Biochemical Analysis of Mutant T7 Primase/Helicase Proteins Defective in DNA Binding, Nucleotide Hydrolysis, and the Coupling of Hydrolysis with DNA Unwinding*

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We characterized nine helicase-deficient mutants of bacteriophage T7 helicase-primase protein (4A) prepared by random mutagenesis as reported in the accompanying paper (Rosenberg, A. H., Griffin, K., Washington, M. T., Patel, S. S., and Studier, F. W. (1996) J. Biol. Chem. 271, 26819–26824). Mutants were selected from each of the helicase-conserved motifs for detailed analysis to understand better their function. In agreement with the in vivo results, the mutants were defective in helicase activity but were active in primase function. dTTP hydrolysis, DNA binding, and hexamer formation were examined. Three classes of defective mutants were observed. Group A mutants (E348K, D424N, and S496F), defective in dTTP hydrolysis, lie in motifs 1a, 2, and 4 and are possibly involved in NTP binding/hydrolysis. Group B mutants (R487C and G488D), defective in DNA binding, lie in motif 4 and are responsible directly or indirectly for DNA binding. Group C mutants (G116D, A257T, S345F, and G451E) were not defective in any of the activities except the helicase function. These mutants, scattered throughout the protein, appear defective in coupling dTTPase activity to helicase function. Secondary structural predictions of 4A and DnaB helicases resemble the known structures of RecA and F1 ATPase enzymes. Alignment shows a striking correlation in the positions of the amino acids that interact with NTP and DNA.

DNA helicases catalyze unwinding of duplex DNA to single-stranded DNA, a process energetically coupled to NTP hydrolysis. Helicases are an important class of proteins required in almost all the processes of DNA and RNA metabolism. Recently, a large number of putative helicases have been identified mainly from amino acid sequence homologies. Known helicases have homologous amino acid sequences, confined to small regions in the protein, that are used as signature motifs for identifying helicases (1, 2). Since a high resolution structure of a helicase is not known at the present time, the roles of these conserved motifs remain largely unclear.

We are studying the mechanism of bacteriophage T7 DNA helicase, which is involved in DNA replication. Bacteriophage T7 is a model system used to study the detailed mechanisms of DNA replication because of its simplicity. A minimum of two proteins, T7 DNA polymerase and T7 DNA primase/helicase, have been shown to reconstitute duplex DNA replication in vitro. T7 gene 4 encodes the two primase/helicase proteins, 4A and 4B (3). The full-length 63-kDa 4A protein has both helicase and primase activities, whereas the shorter 56-kDa 4B protein that begins at a second initiation codon has only helicase activity (4, 5). The helicase activity unwinds double-stranded DNA during leading strand DNA replication, and the primase catalyzes synthesis of tetraribonucleotides that serve as primers for lagging strand DNA replication (4).

T7 DNA helicase belongs to the general class of hexameric helicases. Its low resolution structure, studied in detail using electron microscopy and image averaging, shows that both 4A and 4B proteins form ring-shaped hexamers, and the ssDNA1 binds through the central hole of the ring (6). This mode of DNA binding results in protection of about 25 bases of ssDNA from nuclease digestion (7) and likely confers high processivity to DNA unwinding. The helicase forms hexamers only in the presence of nucleotide ligands such as dTDP, dTTP, ATP, and dTMP-PCP (8, 9). DNA binds tightly only to the hexameric species and requires the presence of dTTP or dTMP-PCP (7). The various activities of the helicase protein such as NTP binding/hydrolysis, protein oligomerization, and DNA binding are linked (9). Therefore, it is likely that amino acids responsible for these activities also may be close in space or perhaps lie in the same motif.

Regions of T7 gene 4 protein show sequence homology to several bacterial and bacteriophage primase/helicase and primase-related helicases that belong to the DnaB family of helicases. Comparison of amino acid sequences in this family of helicases has led to the identification of five conserved motifs denoted 1A, 1a, 2B, 3, and 4 (2). Conserved motif 1A is the well known GXGXXGKS/S sequence found in numerous nucleotide-binding proteins and shown in many ATPases to be involved in binding the diphosphate or the triphosphate moiety of nucleotides (10). Motif 2B is most likely the conserved motif B sequence involved in binding nucleotide via Mg2+ confirmed from this study. The remaining three motifs, 1a, 3, 4 have unknown functions.

Site-directed mutagenesis has been used in the past to probe the function of several of the conserved motifs. Mutations have been made in the 1A motif, GXGKS sequence. Replacement of lysine 318 in this motif with an alanine (11) and replacement of glycine 317 with valine and lysine 318 with methionine (12).

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† The abbreviations used are: ssDNA, single-stranded DNA; bp, base pair(s); bovine serum albumin DEAE, diethylaminoethyl; dTMP-PCP, β-methylene deoxythymidine triphosphate; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; kb, kilobase pair(s).
result in reduction of the dTTP hydrolysis activity and elimina-
tion of the helicase activity. Subsequent mutagenesis efforts focused on motif 4. H475A and D485G mutant proteins display reduced oligomerization ability particularly in the absence of nucleotide (13). These mutants also have reduced dTTPase (lower kcat/Km), ssDNA binding, and duplex DNA unwinding activities; thus, the precise role of this motif was not clear.

