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The Linker Region between the Helicase and Primase Domains of the Gene 4 Protein of Bacteriophage T7

ROLE IN HELICASE CONFORMATION AND ACTIVITY*

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The gene 4 protein of bacteriophage T7 provides both helicase and primase activities. The C-terminal helicase domain is responsible for DNA-dependent dTTP hydrolysis, translocation, and DNA unwinding whereas the N-terminal primase domain is responsible for template-directed oligoribonucleotide synthesis. A 26 amino acid linker region (residues 246–271) connects the two domains and is essential for the formation of functional hexamers. In order to further dissect the role of the linker region, three residues (Ala^{257}, Pro^{259}, and Asp^{263}) that was disordered in the crystal structure of the hexameric helicase fragment were substituted with all amino acids, and the altered proteins were analyzed for their ability to support growth of T7 phage lacking gene 4. The *in vivo* screening revealed Ala^{257} and Asp^{263} to be essential whereas Pro^{259} could be replaced with any amino acid without loss of function. Selected gene 4 proteins with substitution for Ala^{257} or Asp^{263} were purified and examined for their ability to unwind DNA, hydrolyze dTTP, translocate on ssDNA, and oligomerize. In the presence of Mg^{2+}, all of the altered proteins oligomerize. However, in the absence of divalent ion, alterations at position 257 increase the extent of oligomerization whereas those at position 263 reduce oligomer formation. Although dTTP hydrolysis activity is reduced only 2–3-fold, none of the altered gene 4 proteins can translocate effectively on single-strand DNA, and they cannot mediate the unwinding of duplex DNA. Primer synthesis catalyzed by the altered proteins is relatively normal on a short DNA template but it is severely impaired on longer templates where translocation is required. The results suggest that the linker region not only connects the two domains of the gene 4 protein and participates in oligomerization, but also contributes to helicase activity by mediating conformations within the functional hexamer.

The replisomes of essentially all replication systems have five essential components, a DNA polymerase, a processivity factor for the polymerase, a DNA helicase, a DNA primase, and a single-stranded DNA (ssDNA)[1]-binding protein (1). The replisome of bacteriophage T7 is unique in that the proteins that account for these five functions mediate coordinated leading and lagging strand DNA synthesis without the aid of other accessory proteins such as clamp-loading proteins and helicase-loading proteins (2). All of these activities with the exception of the processivity factor are encoded by the phage. The economy of proteins in the phage T7 system is also manifest in the residence of the helicase and primase within a single polypeptide, the T7-encoded gene 4 protein (3, 4). There is a requirement for an association of DNA primase with its cognate helicase in all replication systems, but the two proteins are usually encoded by distinct and separate genes, thus necessitating an association at the replication fork. As shown in Fig. 1, the helicase and primase activities of the gene 4 protein each reside in separate domains located in the C-terminal and N-terminal halves of the 63-kDa gene 4 protein, respectively (5). The DNA sequence encoding each domain has been cloned and the resulting helicase and primase fragments have full helicase and primase activities, respectively (6–8). However, each domain, particularly the primase domain, is impaired in specific reactions when it is not coupled to the other domain. The crystal structures of both the helicase (9, 10) and primase (11) domains have been solved.

Like other DNA helicases of the DnaB family, the gene 4 protein functions as a hexamer (12). The hexameric gene 4 protein binds tightly to single-stranded DNA in the presence of dTTP and uses the energy of hydrolysis of dTTP to translocate unidirectionally 5′ to 3′ on the DNA strand to which it is bound (13, 14). Biochemical analysis of C-terminal helicase fragments of the gene 4 protein demonstrated that the region of the gene 4 protein linking the primase and helicase domains (residues 246–271) is critical for oligomerization (8). The primase and helicase domains of gene 4 protein share extensive homology with primases and helicases from other organisms (5). However, this small portion of residues 246 through 271 does not share amino acid sequence with other members of either the helicase or primase families, and thus was defined as the linker region. Helicase fragments lacking this linker region failed to oligomerize except at extremely high protein concentration and were severely impaired for all helicase activities (8). The crystal structure of the hexameric helicase fragment subsequently revealed that the subunit interface of the helicase ring is stabilized by interactions between an N-terminal region (residues 264–284 or helix A) of one subunit and a pocket on the adjacent subunit (residues 364–395 or helices D2 to D3) (10) (Fig. 1). Thus, a portion of the linker region identified by biochemical studies (residues 246–271) is found in this interaction. However, residues 241–260 in the structure were poorly ordered. In addition to these interactions at the interface, a series of loops near the nucleotide binding site also make contact between adjacent subunits with potential contacts of adjacent subunits with the bound nucleotide.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; pfu, plaque-forming unit.

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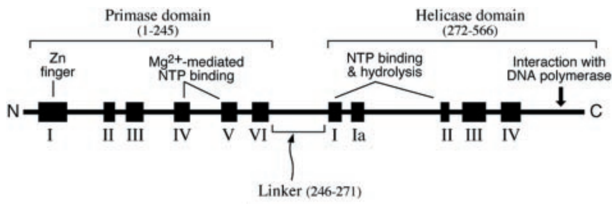

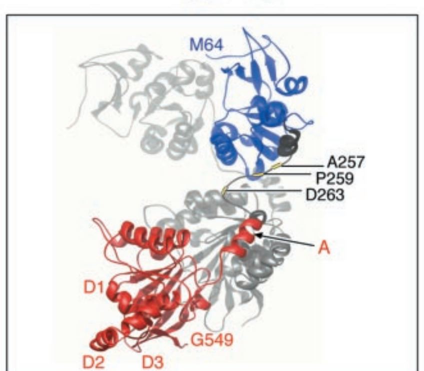

FIG. 1. **Organization of gene 4 protein of bacteriophage T7.** The linear schematic of gene 4 protein shows the helicase and primase domains located in the C- and N-terminal halves of the protein, respectively. The *boxes*, identified by roman numerals, depict conserved motifs found in the corresponding superfamily of helicases and primases. The helicase and primase domains are connected via a 26 amino acid linker region. The crystal structure (10, 11, 38) of two subunits of the hexameric 56-kDa gene 4 protein lacking the zinc finger is shown in the *lower half* of the figure. The helicase and primase domains of one subunit are presented in *red* and *blue*, respectively. Helices A, D1, D2, and D3 are involved in interactions with the adjacent subunit. The linker region (residues 246–271), defined by its lack of homology with proteins in helicase and primase superfamily, is shown in *black*. The three amino acids, Ala^{257}, Pro^{259}, and Asp^{263}, which are the subject of the present study are denoted and colored in *yellow*.

