet related changes in the properties of the gene 4 protein. The H475A and D485G mutations decrease the ability of the proteins to form hexamers, and the R487A mutation affects the ability of the gene 4 protein hexamer to use the energy of nucleotide hydrolysis for translocation on ssDNA.

The H475A and D485G proteins have significantly lower ssDNA-dependent dTTPase activity than does the wild-type protein. Since nucleotide hydrolysis catalyzed by the gene 4 protein is stimulated by ssDNA, and since only gene 4 protein

hexamers bind ssDNA (data not shown, refer to Ref. 41), it appears that the reduced dTTPase activity of these mutants is due to less efficient hexamer formation. Consistent with this is the finding that the loss of nucleotide hydrolysis activity resulting from these mutations is proportional to their decreased ability to form hexamers as measured by native PAGE (refer to Table III). The results of the ssDNA binding and helicase assays also support the conclusion that the primary defect caused by the H475A and D485G mutations is a lower protein-protein binding affinity and not a defect in nucleotide binding or hydrolysis. Based on these findings, we conclude that the activities of the gene 4 protein, ssDNA-dependent nucleotide hydrolysis, ssDNA binding, translocation on ssDNA, and DNA strand separation are all dependent on hexamer formation.

The R487A mutation affects the activity of the gene 4 protein in a different manner. This mutant protein forms hexamers as well as wild-type protein and hydrolyzes nucleotides better than wild-type in the absence of ssDNA. However, nucleotide hydrolysis by this protein is inhibited, instead of stimulated by ssDNA. In addition, the R487A protein does not bind ssDNA tightly or exhibit any ability to unwind dsDNA. Together, these findings strongly suggest that the R487A mutation affects the ability of the monomers within a hexamer to interact properly during nucleotide hydrolysis and translocation on ssDNA.

The 63-kDa gene 4 protein, by virtue of its unique amino-terminal domain, also catalyzes template-dependent synthesis of tetraribonucleotides (10). Each mutant protein is able to synthesize primers for T7 DNA polymerase, indicating that the mutations in this conserved carboxyl-terminal region of the protein do not directly affect its ability to function as a primase. Similar results were observed in our analysis of a NBS mutant gene 4A protein (17). This latter mutant protein could not hydrolyze dTTP, and therefore could not translocate on ssDNA, but it could synthesize template-directed primers, presumably through random interaction with DNA.

The D485G mutation found in our original clone of gene 4 protein can be attributed to the lethality of the wild-type protein to *E. coli*. In fact, in the process of recloning wild-type gene 4, we found that a high frequency of clones contained mutations, at least one of which was defective in nucleotide hydrolysis (data not shown). The primary defect caused by this mutation resulted in our initial inability to demonstrate the oligomeric nature of the gene 4 protein by gel filtration (12, 15). In this report, we have shown that the D485G protein retains all of the catalytic properties of the wild-type protein, but in each assay the specific activity of this mutant protein is lower than that of the wild-type protein. Nonetheless, the overall observations made with this cloned mutant protein are consistent with those made with gene 4 protein purified from phage-infected cells. This is not a surprising result since the mutant protein does support T7 replication and growth (14, 37). Therefore, we do not believe that any of our earlier results obtained with this protein will differ significantly from those of the wild-type protein. Rather, we believe that certain reactions requiring tight protein-protein interactions, such as the coupling of DNA polymerase and helicase/primase activities at the T7 DNA replication fork (35) will be augmented.

A recent EM analysis of the gene 4 protein revealed that ssDNA passes through the center of the gene 4 protein hexamer (38). It was also demonstrated that the *E. coli* RuvB branch migration protein, which has helicase activity, forms double hexameric rings around DNA (25). The ring structure of these protein complexes raises the intriguing question of how these hexagonal rings load onto the DNA. Since the gene 4 protein tightly binds circular ssDNA (40) we can rule out mechanisms requiring loading via free ends. Therefore, in order to bind ssDNA, the gene 4 hexamer must either assemble around the DNA or the preformed hexameric ring must open to load onto the ssDNA. As observed with other hexameric helicases, such as DnaB and T4 gp41 (18, 42), nucleoside triphosphate binding facilitates hexamer formation by the gene 4 proteins (22). In this study, we also observed an increase in hexamer formation by the gene 4 protein upon binding dTTP (Fig. 2*D* and Tables III and IV). The product of the hydrolysis reaction, dTDP, does not induce hexamer formation to the same extent as does dTTP (Fig. 2*B* and Table IV). This result suggests that NTP binding leads to a conformational change in the gene 4 protein that increases the protein-protein binding affinity and that conformational changes during the hydrolysis of the NTP to NDP decreases the association affinity. When bound to ssDNA, these conformational changes are rapid and result in translocation. In the absence of bound ssDNA, the hexamer will have more time to partially dissociate due to the relatively slow rate of the hydrolysis reaction. The decreased protein self-association affinity that occurs as dTTP is hydrolyzed to dTDP may therefore be a necessary step for the hexameric ring formed by the gene 4 protein to open and bind ssDNA.