In the accompanying paper (14), random mutagenesis and genetic selection were used to obtain lethal mutants in the cloned T7 primase/helicase gene (gene 4A). The 76 mutants selected were distributed throughout much of the protein, and most of them provided sufficient primase function for T7 growth but were defective in helicase function. Many of the mutants in the C-terminal half of the protein lie in or close to conserved helicase motifs, and the mutants should be useful for understanding the function of these motifs. In this paper, we present the biochemical characterization of nine of the mutant proteins.

**EXPERIMENTAL PROCEDURES**

**Nucleotides and Other Reagents**—ATP, CTP, dATP, dGTP, dCTP and dTTP were purchased from Sigma. Single-stranded M13 DNA was isolated as described below. T4 polynucleotide kinase (10 units/l) used to tailor DNA synthesis products was purchased from Amersham Life Sciences, Inc. The nucleotide stocks were stored at −70°C. Radioactive nucleotides, [γ-32P]ATP, [α-32P]dCTP, [α-32P]dGTP, and [α-32P]dTTP (3000 Ci/mmol) were obtained from ICN Biochemicals. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Amersham Life Sciences, Inc. The nucleotide (13). These mutants also have reduced dTTPase (lower kcat/Km), ssDNA binding, and duplex DNA unwinding activities; thus, the precise role of this motif was not clear.

**Measurement of Primase and Helicase Activity During DNA Synthesis**—Mutant T7 Primase/Helicase Proteins

The ssM13 DNA/60-mer fork DNA was prepared by a similar procedure. The 60-mer was radiolabeled and annealed to ssM13 DNA to form 30 bp of duplex (M13 positions 6202–6235) and 30-nucleotide nonholomorphic 3′-tail (20). The M13 DNA (50 nM) and the 60-mer (10 nM) annealing mix were heated to 95°C for 1 min and slowly cooled to 37°C. It was incubated at 37°C for 24 h to complete the annealing.

**Measurement of Helicase Activity**—The helicase activity was measured at 22°C in a buffer consisting of 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, and 10% glycerol, 4A or a mutant 4A (2.5 µM) was incubated for 10 min in annealing buffer (50 mM Tris acetate, 10 mM MgO, and 100 mM NaCl). The solution was rapidly heated to 95°C for 5 min and slowly cooled to room temperature over several hours.

The ssM13 DNA/60-mer fork DNA was prepared by a similar procedure. The 60-mer was radiolabeled and annealed to ssM13 DNA to form 30 bp of duplex (M13 positions 6202–6235) and 30-nucleotide nonholomorphic 3′-tail (20). The M13 DNA (50 nM) and the 60-mer (10 nM) annealing mix were heated to 95°C for 1 min and slowly cooled to 37°C. It was incubated at 37°C for 24 h to complete the annealing.

**Measurement of Helicase Activity**—The helicase activity was measured at 22°C in a buffer consisting of 50 mM Tris-Cl, pH 7.5, 50 mM NaCl, 10 mM MgCl₂, and 10% glycerol, 4A or a mutant 4A (2.5 µM) was incubated for 2 min in 50 mM dTTP. The reaction was initiated by adding 5′-32P-labeled fork (2 nM). Low DNA concentration was used to reduce the rate of reannealing of unwound DNAs. At time points from 0 to 60 min, reaction aliquots were mixed with SDS Quench dye (3% SDS, 100 mM EDTA, 40% glycerol, and 0.1% bromphenol blue), and the samples were immediately loaded on a 7% native polyacrylamide gel prepared in TBE buffer and run at 80 V. The remaining fork DNA and unwound ssDNA were quantitated on the PhosphorImager instrument (Molecular Dynamics). The quantitated DNAs at various time points were normalized to the heat-denatured sample, and the zero point (no protein) was subtracted from each.

The same procedure was used for the M13/60-mer fork DNA substrate. This reaction was initiated by the addition of the M13/60-mer fork DNA to a final concentration of 5 nM M13 DNA (1 nM of M13 with the radiolabeled 60-mer annealed).
Measurement of dTTP Hydrolysis—The dTTPase activity of the wild-type 4A (1 μM) and the mutant proteins (1 μM) was measured at 22 °C. Proteins were preincubated with 100 μM dTTP for 10 min with or without M13 DNA (50 nM). The reactions were initiated by adding a mixture of 5 mM dTTP and [α-32P]dTTP (2 μCi). At various time intervals, aliquots were quenched with an equal volume of 0.5 M EDTA. The unreacted dTTP and the hydrolysis product, dTDP, were separated on polyethyleneimine-cellulose thin layer chromatography plates (polyethyleneimine-cellulose TLC plates, Whatman) using 0.3 M potassium phosphate, pH 3.4, as the running buffer. The separated dTTP and dTDP were quantitated using the Molecular Dynamics PhosphorImager instrument.

Native PAGE to Assay for DNA Binding and Hexamer Formation—Hexamer formation and DNA binding activities were assayed by native PAGE (6%) containing Mg-dTMP-PCP as described (7). The protein samples (10 μl) contained 12 μM 4A or mutant protein, 2 μM 5′-radiolabeled 30-mer DNA, 1 mM dTMP-PCP in binding buffer (50 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 5 mM sodium acetate, and 1 mM DTT). Native gel loading buffer (2 μl of 50% glycerol, 0.25% bromphenol blue) was added to each sample, and the gel was run at constant current (18 mA) for 2 h. The protein species were detected by Coomassie Blue staining, and the DNA-bound species were observed by using the PhosphorImager instrument. Hexamer formation in the absence of DNA was also assayed by the same native gel experiment. The running conditions and sample composition were identical except that 30-mer DNA was omitted.