While it is clear from the biochemical and structural data that the linker region is essential for oligomerization of the gene 4 protein, the identity of the key residues critical for protein-protein interaction are unknown since previous studies have made use of truncated helicase domains. Furthermore, it is not known if the linker region via its contact with adjacent subunits assists in coordinating helicase and primase activities at the replication fork. One study (15) involving a random mutagenesis of the entire T7 gene 4 protein did find that substitutions of residues within this region such as A257T, A257V, and G258D give rise to proteins that cannot support T7 phage lacking gene 4. One of these altered proteins, a gene 4 protein with the substitution of threonine for alanine at position 257 (gp4-A257T), had reduced dTTPase activity and no DNA unwinding activity. In order to define more precisely the role of the linker, we have carried out an extensive study of three amino acid residues (Ala^{257}, Pro^{259}, and Asp^{263}) all located in the linker region.

Three libraries of gene 4 were generated such that each of the three amino acids was replaced with all other amino acids, and the resulting proteins were analyzed for their ability to support T7 phage lacking gene 4. We find that two of these residues, Ala^{257} and Asp^{263}, play a critical role in oligomerization and translocation of the gene 4 protein.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from Invitrogen. Plasmid DNA purification kits were from Qiagen. Restriction endonucleases, alkaline phosphatase, and Deep Vent® polymerase were purchased from New England Biolabs. T4 polynucleotide kinase, T4 DNA ligase, radiolabeled nucleotides, high molecular weight protein markers, and desalting column S-400HR were purchased from Amersham Biosciences. Agarose and β,γ-methylene dTTP were from USB Corp. Polyethyleneimine cellulose thin layer chromatography (TLC) plates were from J. T. Baker. T7 DNA polymerase (T7 gene 5 protein-*Escherichia coli* thioredoxin complex), M13mp18 ssDNA, and a primer used for strand displacement DNA synthesis assay were kindly provided by Donald Johnson (Harvard Medical School).

Random Mutagenesis of Selected Codons—Random mutagenesis of the codons specifying Ala^{257}, Pro^{259}, and Asp^{263} in gene 4 protein was carried out using primers containing mixed nucleotides at these positions. The codon site was flanked by 10–12 nucleotides of the wild-type sequence. For example, a set of primers for Ala^{257} was 5′-CAAGTGTGGAATNNNGGTCCTTGGATT-3′ and 5′-AATCCAAGGACCNNNATTCCACACTTG-3′ (N, any base). Using these mutagenic primers and outside primers in a two-step overlap PCR procedure (16), a pool of DNA containing mutated codons was generated. After digestion with BstBI and Bsu36I, the DNA (0.62 pmol) was ligated into plasmid pET24gp4-63 (0.049 pmol) previously cut with the same restriction enzymes. One-third of the ligation reaction mixture was transformed into 0.1 ml of *E. coli* strain DH5α (2.6×10^8 cells) by heat shock at 42 °C for 1 min followed by the addition of 0.9 ml of SOC medium. The cells were incubated at 37 °C for 1 h, and 0.1 ml of the culture was spread onto agar plates layered with either T7Δ4 phage or no phage. After overnight growth, bacterial colonies that emerged from plates infected with T7Δ4 phage (1×10^7 pfu) were picked and re-streaked on a new plate. Inability of the isolated colonies to support T7Δ4 phage was confirmed by infecting separate bacterial cultures with the phage. Plasmid DNA from the surviving bacteria was prepared and the integrity of the gene 4 coding region near the substitution site was examined by restriction enzyme analysis with SnaBI and AflII. Finally, the DNA sequence of the gene 4 coding from Gly^{179} through Ser^{312} of the protein was determined by the DNA sequencing facility at The Dana-Farber/Harvard Cancer Center.

Protein Overproduction and Purification—The entire gene 4 coding region of selected gene 4 proteins was confirmed by DNA sequence analysis. The plasmids were transformed into *E. coli* HMS 174(DE3), and gene 4 proteins were overproduced by isopropyl-1-thio-β-D-galactopyranoside induction. Genetically altered gene 4 proteins were purified following procedures described previously (16). The purified proteins were all greater than 95% pure as determined by SDS-PAGE analysis and staining with Coomassie Blue.

Biochemical Assays of Gene 4 Protein—Most of the assays used in this study have been described previously in detail (16). All assays (DNA unwinding, DNA binding, dTTP hydrolysis, oligomerization of gene 4 protein, primer synthesis, and DNA synthesis assays) used a reaction buffer containing 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM potassium glutamate plus additional components described in each assay. All reactions were carried out at 37 °C for the indicated time period.

DNA Unwinding Assay—The DNA substrate for DNA unwinding assay was prepared by annealing a 5′-^{32}P radiolabeled 45-mer (5′-ATAAC TCTAT GCACA TTGAC CATGC TTCAG ATTCG TATTG TTACT-3′) to a 65-mer (5′-TTTTT TTTTT TTTTT TTTTT ATTCG TAATC CGACC TCGAG GCATG GTCAA TGTGC ATAGA GTTAT-3′) in 50 mM NaCl. The DNA substrate (100 nM) was incubated with the indicated amounts of gene 4 protein for 5 min in the presence of 0.5 mM dTTP. After termination of the reaction by the addition of EDTA to a final concentration of 25 mM, the reaction mixture was loaded onto a 10% non-denaturing gel. Oligonucleotides separated from the partial duplex substrate by the helicase were measured using a Fuji BAS 1000 Bioimaging analyzer. In a control experiment in which 100 nM each of ssDNA 45- and 65-mer were incubated, we found that there is significant annealing (~60%) of the DNA strands. Therefore, it is likely that some reannealing of the ssDNA strands arising during the helicase reaction occurs thus leading to an underestimation of DNA unwinding. However, lowering the DNA concentration 10-fold to reduce the annealing does not alter the results.

DNA Binding Assay—DNA binding affinity of gene 4 protein was measured by nitrocellulose (NC) filter binding. The reaction (10 μl) containing 1.3 nM of 5′-^{32}P-radiolabeled DNA (5′-GGGTCA$_{10}$-3′) was incubated with various amounts of gene 4 protein in the presence of 1 mM non-hydrolyzable β,γ-methylene dTTP for 30 min. The reaction mixture was loaded onto two layers of filters, a NC membrane (0.45 μm, Bio-Rad) laid atop a Zeta-Probe® membrane (Bio-Rad) fixed on a Dot microfiltration apparatus (Bio-Rad). The protein-DNA complex bound to the NC membrane and free DNA on the Zeta-Probe® membrane was measured using a Fuji BAS 1000 Bioimaging analyzer.