The ssDNA binding experiments demonstrate that nucleotide binding and hexamer formation are not sufficient for tight DNA binding, nor is nucleotide hydrolysis required for the gene 4 protein to bind ssDNA, since the nonhydrolyzable analog β,γ-methylene dTTP promotes strong binding (40). The R487A protein forms hexamers as well as wild-type protein and binds ssDNA almost as well in the cross-linking assay. It may be that the hexamers formed by the R487A protein interact with DNA, but cannot undergo the conformational changes required for the hexamer to "grip" the DNA and translocate. Consequently, it slips off the end of the oligonucleotide during electrophoresis, and, thus, binding cannot be detected in the gel-shift assay.

We find it of interest that the two closely spaced mutations D485G and R487A have such differing effects on the oligomerization and enzymatic activities of the gene 4 protein. The residues Asp^{485} and Arg^{487} are at the center of the core region of homology with the DnaB family of helicases and so suggest a common mechanism of action for hexameric helicases. Thus, we speculate that interactions mediated by this domain between the subunits in a hexamer may be responsible for the presence of three high and three low affinity NBS observed in studies of the DnaB hexamer (43). This hypothesis is supported by the close proximity of the residues directly involved in the protein-protein interactions. It is very likely that the protein domain responsible for communication of conformational changes between the hexamer subunits during nucleotide hydrolysis and the domain responsible for protein-protein interactions would be integrated.

It should be noted that the gene 4 proteins H475A and D485G, while defective in hexamer formation, do not migrate as monomers in native PAGE analysis. Although it is possible that the fastest migrating protein band visible on the silver-stained native gels is actually a monomer with aberrant migration, this seems unlikely since a "Ferguson" analysis (44) of both the protein standards and the estimated molecular weight of each of the visible gene 4 protein bands generates a linear plot. Additionally, small zone gel filtration analysis of gene 4 protein at various concentrations in the presence and absence of nucleotide detected only two species of oligomer, estimated to be dimer and hexamer (22). If the proteins interact in a "head-to-tail" configuration, it is difficult to imagine how mutations that influence hexamer formation would not affect dimerization. This head-to-tail configuration predicts a single interface, the disruption of which would lead to the appearance of monomers (Fig. 9). On the other hand, if dimer formation is repre-

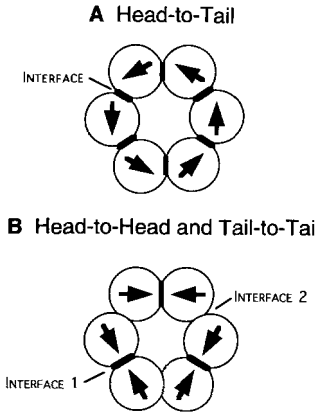

FIG. 9. **Model for association of gene 4 proteins in a hexamer.** *A*, head-to-tail association of monomers showing six protein-protein interfaces of a single type. *B*, head-to-head and tail-to-tail association of three dimer pairs of gene 4 protein with two types of interface.

sented as a "head-to-head" interaction of the monomers, and the dimers then interact through "tail-to-tail" contacts to form hexamers, we would predict two types of protein-protein interface (Fig. 9). Mutations at one interface would affect hexamer formation, but would have no influence on dimer formation. Precisely such a model was proposed by Dong *et al.* (18) for nucleotide-induced hexamer formation by the phage T4 gp41 helicase. They observed the formation of dimers at low protein concentrations and hexamers at higher concentrations and proposed the existence of two interfaces with different protein association strengths. The gene 4 protein-protein interactions appear to be much stronger than those of T4 gp41 as hexamers of gene 4 protein were detected in the absence of nucleotide by native PAGE at protein concentrations of 2 μM and by electron microscopy at very low protein concentrations (175 nM), whereas T4 gp41 does not form hexamers at low protein concentrations unless a nucleotide is present (18).

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