Nitrocellulose-DEAE DNA Binding Assay—DNA binding by nitrocellulose/DEAE filter binding assay was performed as described (7). Radiolabeled 30-mer (1 μM) was mixed with increasing 4A or the mutant protein (1–20 μM). The buffer contained 50 mM Tris acetate, pH 7.5, 10 mM magnesium acetate, 5 mM sodium acetate, 1 mM DTT, and 1 mM TMP-PCP. After 20 min incubation, samples were filtered through the membrane assembly and washed with 100 μl of buffer. The protein-bound DNA on the nitrocellulose membrane and the free DNA on the DEAE membrane were quantitated using the PhosphorImager instrument.

RESULTS

Expression and Purification of the 4A Mutant Proteins—Representative mutants were chosen from the N terminus half of 4A (the presumed primase domain), the linking region between the primase and helicase domains, and the C terminus helicase domain for purification and biochemical analysis. Mutants from the C-terminal helicase domain were chosen from each of the conserved helicase motifs, identified from amino acid sequence homology between the DnaB family of helicases (Fig. 1), with the hope that the biochemical activities of these mutants will lead to a better understanding of the role of these motifs. The selected 4A mutants were overexpressed under the T7lac promoter in BL21 (DE3) cells using the T7 expression system (18). The level of overexpression of all the mutants was approximately two to five times greater than that of wild-type 4A protein. The proteins were purified to greater than 95% purity as judged by SDS-PAGE (Fig. 2) using the modified 4A purification scheme. The 4A mutant proteins behaved similarly to the wild-type protein during the entire purification process.

Primase Activity of the 4A Mutant Proteins—Since all the mutants complemented T7Δ4A phage (14), it suggested that the mutants either have sufficient primase activity to support phage growth or are capable of restoring the primase activity to 4B protein. The primase activity of the mutant proteins was assayed by measuring the steady-state kinetics of RNA primer synthesis. A 60-mer ssDNA containing the primase recognition sequence 3′-CTGGG/T and requires the presence of either dTTP or dTMP-PCP, which promote stable hexamer formation and DNA binding. At 22 °C in the presence of dTTP, 4A synthesizes 2- to 4-mer RNAs with a steady-state rate constant of 3.3 × 10−3 s−1, and 26% of the RNA products synthesized by 4A were full-length 4-mer products. All the mutant proteins were competent in their primase function (Table I). The rates ranged from close to wild-type (3.5 × 10−3 s−1) for D424N mutant to about 10-fold lower (3.9 × 10−4 s−1) for G488D mutant protein. Similarly, the processivities ranged from close to wild-type (92% of the wild-type) for S345F mutant to 2-fold lower for D424N (46% of wild-type). The RNA-primed DNA synthesis assay also showed that the mutant proteins are capable of synthesizing RNA primers that support DNA synthesis by T7 DNA polymerase (Fig. 4).

The mutants with low primase activity, R487C, G488D, and S496F (16, 12, and 26% of 4A, respectively), all lie in the conserved helicase motif 4. Interestingly, this low primase activity is still enough to complement the T7Δ4A phage in vivo (14). The lower primase activity of these mutants we believe is due to their defect in DNA binding as shown below. Consistent with this, when the primase activity was remeasured in the presence of a higher concentration of the template DNA, the activity of R487C and S496F increased to 40 and 50% of 4A. The activity of G488D, however, remained low at 10%. Overall, there does not appear to be a severe defect in the primase function in any of the mutant proteins, consistent with the ability of the mutant proteins to provide primase activity in vivo (14).

Helicase Activity of the 4A Mutant Proteins—We have used three different DNA substrates to assay for the helicase activity of the mutant proteins. Two assays measured the intrinsic helicase activity of the mutant protein, and one assay measured the helicase activity in the presence of DNA polymerase. T7 DNA helicase requires two noncomplementary ssDNA tails at one end of a duplex DNA (fork DNA) to initiate DNA strand sepa-
The kinetic assay showed that 4A' protein completely unwound the fork DNA in 2 min, the shortest time point used in the assay. However, none of the mutant proteins showed any DNA unwinding activity, even after 1 h of reaction, indicating that the mutant proteins needed a “running start” or needed to translocate on ssDNA prior to DNA unwinding. The third assay consisted of measuring strand displacement coupled to DNA synthesis by T7 DNA polymerase.

**Oligo-fork Unwinding Assay—Helicase activity was measured using radiolabeled fork DNA at 22 °C in the presence of 5 mM dTTP. Unwound ssDNA strands were resolved from the fork DNA using native PAGE, as shown in Fig. 3. The kinetic assay showed that 4A' protein completely unwound the fork DNA in 2 min, the shortest time point used in the assay. However, none of the mutant proteins showed any DNA unwinding activity, even after 1 h of reaction, indicating that the mutants are severely defective in helicase function. Since reannealing of the fork at 1 nM DNA concentration occurs with a rate of $2 \times 10^{-4}$ s$^{-1}$ or with a half-time of 70 min, unwinding occurring with a rate comparable with reannealing would be barely detectable. Therefore, relative to the unwinding rate of 4A' equal to 3.2 bps/s at 18 °C,2 we estimate that the helicase activity of the mutants is at least 600-fold lower than 4A'.