DNA-dependent Hydrolysis of dTTP—ssDNA-dependent hydrolysis of dTTP by gene 4 protein was determined by incubating 0.25 mM

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[α-^{32}P]dTTP (0.1 μCi), 8 nM M13 ssDNA with the indicated concentration of the protein for 20 min. After termination of the reaction by the addition of EDTA to a final concentration of 25 mM, the reaction mixture was spotted onto a polyethyleneimine cellulose TLC plate. The TLC plate was developed with a solution containing 1 M formic acid and 0.8 M LiCl. The amount of [α-^{32}P]dTDP formed in the reaction was measured using a Fuji BAS 1000 Bioimaging analyzer.

Oligomerization of Gene 4 Proteins—The ability of gene 4 protein to oligomerize was determined by analyzing the oligomerized protein by electrophoresis on a non-denaturing polyacrylamide gel. The complete reaction (20 μl) contained 1 μM gene 4 protein, 1 mM β,γ-methylene dTTP, and 0.1 μM 45-mer oligonucleotide (5′-AGAGC GTCAC TCTTG TGACT ACCAG TGGTC GCAAA GTTCT TATCT-3′). After incubation for 20 min at 37 °C, the reaction mixture was loaded onto a 10% non-denaturing polyacrylamide gel and electrophoresed at 4 °C for 5 h. Gel running buffer (25 mM Tris-HCl, pH 7.0, 190 mM glycine) contained 10 mM $Mg(OAc)_2$ or did not. The protein was stained with Coomassie Blue in order to visualize the oligomerized protein.

Primase Assay—Template-directed oligoribonucleotide synthesis was determined by measuring the incorporation of [α-^{32}P]CMP into oligoribonucleotides using a synthetic DNA template containing a primase recognition site. The reaction (10 μl) included the indicated template, 0.1 mM each of ATP and [α-^{32}P]CTP (0.1 μCi), and the indicated amount of gene 4 protein. The DNA template was 100 μM 6-mer (5′-GGGTCA-3′) or 1 μM 65-mer used for DNA unwinding substrate. In the case of the 65-mer, the indicated amounts of dTTP or β,γ-methylene dTTP were added in the reaction. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 3 μl of sequencing dye and loaded onto a 25% denaturing polyacrylamide sequencing gel containing 3 M urea. Electrophoresis was carried out at 1800 V for 3 h, and the gel was dried. Radioactive oligoribonucleotide products were analyzed using a Fuji BAS 1000 Bioimaging analyzer.

Strand Displacement DNA Synthesis by T7 DNA Polymerase—A DNA template used to measure DNA strand displacement DNA synthesis was prepared by annealing M13 ssDNA in 10 mM NaCl to a 5′-^{32}P radiolabeled 66-mer primer (5′-T_{36}AATTC GTAAT CATGG TCATA GCTGT TTCCT-3′) to create a non-complementary T_{36} tail at the 5′-end of a 30-bp duplex region. Excess primers in the annealing mixture were removed using a desalting column S-400HR. The reaction containing 5 nM template DNA, 0.3 mM of all four dNTPs, and 10 nM T7 DNA polymerase was initiated for 2 min before the indicated amounts of gene 4 protein were added. After incubation for 30 min, the reaction was terminated by the addition of EDTA to a final concentration of 20 mM and loaded on a 1% agarose gel containing 0.5 mg/ml ethidium bromide. The gel was run in a buffer (45 mM Tris borate, pH 8.3, 1 mM EDTA) and dried for autoradiography.

RNA-primed DNA Synthesis by T7 DNA Polymerase—The ability of gene 4 protein to prime DNA synthesis catalyzed by T7 DNA polymerase on M13 ssDNA was measured by incubating 9.8 nM M13 ssDNA, 0.3 mM each of dTTP, dCTP, dATP, and [α-^{32}P]dGTP (0.1 μCi), 0.1 mM each of ATP and CTP, 100 nM gene 4 protein, and 20 nM T7 DNA polymerase. After incubation for 5 min, the reaction was terminated by the addition of EDTA to a final concentration of 20 mM and spotted onto a DE-81 membrane (Whatman). The amount of radioactively synthesized DNA was determined by measuring the radioactive products retained on the membrane after washing the membrane three times with 10 ml of 0.3 M ammonium formate (pH 8.0).

RESULTS

Screening of Permutable Amino Acid Changes at Specific Positions in the Linker Region

A short segment of 26 amino acids lacking homology to members of either the helicase or primase families has been termed the "linker region" (5). Biochemical data suggested that this linker region is critical for the functioning of the gene 4 protein (8). As anticipated from the biochemical data, crystal structures of the helicase domains revealed residues in the linker of one subunit of the hexamer contacting residues in the adjacent subunit, thus contributing to the stability of the hexamer (9, 10). Although the structure showed clearly the participation of the linker in subunit interaction, 9 of 26 residues were disordered in the structure. In order to examine the role of these 9 amino acids in helicase-primase function, we selected three for detailed analysis. Residues Pro^{259} and Asp^{263} were chosen since they are conserved in gene 4 protein of phage T3 where the primase is also fused to the helicase. Residue Ala^{257} was included in this study since alteration at this position had been shown previously to affect DNA-dependent dTTP hydrolysis and DNA unwinding (17).

TABLE I

Selection of gene 4 clones that do not support growth of T7Δ4

The codons for each of the selected amino acids (Ala^{257}, Pro^{259}, and Asp^{263}) were randomly mutated in a cloned gene 4 so as to give three libraries, each containing all possible amino acid replacements in the respective codons. After transformation of plasmids from the library into *E. coli* DH5α, bacterial cells were spread on plates containing the indicated number of T7Δ4. The numbers of colonies were determined by plating 1/10 of the transformation reactions.

	Position of random mutation		
	Ala^{257}	Pro^{259}	Asp^{263}
No phage	74	123	65
T7Δ4 (1.3 × 10^6 pfu)	43	10	46
T7Δ4 (1.3 × 10^7 pfu)	31 ± 3	5 ± 1	43 ± 2
T7Δ4 (1.3 × 10^8 pfu)	7	2	5

The codons for each of the three selected amino acid residues were mutated randomly in gene 4 and cloned into a plasmid so as to give rise to all possible amino acid substitutions at these three sites (see "Experimental Procedure"). In order to select for altered gene 4 proteins that were defective in primase and/or helicase activity, we first selected for gene 4 proteins that could not support the growth of T7Δ4 lacking gene 4. In this selection, *E. coli* cells transformed with the pools of plasmids harboring the mutated gene 4 are plated onto plates previously layered with T7Δ4 phage. If the appropriate phage concentration is present on the plate, *E. coli* cells expressing gene 4 proteins that are defective in supporting T7Δ4 phage will survive. Cells that have functional gene 4 protein expressed will allow T7 phage to propagate, resulting in lysis of the colony. Since too high a concentration of phage can kill all of the cells lacking a functional gene 4 protein, it was first necessary to select an appropriate number of T7Δ4 phage to layer on each plate. As shown in Table I, infection with 1.3 × 10^8 pfu/plate resulted in lysis of ~90% of cells regardless of the position mutated, suggesting that lysis of cells occurred even in the absence of a functional gene 4 protein. However, at 1.3 × 10^7 pfu/plate, a clear distinction in survival among the three libraries was apparent (42% for Ala^{257}, 4% for Pro^{257}, and 66% for Asp^{263}). Since further reduction (1.3 × 10^6 pfu/plate) of infecting phage did not significantly change the number of surviving cells, we chose 1.3 × 10^7 pfu/plate for the screening procedure.

Using the screen described above, individual colonies that survived phage infection were isolated. Lack of ability to support T7Δ4 phage was confirmed by infecting cell cultures derived from individual colonies with T7Δ4. Plasmid DNAs from surviving colonies were purified and subjected to restriction enzyme analysis with SnaBI and AflII and only those that contained the intact coding segment for gene 4 were retained. Initially 151, 20, and 136 colonies survived from plating on the libraries of gene 4 with altered amino acids at Ala^{257}, Pro^{259}, and Asp^{263}, respectively. After restriction enzyme analysis, 124 (Ala^{257}), 8 (Pro^{259}), and 105 (Asp^{263}) plasmid DNAs remained.

In order to identify the amino acid replacements that led to gene 4 proteins that could not support T7Δ4 phage growth, the sequence of each plasmid DNA selected in the screen was determined. The results of the DNA sequence analysis are presented in Table II where the amino acid substitutions are tabulated. The striking observation is that only 8 plasmids were identified in the Pro^{259} library, and none of these had single amino acid substitution at position 259; they consisted of stop codons, deletions, an insertion, or multiple amino acid

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TABLE II
Non-viable amino acid substitutions in linker region of gene 4 protein

Plasmids encoding genetically altered gene 4 proteins at residues Ala^{257}, Pro^{259}, and Asp^{263} that could not support T7Δ4 phage were analyzed by DNA sequence analysis. The DNA sequence encoding the gene 4 sequence from Gly^{179} to Ser^{312} was determined, and the identity of the amino acid replacement at positions 257, 259, and 263 was deduced from the codon for those residues. The amino acids identified are listed in decreasing order of their frequency.

Position of random mutation					
Ala^{257}		Pro^{259}		Asp^{263}	
Replacement	Occurrence	Replacement	Occurrence	Replacement	Occurrence
Arg	24	Stop	2	Ala	25
Val	21	Deletion	2	Arg	18
Leu	19	Insertion	1	Pro	16
Pro	18			Cys	8
Glu	11	Tyr	0	Thr	6
Asp	4	Trp	0	Ser	6
Gln	4	Val	0	Val	5
Trp	4	Thr	0	His	4
Stop	4	Ser	0	Leu	4
Deletion	4	Arg	0	Met	3
Ile	3	Gln	0	Gln	3
Thr	3	Asn	0	Phe	2
His	2	Met	0	Deletion	1
Tyr	1	Leu	0	Lys	1
		Ile	0	Asn	1
Cys	0	His	0		
Phe	0	Gly	0	Glu	0
Gly	0	Phe	0	Stop	0
Lys	0	Glu	0	Ile	0
Met	0	Asp	0	Trp	0
Asn	0	Cys	0	Gly	0
Ser	0	Ala	0	Tyr	0
Multiple mutations	2	Multiple mutations	3	Multiple mutations	2
Total	124	Total	8	Total	105

replacements (Table II). In order to assure that amino acid substitutions were occurring at position 259, we isolated several colonies prior to phage infection and sequenced the DNA to find that Val, Gln, His, Cys, and Gly had replaced Pro. None of these substitutions change the ability to support T7Δ4 (data not shown). We conclude that Pro^{259} is not a critical amino acid for gene 4 function, because it can be replaced with any amino acid. Although it is difficult to make generalizations regarding the substitutions for Ala^{257} and Asp^{263}, it does appear that aliphatic residues such as valine, leucine, and isoleucine cannot replace the alanine at position 257, suggesting that the size of the residue at this position is important. Arginine, leucine, valine, and proline were found at a relatively high frequency at position 257. This finding is not unexpected since these amino acids have multiple codons (6, 6, 4, and 4, respectively). Interestingly, serine and glycine, having 6 and 4 codons, respectively, were not substituted at position 257. Since the side chains of these residues are relatively small, it appears likely that the size of the residue at position 257 is critical. Similarly, amino acids coded by multiple codons (alanine, arginine, and proline) were abundant at position 263. In this position, it is noteworthy that asparagine, lacking a negative charge, cannot substitute for aspartic acid. Some amino acid substitutions were not observed presumably because the numbers in the screen are not sufficiently large to be statistically significant.

Overproduction and Purification of Altered Gene 4 Proteins

From the data in Table II, we identified the least drastic amino acid substitutions at positions 257 and 263. We chose three gene 4 proteins with the substitutions of leucine, proline, and valine for Ala^{257} and three with the substitutions of alanine, asparagine, and serine for Asp^{263}. Prior to purification, the ability of these altered gene 4 proteins were examined for their ability to support the growth of T7Δ4 phage and for their effect on the growth of wild-type T7 phage (Table III). None of the altered proteins with single amino acid changes at positions 257 and 263 could support the growth of T7Δ4 phage, and none of the proteins demonstrated a dominant negative effect on the growth of wild-type T7 phage.

TABLE III
Ability of recombinant gene 4 protein to complement growth of T7 phage

Gene 4 proteins containing the indicated amino acid substitution were expressed in *E. coli* DH5α. After infection with either T7Δ4 or wild-type T7 phage, the number of plaques were counted and normalized to the value obtained with the wild-type gene 4 protein.

Alteration in gene 4 protein	T7 phage	
	Δ4	WT[a]
WT	1.0[b]	1.0
A257L	1.5×10^{-8}	0.39
A257P	1.5×10^{-8}	0.45
A257V	5.0×10^{-8}	0.42
D263A	$< 10^{-9}$	0.49
D263N	$< 10^{-9}$	0.64
D263S	5.4×10^{-8}	0.51

[a] Wild-type.
[b] Relative efficiency of plating.