**RNA-primed DNA Synthesis and Strand Separation Assay for Helicase Activity—RNA-primed DNA synthesis assay was used to investigate the ability of the mutant proteins to provide helicase activity to support DNA synthesis by T7 DNA polymerase.**

In this assay, ssM13 was used as the template. All the mutant proteins had primase activity. DNA products up to 7 kb in length can be formed by elongation of RNA primers by T7 DNA polymerase (Fig. 4). The polymerase does not perform strand displacement DNA synthesis (see control reaction in Fig. 4), and it needs the helicase activity to synthesize DNA products longer than 7 kb. These longer DNA products (>40 kb) are formed in reactions containing 4A' but not present in reactions containing some of the mutant proteins. A few mutant proteins, however, did show helicase activity in this assay. These mutant proteins included the ones in the N-terminal domain, the linker region, and some in the C-terminal domain, G116D (N-terminal), A257T (linker region), S345F (motif 1a), and G451E (motif 3). The mutant proteins that showed no helicase activity are all in the C-terminal domain, D424N (motif 2b), R487C, G488D, and S496F (all in motif 4). Note that the latter three are also the ones that have reduced primase activity.

By quantitating the amount of the >7-kb products, we can determine the relative helicase activity of the mutants. Note that this is a very rough estimate of the defect in helicase activity, as this helicase assay is indirect and not quantitative. The mutant proteins that showed helicase activities, G116D, A257T, S345F, and G451E, have 45, 46, 25, and 16% activity, respectively, relative to wild-type 4A' (●), D424N (○), and the mutants G116D, A257T, S345F, G451E, R487C, G488D, and S496F (other symbols).

**Fig. 3. Oligodeoxynucleotide fork assay of DNA unwinding assay.** A shows a native polyacrylamide gel (7%) that was used to separate the unwound DNA from the less mobile, partially duplex fork DNA. The unwinding reactions were carried out at 22 °C using 2.5 μM protein, 2 µM fork DNA, and 5 mM dTTP as described under “Experimental Procedures.” Lanes 1, duplex fork DNA; lane 2, heat-denatured single-stranded DNA; lanes 3–6, 4A' reaction quenched at 10, 20, 30, and 60 min; and lanes 7–10, G116D reaction quenched at the same times. B compares the helicase activity of the wild-type 4A' (●), D424N (○), and the mutants G116D, A257T, S345F, G451E, R487C, G488D, and S496F (other symbols).

**Fig. 4. RNA-primed DNA synthesis assay of DNA unwinding.** PhosphorImager scan of a 0.6% agarose gel used to resolve the DNA products from the primase-dependent helicase assay that was performed as described under “Experimental Procedures.” Lanes labeled 1, control reactions quenched at 20 and 40 min containing no 4A' protein. Lanes labeled 2–9 show DNA products from reaction performed with 4A', G116D, A257T, S345F, G451E, R487C, G488D, and S496F, respectively, quenched at 20 and 40 min. The DNA products of lengths longer than 7 kb indicate the presence of helicase activity.

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**TABLE I**

**Mutant T7 Primase/Helicase Proteins**

| Mutant   | Rate constant $k_p$ (s$^{-1}$) | Processivity $\eta$ (%) |
|----------|-------------------------------|-------------------------|
| 4A'      | 0.0033 ± 0.0003               | 26                      |
| G116D    | 0.0014 ± 0.0001               | 19                      |
| A257T    | 0.00083 ± 0.0002              | 18                      |
| S345F    | 0.0020 ± 0.0001               | 24                      |
| D424N    | 0.0035 ± 0.0002               | 12                      |
| G451E    | 0.0015 ± 0.00042              | 17                      |
| R487C    | 0.00053 ± 0.000025            | 16                      |
| G488D    | 0.00039 ± 0.000052            | 14                      |
| S496F    | 0.00083 ± 0.000057            | 19                      |

$^2$ Processivity was calculated by dividing the amount of 4-mer (ACCA) synthesized by the total amount of RNA products synthesized (AC, ACC, and ACCA).
mutant proteins are scattered throughout the primary sequence of the protein, it is difficult to postulate the nature of this restoration in helicase activity. More detailed biochemical analysis of the mutant proteins will be needed to fully understand the result.

M13/Oligodeoxynucleotide Helicase Assay—It is clear that mutants, D424N (motif 2B), R487C, G488D, and S496F (all in motif 4) had no helicase activity by both assays described above. The mutants G116D (N terminus), A257T (linker region), S345F (motif 1a), G451E (motif 3) show no activity with the small fork DNA unwinding substrate but showed measurable helicase activity in the presence of the polymerase. To understand the different results, we measured the helicase activity of the four mutants using a third assay. One potential problem with using the small fork DNA as the substrate can be the short ssDNA tail region available to initiate DNA unwinding. The other problem might be the reannealing of the unwound DNA which would preclude detection of low levels of helicase activity. The ssM13/60-mer fork DNA substrate should circumvent both of these problems. This assay is, however, not quantitative and does not provide the intrinsic rate of DNA unwinding, as unwinding is dependent on a number of steps prior to strand separation.

Fig. 5 shows the helicase activity of 4A', G116D, A257T, S345F, and G451E using the ssM13/60-mer DNA substrate. 4A' unwound the 60-mer primer with a rate constant of 0.06 bp/s. Note that the measured unwinding rate of wild-type 4A' using this assay was about 50-fold lower than with the small fork DNA unwinding assay. Mutant proteins, G116D (N-terminal) and G451E (motif 3), showed no detectable helicase activity even in this assay. But the mutant proteins A257T (linker region) and S345F (motif 1a) did show detectable unwinding with a rate 10-fold slower (0.006 bp/s) than 4A'. Detection of helicase activity by this assay compared with the small fork assay suggests that these mutant proteins may need a longer ssDNA 5'-tail to initiate unwinding.

dTTPase Hydrolysis Activity of the 4A' Mutant Proteins—Nucleotide triphosphate binding and hydrolysis are necessary for the helicase activity. Since all 4A' mutants had significant defects in their helicase function, we have investigated the dTTPase activity of all the mutant proteins. (T7 DNA helicase prefers dTTP as the nucleotide substrate for its helicase activity.) The dTTPase activity of the mutant proteins was measured both in the absence and in the presence of ssM13 DNA. It is necessary to measure the DNA-independent dTTPase activity as this provides information about the mutant's intrinsic ability to bind and hydrolyze dTTP. The dTTPase activity in the presence of ssM13 DNA is a measure of the DNA-stimulated activity of the mutant protein. To estimate the kcat value, all the assays have been carried out at very high dTTP concentrations (5 mM). The 4A' dTTPase Km in the absence of DNA was 5 μM and in the presence of DNA about 100 μM at 22 °C (data not shown).

Table II lists the dTTPase activities of the mutant proteins. To our surprise, several mutant proteins including G116D (N-terminal), A257T (linker region), S345F (motif 1a), and G451E (motif 3) hydrolyzed dTTP in the absence of DNA with rate constants about 10-fold higher than 4A'. The reason for the increased intrinsic rate of dTTPase activity is unclear but suggestive of an uncoupling between NTPase and DNA unwinding activities as the mutant proteins are defective in helicase function. One mutant, R487C (motif 4), also had a higher intrinsic dTTPase activity, but the neighboring mutant, G488D, had the same intrinsic dTTPase activity as the wild type. The only mutants that had extreme defects of their dTTPase activity are the E348K (motif 1a), D424N (motif 2B), and S496F (motif 4). These mutants are most likely involved in direct interactions with the dTTP nucleotide.

The DNA-dependent dTTPase activity of the mutants was assayed in the presence of ssM13 DNA. The wild-type 4A' protein showed about a 100-fold stimulation of dTTPase activity in the presence of ssM13 DNA (Table II). Mutant proteins, G116D (N terminus), A257T (linker region), S345F (motif 1a), and G451E (motif 3), which had elevated intrinsic dTTPase activity all showed DNA-stimulated dTTPase activity. All hydrolyzed dTTP with slightly lower (2–5-fold) DNA-stimulated dTTPase rates than 4A'. The reduction in DNA-stimulated dTTPase activity of the above mutants was relatively slight compared with the large decrease in the helicase activity of these mutant proteins. The inability of these four mutants to unwind duplex DNA, therefore, cannot be due to their inability to hydrolyze the dTTP substrate.

All the mutants in motif 4, R487C, G488D, and S496F, showed no DNA-stimulated dTTPase activity. Interestingly, the dTTPase activities of R487C and G488D mutants were actually lower in the presence of DNA. Evidently DNA somehow inhibits their dTTPase activity. A similar observation was made with the site-directed mutant R487A (13). These two mutant proteins are the ones that had lower affinity for ssDNA (see Fig. 6 and Fig. 7), and the failure of M13 ssDNA to stimulate dTTP hydrolysis activity correlates with their DNA binding defect. Finally, E348K (motif 1a), S496F (motif 4), and D424N (motif 2B) mutants that were defective in intrinsic dTTPase activity were also defective in dTTPase activity in the presence of ssDNA consistent with their role in interactions with dTTP.

| Mutant     | Rate constant + M13 ssDNA (s⁻¹) | Rate constant - M13 ssDNA (s⁻¹) |
|------------|--------------------------------|---------------------------------|
| 4A'        | 4.7 ± 1.0                       | 0.036 ± 0.006                   |
| G116D      | 1.65 ± 0.13                     | 0.42 ± 0.028                    |
| A257T      | 1.38 ± 0.92                     | 0.37 ± 0.15                     |
| K318A      | 0.04 ± 0.01                     | 0.0095                          |
| S345F      | 2.57 ± 0.37                     | 0.38 ± 0.081                    |
| E348K      | 0.056                           | 0.0008                          |
| D424N      | 0.016                           | 0.002 ± 0.0002                  |
| G451E      | 0.97 ± 0.54                     | 0.28 ± 0.040                    |
| R487C      | 0.031 ± 0.0072                  | 0.25 ± 0.030                    |
| G488D      | 0.0153 ± 0.0012                 | 0.038 ± 0.0066                  |
| S496F      | 0                               | 0.001                           |

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Protein Oligomerization and DNA Binding—Both helicase and primase activities require hexamer formation and DNA binding. The 4A9 mutants were assayed for these functions by native PAGE in the presence of dTMP-PCP, since the nonhydrolizable TTP analogue promotes stable protein oligomerization and DNA binding by 4A9 (7). All the mutants assembled into oligomers ranging from dimers to hexamers and beyond similar to 4A9 (Fig. 6a). These results indicate that none of the mutant proteins are defective in oligomerization. Interestingly, one mutant, R487C, appeared to form more stable hexamers relative to 4A9.