Each of the six altered gene 4 proteins as well as wild-type gene 4 protein were purified by a standard protocol used for purification of wild-type gene 4 protein described previously (16). All of the altered proteins had similar chromatographic properties to the wild-type gene 4 protein. However, all of the altered proteins were more abundant in extracts and the overall yield after purification was 2–5-fold greater than for wild-type gene 4 protein. Similar increases in overexpression have been observed with altered gene 4 proteins defective in dTTPase and helicase activity (8, 17).

Biochemical Assays

DNA Unwinding—In order to analyze defects in the function of the altered gene 4 proteins, we first examined the ability of the proteins to unwind DNA. Upon encountering duplex DNA

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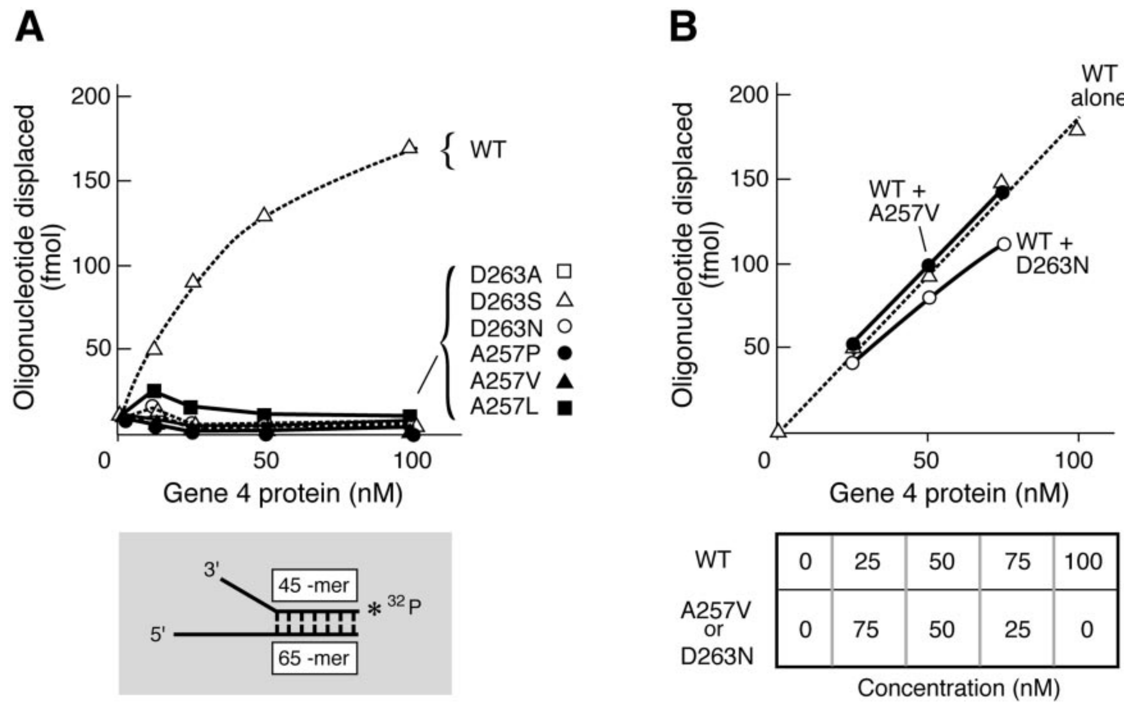

WT	0	25	50	75	100
A257V or D263N	0	75	50	25	0

FIG. 2. **DNA unwinding activity.** DNA unwinding activities of the gene 4 proteins were measured by monitoring the amount of ssDNA displaced from the duplex DNA substrate depicted in the *inset*. The DNA substrate for measuring helicase activity consists of a 45-mer oligonucleotide annealed to a 65-mer oligonucleotide; 20 nucleotides at the 3′-end of the 45-mer are not complementary to the 65-mer. The location of the ^{32}P label on the 45-mer is indicated. *A*, the DNA substrate (100 nM) was incubated with 0.5 mM dTTP, 10 mM Mg^{2+}, and the indicated amounts of each gene 4 protein. After 5 min of incubation at 37 °C, the amount of 5′-^{32}P radiolabeled 45-mer displaced from the 65-mer was determined by non-denaturing PAGE and phosphorimage analysis. *B*, the DNA unwinding activity of wild-type gene 4 protein alone and in mixture with the indicated amounts of either gp4-A257V or gp4-D263N was determined in an assay identical to that described for *panel A* above. The data presented here are from a single experiment although multiple assays gave results consistent with the above.

during translocation on ssDNA, the gene 4 protein will unwind the DNA provided the duplex has a 3′-displaced tail of at least 7 nucleotides (18, 19). The unwinding reaction is dependent on multiple properties of the gene 4 protein in that the protein must form a functional hexamer, bind ssDNA, and efficiently hydrolyze dTTP in order to translocate on ssDNA. In the experiment shown in Fig. 2, we have used a partially duplex DNA with a 3′-ssDNA tail (see *inset*) to measure helicase activity of the wild-type and the altered gene 4 proteins. In this assay, one of the two strands is radioactively labeled such that its displacement from the complementary strand can be measured after electrophoresis of the products on non-denaturing gels. The substrate concentration (100 nM) was in excess of the hexameric enzyme concentrations (2, 4, 8, and 17 nM). Whereas wild-type gene 4 protein mediates the unwinding of the duplex DNA substrate in a reaction proportional to the amount of gene 4 protein, none of the altered gene 4 proteins have significant helicase activity (Fig. 2*A*). This result is particularly noteworthy in view of the finding, shown below, that all of the altered proteins exhibit only a 2–3-fold decrease in ssDNA-dependent dTTPase activity (See Fig. 3*B*).

The addition of either gp4-A257V or gp4-D263N to wild-type gene 4 protein has no effect on the activity of the wild-type gene 4 protein (Fig. 2*B*). Even when the altered gene 4 proteins are present in a 3-fold excess over wild-type gene 4 protein, the activity of the wild-type gene 4 protein is unaffected relative to the activity of the wild-type gene 4 protein alone. Similar results were obtained when the wild-type gene 4 protein and altered gene 4 proteins were preincubated at 37 °C for 5 min prior to assay. Although the preincubation reduced the activity of the wild-type gene 4 protein alone, presumably due to dissociation of functional hexamer into monomer, the presence of either gp4-A257V or gp4-D263N had no effect (data not shown). This result is in agreement with the *in vivo* studies described earlier in this report where the altered gene 4 proteins did not have a dominant negative effect on the growth of T7 phage expressing wild-type gene 4 (Table III).

ssDNA Binding Property—Binding of gene 4 protein to ssDNA is dependent on the ability of the protein to bind nucleotide (14, 20) and to form oligomers (21). We have compared binding of the gene 4 proteins containing a substitution at position 257 or 263 to ssDNA with the wild-type gene 4 protein using a filter binding assay (Fig. 3*A*). We have used β,γ-methylene dTTP since this non-hydrolyzable nucleotide provides for efficient binding to ssDNA in this assay (22). Results from three independent binding assays show that the substitution of leucine, proline, and valine for alanine at position 257 slightly decreases the affinity for ssDNA whereas the substitution of alanine, asparagine, and serine for aspartic acid at position 263 slightly increases the affinity (Fig. 3*A*). However, the maximal deviation from the binding observed with wild-type gene 4 protein was only 2-fold, occurring with gp4-A257L.

dTTP Hydrolysis—The 5′ to 3′ unidirectional translocation of gene 4 protein on ssDNA is coupled to the hydrolysis of dTTP to dTDP and P_i (14). Consequently, the hydrolysis of dTTP by gene 4 protein provides an indirect measurement of its ability to translocate on ssDNA and to unwind duplex DNA. Substitutions of either residue 257 or 263 significantly reduce the rate of ssDNA-dependent dTTP hydrolysis (Fig. 3*B*). The gene 4 proteins altered at position 257 display the greater reduction in activity, perhaps reflective of their decreased binding to ssDNA. Although the alterations at position 263 increase affinity for ssDNA (Fig. 3*A*), they do not increase dTTP hydrolysis; all the substitutions reduce activity below that of wild-type gene 4 protein.

Oligomerization of Gene 4 Protein—The functional state of the gene 4 protein is a hexamer, and the linker region has been shown previously to be critical for oligomerization of the pro-

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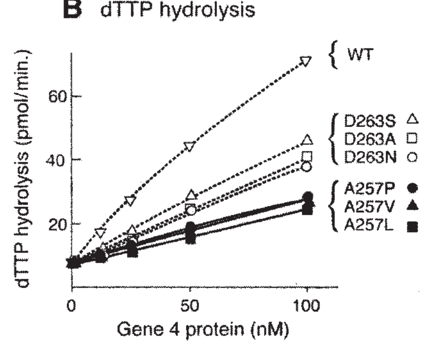

FIG. 3. **ssDNA binding affinity and dTTP hydrolysis activity.** Wild-type gene 4 protein and each of the gene 4 proteins with amino acid substitutions at position 257 (A257L, A257P, and A257V) or 263 (D263A, D263N, and D263S) were assayed for their ability to bind to ssDNA (*A*) and for their ability to hydrolyze dTTP in the presence of ssDNA (*B*). *A*, increasing concentrations of each of the gene 4 proteins were incubated with a constant amount of 5′-^{32}P-radiolabeled 15-mer DNA in the presence of 1 mM β,γ-methylene dTTP, and the protein-DNA complex was measured using a filter binding assay. An average of three independent experiments is presented. *B*, increasing amounts of each of the proteins used in *panel A* were assayed for their ability to hydrolyze [α-^{32}P]dTTP in the presence of 8 nM M13 ssDNA. After incubation for 20 min at 37 °C, the formation of [α-^{32}P]dTDP was measured using thin layer chromatography as described under "Experimental Procedures." An average of two independent experiments is presented. The results from the two or three experiments are within 30% of the values shown in the figure. Because error bars did not change the relative positions of the curves, those are not included in the figures, for clarity.

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tein (8). In the presence of β,γ-methylene dTTP and Mg^{2+}, the majority of the wild-type gene 4 protein is present as oligomers as measured by electrophoresis on native polyacrylamide (Fig. 4*A*). Contrary to our expectations, all altered proteins oligomerized to the same extent as did the wild-type protein (Fig. 4*A*).

In an attempt to detect differences in the ability of the altered proteins to oligomerize, we examined their size distribution in the absence of Mg^{2+}, which is known to stabilize oligomers (23). As shown in Fig. 4*B*, in the absence of Mg^{2+}, the majority of the wild-type gene 4 protein remains monomeric with the minority migrating as oligomers. Addition of dTTP or β,γ-methylene dTTP all enhanced oligomerization in the absence of the divalent cation but the presence of both ssDNA and β,γ-methylene dTTP is required for maximal oligomerization. Under these conditions a clear difference is observed with the altered gene 4 proteins.

In the case of gp4-A257V, the protein forms oligomers to a far greater extent than does the wild-type gene 4 protein under all these conditions (Fig. 4*B*). In the presence of dTTP or β,γ-methylene dTTP, the majority of the gp4-A257V oligomerizes. Equally significant, a high percentage of oligomers that migrate more slowly than hexamers were detected. Essentially identical results were obtained with gp4-A257L but gp4-A257P behaved more like the wild-type (data not shown). When either gp4-A257V or gp4-A257L were mixed with the wild-type protein in equal amounts, the higher molecular weight species observed with gp4-A257V or gp4-A257L alone disappeared (Fig. 4*C*). This result suggests that hetero-oligomers are formed and that the presence of a wild-type linker region can dissipate the extensive oligomerization. These results are interesting in view of the earlier finding that a T7 gene 4 helicase domain having a deletion in this portion of the linker region formed extended oligomers in the crystal structure rather than hexamers (9) whereas the helicase domains containing this portion of the linker region crystallized as hexamers (10).

Gp4-D263N exhibits properties quite distinct from the gene 4 proteins with alterations at position 257. Although gp4-D263N formed oligomers to the same extent as the wild-type gene 4 protein in the presence of Mg^{2+}, in the absence of this divalent cation it oligomerizes to a lesser extent than does the wild-type gene 4 protein except in the presence of both ssDNA and β,γ-methylene dTTP (Fig. 4*B*). Essentially identical results were obtained when D263 was replaced with alanine or serine (data not shown).

Primase Activity—In addition to its helicase activity, T7 gene 4 protein also catalyzes the synthesis of di-, tri-, and tetraribonucleotides at specific recognition sequences on ssDNA (24). The tetraribonucleotides can be used as primers by T7 DNA polymerase. The primase domain of gene 4 protein, located in the N-terminal half of the polypeptide chain catalyzes the template-directed synthesis of oligoribonucleotides. Template-directed oligoribonucleotide synthesis catalyzed by the altered proteins is relatively normal as shown in Fig. 5*A*. In this assay, a 6-nucleotide template containing a primase recognition sequence 5′-GGGTC-3′ was incubated with [α-^{32}P]CTP, ATP, and the gene 4 proteins. Oligoribonucleotide synthesis at this recognition site yielded radioactively labeled pppAC, pppACC, and pppACCC, which can be detected on denaturing polyacrylamide gels. Quantitation of the major product, pppACCC, indicates that only gp4-A257L and gp4-A257P show reduced activity in this assay relative to wild-type gene 4 protein although activity is reduced only 2-fold.