Previous studies have shown that ssDNA binding and hexamer formation are linked processes, that is hexamer formation is necessary for DNA binding (7). In addition, DNA binds to 4A9 on a native gel only in the presence of dTMP-PCP, most likely because dTMP-PCP does not get hydrolyzed and thus promotes stable hexamer formation. In addition, hexamers are stabilized on the native gel in the presence of ssDNA. To assay for DNA binding, the native PAGE experiment was carried out in the presence of radiolabeled 30-mer DNA and Mg(dTMP-PCP). The protein species were detected by Coomassie Blue staining, and DNA binding was detected by PhosphorImaging. Fig. 6b shows the Coomassie-stained gel showing oligomerization of the mutant proteins in the presence of DNA. All the mutant proteins oligomerized in the presence of dTMP-PCP and DNA. In the presence of DNA, the mutant proteins G116D (N terminus), A257T (linker region), and S345F (motif 1a) formed more stable hexamers and fewer lower order oligomers similar to 4A9. However, oligomerization of the mutant proteins, E348K (motif 1a), G451E (motif 3), D424N (motif 2B), R487C, G488D, and S496F (all motif 4), was unaffected by the presence of DNA.

**Fig. 6.** Native polyacrylamide gel electrophoresis of 4A9 mutants. a shows protein oligomers resolved on a Coomassie-stained 6% nondenaturing polyacrylamide gel in the presence of Mg(dTMP-PCP) and the absence of DNA. Lanes 1–10, 4A9, G116D, A257T, S345F, G451E, R487C, G488D, S496F, E348K, and D424N (12 μM). b shows oligomers resolved on a Coomassie-stained 6% nondenaturing polyacrylamide gel in the presence of dTMP-PCP and DNA. Lanes 1–10, 30-mer DNA (2 μM) with 4A9, G116D, A257T, S345F, G451E, R487C, G488D, S496F, E348K, and D424N (12 μM). c shows a PhosphorImager scan of the native gel in b. Radiolabeled free 30-mer DNA and 30-mer DNA bound to the 4A9 or mutant hexamers is indicated. Lane 0, 30-mer DNA (2 μM) with no 4A9 protein. Lanes 1–10 are the same as in b.

Protein Oligomerization and DNA Binding—Both helicase and primase activities require hexamer formation and DNA binding. The 4A9 mutants were assayed for these functions by native PAGE in the presence of dTMP-PCP, since the nonhydrolyzable dTTP analogue promotes stable protein oligomerization and DNA binding by 4A9 (7). All the mutants assembled into oligomers ranging from dimers to hexamers and beyond similar to 4A9 (Fig. 6a). These results indicate that none of the mutant proteins are defective in oligomerization. Interestingly, one mutant, R487C, appeared to form more stable hexamers relative to 4A9.

Previous studies have shown that ssDNA binding and hexamer formation are linked processes, that is hexamer formation is necessary for DNA binding (7). In addition, DNA binds to 4A9 on a native gel only in the presence of dTMP-PCP, most likely because dTMP-PCP does not get hydrolyzed and thus promotes stable hexamer formation. In addition, hexamers are stabilized on the native gel in the presence of ssDNA. To assay for DNA binding, the native PAGE experiment was carried out in the presence of radiolabeled 30-mer DNA and Mg(dTMP-PCP). The protein species were detected by Coomassie Blue staining, and DNA binding was detected by PhosphorImaging. Fig. 6b shows the Coomassie-stained gel showing oligomerization of the mutant proteins in the presence of DNA. All the mutant proteins oligomerized in the presence of dTMP-PCP and DNA. In the presence of DNA, the mutant proteins G116D (N terminus), A257T (linker region), and S345F (motif 1a) formed more stable hexamers and fewer lower order oligomers similar to 4A9. However, oligomerization of the mutant proteins, E348K (motif 1a), G451E (motif 3), D424N (motif 2B), R487C, G488D, and S496F (all motif 4), was unaffected by the presence of DNA.

**Fig. 7.** Nitrocellulose DNA binding assay. Membrane binding assays of 4A9 and the mutants were performed using a constant amount of the 30-mer DNA (1 μM) and increasing amounts of protein (1–20 μM). Titrations were performed as described under “Experimental Procedures.” Binding isotherms are shown for 4A9 (●), E348K (□), D424N (■), G451E (□), R487C (▲), G488D (△), and S496F (▲).
DNA binding by the mutant proteins was analyzed by PhosphorImaging (Fig. 6c). The mutant proteins, G116D (N-terminal) and A257T (linker region), bound DNA as well as 4A'. S345F (motif 1a) bound DNA with a higher affinity than 4A' (257%), and G451E (motif 3) bound DNA weakly (10%). Others such as E348K (motif 1a), D424N (motif 2B), and all the mutants in motif 4, R487C, G488D, and S496F showed no DNA binding.

**Discussion**

To unwind duplex DNA, a helicase requires a number of subactivities, including NTP binding, NTP hydrolysis, oligomerization, DNA binding, and coordination among these various subactivities. A defect in any of these functions can lead to a defect in the unwinding activity. We have used random mutagenesis and genetic selection to obtain helicase-defective mutants of T7 primase/helicase with the goal of identifying amino acids or regions of the protein participating in these various activities. A detailed biochemical analysis of these mutants has allowed us to understand the basis for the helicase defect at the level of these various subactivities.

Random mutagenesis created a number of single amino acid mutations distributed throughout the protein that produced defects in the helicase function required in vivo (14). All of the mutants provided sufficient primase function, except those that produced shortened proteins. The biochemical properties of the nine mutant proteins analyzed in this paper are consistent with their in vivo properties; all were able to synthesize RNA primers but had severe defect in helicase activity.