Primase activity on longer DNA templates is dependent on the translocation of the protein to the recognition site after a random binding event (13). The translocation mediated by the hexameric helicase domain requires hydrolysis of dTTP (14). As shown in Fig. 5*B*, the wild-type gene 4 protein requires the presence of dTTP to synthesize primers effectively on a 65-mer oligonucleotide, 5′-$(N)_{43}$TGGTC$(N)_{17}$-3′. In this assay where translocation is important, the primase activities of gp4-A257V and gp4-D263N are not stimulated by dTTP. This result is important because it suggests not only that the helicase activity of the altered gene 4 proteins is defective but that the ability of the proteins to translocate on ssDNA is equally impaired.

Interactions of Gene 4 Protein with T7 DNA Polymerase—T7 gene 4 protein has two major interactions with T7 DNA polymerase at the replication fork. T7 DNA polymerase alone is not able to catalyze strand displacement synthesis of duplex DNA until the gene 4 protein provides the helicase activity for unwinding the duplex DNA ahead of the polymerase (25). During this reaction, the gene 4 protein maintains an association with the polymerase, an interaction that involves the acidic C terminus of the gene 4 protein (26). We have examined the ability of the altered gene 4 proteins to enable T7 DNA polymerase to mediate DNA synthesis on a duplex DNA with a preformed fork. In the absence or presence of gene 4 protein, T7 DNA polymerase catalyzed the synthesis of full-length M13 dsDNA. However, only wild-type gene 4 protein enabled T7 DNA polymerase to catalyze strand displacement synthesis (Fig. 6*A*). This result is not surprising, in view of the absence of

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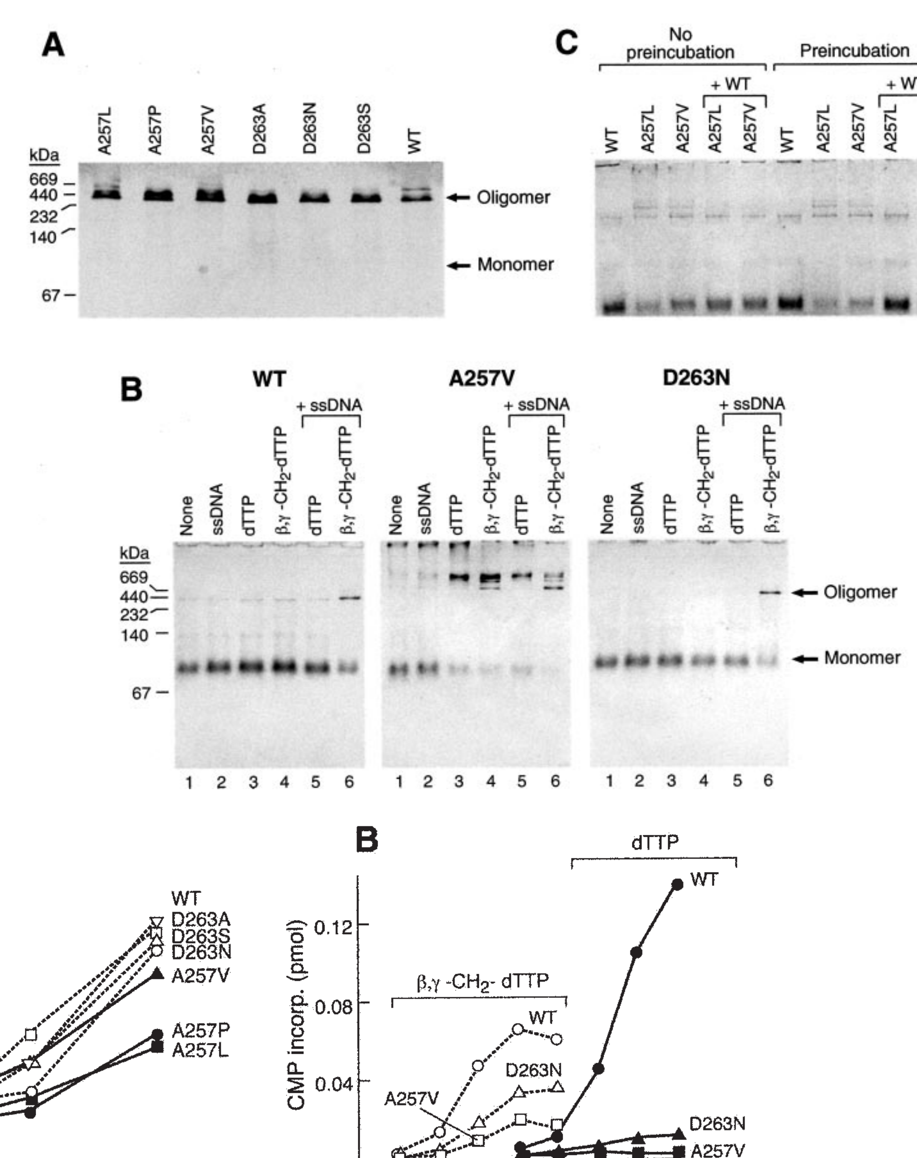

FIG. 4. **Oligomerization.** Gene 4 proteins were incubated with the indicated components for 20 min at 37 °C, and the resulting oligomeric state of the proteins were analyzed by electrophoresis at 4 °C through non-denaturing 10% polyacrylamide gels. *A*, migration of proteins in the presence of Mg^{2+}. After incubation with both β,γ-methylene dTTP and 45-mer DNA, the protein mixture was run in electrophoresis running buffer containing 10 mM Mg^{2+}. *B*, migration of proteins in the absence of Mg^{2+}. After incubation with indicated components, protein mixture was run in electrophoresis running buffer without 10 mM Mg^{2+}. The components incubated with the gene 4 proteins are: *lane 1*, none; *lane 2*, 0.1 μM 45-mer DNA; *lane 3*, 1 mM dTTP, *lane 4*, 1 mM β,γ-methylene dTTP; *lane 5*, 0.1 μM 45-mer DNA and 1 mM dTTP; *lane 6*, 0.1 μM 45-mer DNA and 1 mM β,γ-methylene dTTP. *C*, the indicated gene 4 proteins were either preincubated at 37 °C for 5 min (*right set*) or not incubated (*left set*) prior to the addition of 1 mM dTTP. The proteins were present at a concentration of 1 μM, and protein mixtures consist of 0.5 μM each. After incubation, the proteins were analyzed as described in *panel B*.