**Relationship between Primase and Helicase Activities**—In bacteriophage T7, both the helicase and primase activities can be provided by the same protein (4A'), and from amino acid sequence homology to primases and helicases, it appears that the N-terminal half of the protein contains all the primase motifs and the C-terminal domain the helicase motifs. The two activities are coupled to a certain extent because amino acid changes in the N-terminal domain affected the helicase activity and vice versa. The mutants in the C-terminal domain, R487C, G488D, and S496F, that had lower affinity for ssDNA showed a measurable reduction in primase activity. This suggests that motif 4, likely involved in DNA binding necessary for the helicase function, may also be important for efficient RNA primer synthesis. The zinc-binding motif (N-terminal 63 amino acids) that recognizes the primase site may not be the only DNA binding site necessary for primase activity. Previous work (6) has shown that ssDNA binds through the central hole of the hexamer as shown in Fig. 8. The 3'-end of the DNA binds on the side of the hexamer containing the larger domain, which we have identified using limited proteolysis as the C-terminal domain or the helicase domain. 3 It has been noted that a 5-base tail on the 3'-side of the primase recognition site is not sufficient for primer synthesis, and a 10-base tail on the 3'-side of the primase recognition site is required (24). We therefore propose that the 10-base tail on the 3'-side is bound by the DNA-binding site inside the hole of the hexamer when the zinc-binding domain binds to the primase recognition sequence (Fig. 8). This mode of DNA binding explains the lower primase activity of the mutants, since R487C, G488D, and S496F have impaired DNA binding ability. This may also partly explain the activation of DnaG primase by the DnaB helicase in *E. coli* and the activation of gp61 primase by the gp41 helicase in bacteriophage T4.

**Functional Classification of the Mutants**—We have chosen nine representative mutants from both the N terminus and from the various conserved motifs of the C terminus domain for biochemical studies. Based on their defects with respect to the various subactivities, the nine 4A' mutants have been classified into three groups (see Table III). A previously character-
ized mutant, K318A, is also included for discussion. Group A mutants are defective in intrinsic dTTPase activity. These mutants are confined to the C-terminal domain and lie in the various conserved helicase motifs. Group B mutants are defective in DNA binding but competent in intrinsic dTTPase activity. These mutants are also confined to the C-terminal domain. Group C mutants are defective in coupling dTTPase and helicase activity. These mutants have normal subactivities, and the mutants are not confined to the C-terminal domain but are found throughout the protein. We have not identified any mutants defective in oligomerization. Perhaps protein-protein interactions that stabilize the hexamer occur over a large surface area; hence, multiple mutations would be required to affect oligomerization to a significant level.

Nucleotide Triphosphate Binding—The group A mutants defective in dTTP binding/hydrolysis are found in four different helicase motifs. Mutants K318A, E348K, D424N, and S496F lie within motifs 1A, 1A, 2B, and 4, respectively. Motifs 1A and 2B are the well known Walker A and B sequences that have been observed in GTPases such as EF-Tu (25). We postulate that binding site and the DNA-binding site, similar to the switches region that allows communication between the nucleotide-phosphate, affects the affinity for DNA, there is probably a switch site. Since the state of the nucleotide bound, triphosphate or diphosphate, affects the affinity for DNA, there is probably a switch region that allows communication between the nucleotide-binding site and the DNA-binding site, similar to the switches observed in GTPases such as EF-Tu (25). We postulate that motif 4 involved in both dTTP binding/hydrolysis and DNA binding may be part of that switch region.

A previous report hypothesized that motif 4 is important for hexamer formation based on characterization of three site-directed mutants, H475A, D485G, and R487A (13). It is known that nucleotide binding, hexamer formation, and DNA binding are all linked processes. Therefore, the idea that this domain is involved in hexamer formation may be an oversimplification of the function of the residues in this motif. In addition, the role of this motif in hexamer formation is not consistent with the characterization of random mutants reported in this paper. We have shown that the R487C and G488D mutants were not defective in oligomerization. In fact R487C formed hexamers in the absence of DNA that are more stable than the wild-type 4A. Note that the site-directed mutant R487A was reported to have no defect in oligomerization but clearly a defect in DNA binding. It was suggested that these defects were due to improper orientation of the subunit within the hexamer (13). However, since this is the most severely affected mutant of the three site-directed ones, we prefer the explanation that the Arg-487 and Gly-488 residues play a role in DNA binding. The effect of mutating residues in this region on hexamer formation may be an indirect one because of the coupling between DNA binding and hexamer formation.

Energy Transduction—Group C mutants did not show a severe defect in any of the subactivities. These mutants hydrolyzed dTTP and bound DNA and formed stable hexamers. However, none had the ability to unwind the small fork DNA. We have classified these mutants as having a defect in coupling the subactivities to helicase function. It is not uncommon in helicases that point mutations result in a defect in coupling nucleotide hydrolysis and DNA unwinding. Mutations affecting coupling have been reported recently for E. coli transcription termination factor rho (28) and RNA helicase eukaryotic initiation factor-4A (29). In eukaryotic initiation factor-4A, the mutants defective in coupling ATPase to helicase activity are found among the A motif, B motif, and a C-terminal region (region III) conserved among DEAD box RNA helicases.

The group C mutants are either intrinsically defective in DNA unwinding or have a defect in coupling the energy of nucleotide hydrolysis to DNA unwinding. Because of their large number and their distribution throughout the protein in both domains, it is more likely that these mutants are defective in energy transduction brought about by a defect in conformational changes of the protein. Uncoupling of activities can occur through a variety of mechanisms. The dTTPase activity may be uncoupled from translocation activity necessary for DNA unwinding. In such a case, the proteins will hydrolyze dTTP but fail to move along the DNA, since hydrolysis fails to cause the necessary conformational changes. Alternatively, these mutants may be severely defective in processive translocation. This would lead to frequent dissociation of the protein which would impair both translocation and DNA unwinding. The two types of defects described above may be caused by improper coordination of dTTP binding and/or DNA binding within the same subunit of the hexamer, or improper coordination among the various subunits. Interestingly, all group C mutants have a characteristic 10-fold increase in the intrinsic dTTPase activity relative to wild-type 4A. The increased dTTPase activity is either the consequence of or the cause for the uncoupling of activities. More detailed experiments will be required to determine the exact reason for the uncoupling and the increased dTTPase activity of this group of mutants.

One peculiar property of the group C mutants is that they possessed helicase activity in the presence of the DNA polymerase, although somewhat reduced compared with the wild-type. This was surprising as these mutants were completely defective in the fork unwinding assay. Since T7 DNA polymer-
F1-ATPase as described in the text. There could be a difference in the activity to complement the T7 helicase/primase deletion phage. The mutants are likely complexed with the T7 protein which is a hexameric protein that has RNA/DNA unwinding activity (34). Both F1-ATPase and RecA proteins are structurally related to the 4A' protein. The F1-ATPase is a ring-shaped hexamer with six nucleotide-binding sites, and it interacts with the γ subunit of the ATPase within the central cavity of the ring, in an analogous manner to the mode of ssDNA binding to 4A'. The RecA protein forms helical filaments around ssDNA which binds in the interior cavity of the protein helix. In addition, the loops of RecA protein that interact with the DNA correspond to the loops of the F1-ATPase that contact the γ subunit (35, 36).

The secondary structures of 4A' and the DNA binding motifs, has been aligned with the known secondary structure of the F1-ATPase and the RecA protein, both structures are very similar in their secondary structures and tertiary folds (26, 33). The secondary structural prediction of 4A' also corresponded well to that of F1-ATPase and RecA, particularly around the Walker A and B nucleotide binding motifs.

Only the C-terminal half of 4A', which contains the NTP and proposed DNA binding motifs, has been aligned with the known secondary structure of the F1-ATPase. Residues around Walker A motif (motif 1A) and Walker B motif (motif 2B) have been used as guides for alignment. The resulting alignment is shown in Fig. 9. The motif 1A begins with a sheet spanning residues 306–311 and is followed by the P-loop containing the GKS sequence. Conserved motif 1a follows as a sheet spanning residues 337–342 and the following loop. The secondary structural prediction of 4A' also corresponded well to that of F1-ATPase and RecA, particularly around the Walker A and B nucleotide binding motifs.

Possible Structure of the NTP-binding Site—No high resolution structure of a helicase is known at the present time. An attempt has been made here to predict the location of the above mutants with respect to the NTP-binding site by secondary structure predictions of 4A' and alignment with the known structure of RecA (33) and F1-ATPase (26). Interestingly, F1-ATPase shows a great deal of sequence homology to E. coli rho protein which is a hexameric protein that has RNA/DNA unwinding activity (34). Both F1-ATPase and RecA proteins are structurally related to the 4A' protein. The F1-ATPase is a ring-shaped hexamer with six nucleotide-binding sites, and it interacts with the γ subunit of the ATPase within the central cavity of the ring, in an analogous manner to the mode of ssDNA binding to 4A'. The RecA protein forms helical filaments around ssDNA which binds in the interior cavity of the protein helix. In addition, the loops of RecA protein that interact with the DNA correspond to the loops of the F1-ATPase that contact the γ subunit (35, 36).

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dicted structure of 4A' correspond well also from residue 481 to 504 that constitutes motif 4. The coil of residues 486–490 corresponds to loop L2 in the RecA structure and the R-loop in the F1-ATPase structure. This predicted structure is entirely consistent with the biochemical properties of the mutants in these conserved motifs.

The four group A mutants K318A, E348K, D424N, and S496F (defective in nucleotide hydrolysis) are in regions that correspond to residues of the F1-ATPase protein that directly contact the nucleotide. There are five regions in F1-ATPase that contact ATP, the Walker A motif, residues forming a loop and a helix analogous to conserved motif 1a, the Walker B motif; the Asp-424 is in the Walker B motif; Glu-348 is in motif 1a; and the Ser-496 is in the helix in motif 4. Analogous to F1-ATPase, it is also possible that 4A' may bind NTP at the interface. This would be consistent with the fact that nucleotide binding is linked to hexamer formation. If such is the case, some of the above residues, especially the ones in motif 4 in 4A', may also be involved in interactions with the nucleotide binding to the adjacent subunit. Examination of the above structural prediction makes assignment of motif 4 as part of the DNA-binding site and the effector switch region between the nucleotide-binding site and the DNA-binding site even more likely. The corresponding loop where these two mutations occur is the R-loop in the F1-ATPase, which contacts the γ subunit in the central cavity of the ring-shaped hexamer, and the L2 loop in the RecA protein that interacts with the DNA.

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