FIG. 5. **Oligoribonucleotide synthesis.** *A*, template-directed oligoribonucleotide synthesis on a 6-nucleotide template. A 6-mer (5′-GGGTCA-3′) containing a primase recognition sequence 5′-GGGTC-3′ was incubated with the indicated concentrations of the wild-type gene 4 protein or the altered gene 4 proteins in the presence of CTP and ATP. After incubation for 20 min at 37 °C, the major product of the reaction, pppACCC were detected on 25% denaturing polyacrylamide gels. The amount of synthesis is expressed as the amount of CMP incorporated. *B*, template-directed oligoribonucleotide synthesis on a 65-nucleotide template. On relatively long templates primase activity is dependent on the translocation of the protein to the recognition site, a reaction mediated by the helicase domain of gene 4 protein. A 65-mer (5′-$(N)_{43}$TGGTC$(N)_{17}$-3′) containing a primase recognition site 5′-TGGTC-3′ was incubated with CTP, ATP, and the indicated concentrations of dTTP or its non-hydrolyzable analog β,γ-methylene dTTP. The reactions contained 20 nM wild-type gene 4 protein, gp4-A257V, or gp4-D263N. After incubation for 20 min at 37 °C, the major product of the reaction, pppACCC were detected on 25% denaturing polyacrylamide gels. The amount of synthesis is expressed as the amount of CMP incorporated. The data presented here are from a single experiment although multiple assays gave results consistent with the above.

unwinding activity observed with the altered gene 4 proteins alone (Fig. 2*A*).

The second interaction of gene 4 protein with T7 DNA polymerase occurs during the delivery of oligoribonucleotides synthesized from ATP and CTP precursors by the primase domain of the gene 4 protein to the polymerase (27). In the absence of gene 4 protein, T7 DNA polymerase is unable to initiate DNA synthesis on M13 ssDNA (Fig. 6*B*). In the presence of wild-type gene 4 protein, there is extensive DNA synthesis. In a control experiment, the 56-kDa gene 4 protein, lacking the Cys_4 zinc finger, is unable to synthesize primers and therefore unable to stimulate T7 DNA polymerase on the unprimed M13 ssDNA. Although a slight but significant stimulation of DNA synthesis by the polymerase complex was observed with altered gene 4 proteins, the stimulation is at least 5-fold less than that observed with wild-type gene 4 protein. This reduced stimulation is in agreement with our finding that the primase is unable to access primase recognition sites on large DNA molecules due to the defective translocation activity of the altered gene 4 proteins.

DISCUSSION

One of the critical tasks during DNA replication is the precise coordination of leading and lagging strand synthesis. In the bacteriophage T7 replication system, only four proteins are required for a functional replisome that meets all the criteria for mediating coordinated DNA replication (2, 28). Even with this economy of proteins, it has been difficult to dissect the

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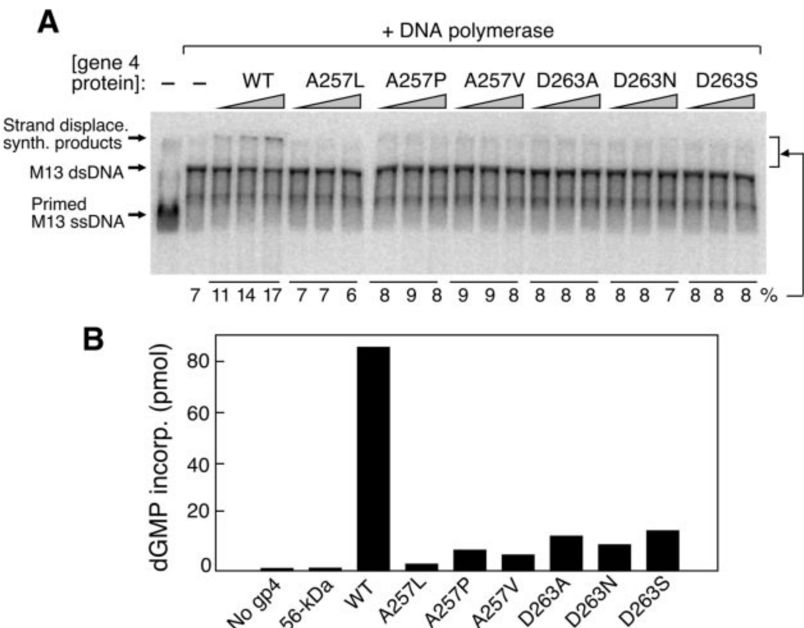

FIG. 6. **Interaction of gene 4 protein with T7 DNA polymerase.** *A*, strand displacement DNA synthesis. On primed M13 ssDNA, T7 DNA polymerase is able to catalyze strand displacement DNA synthesis in the presence of gene 4 protein (25). The reaction contained M13 ssDNA template annealed to the 5′-^{32}P-labeled 66-mer primer, dATP, dGTP, dTTP, and dCTP. The reaction was initiated by the addition of T7 DNA polymerase. After incubation for 2 min at 37 °C, increasing amounts of gene 4 protein (8, 17, and 33 nM) were added, and the reaction was incubated for 30 min. Reactions were terminated by the addition of EDTA, and the radioactive products of DNA synthesis were detected by electrophoresis on 1% agarose gel. The product corresponding to full-length M13 DNA and the product of strand displacement synthesis are indicated. The amount of radioactivity having a mobility less than that of M13 dsDNA is shown as a percentage of the total radioactivity *below* each lane of the gel. *B*, RNA-primed DNA synthesis, T7 DNA polymerase requires the synthesis of oligoribonucleotides by gene 4 protein in order to initiate DNA synthesis on M13 ssDNA. The reaction contained ATP, CTP, dTTP, dCTP, dATP, [α-^{32}P]dGTP, M13 ssDNA, T7 DNA polymerase, and 100 nM of the indicated gene 4 proteins. The 56-kDa gene 4 protein, lacking the Cys_4 zinc finger, was used as a negative control because it is unable to synthesize primers and therefore unable to prime DNA synthesis by T7 DNA polymerase. After incubation for 5 min at 37 °C, the products of the reaction were spotted on DE-81 membrane. The amount of DNA synthesis was determined by measuring the amount of radioactive dGMP incorporated into DNA as described under "Experimental Procedures."

multiple protein-protein interactions and their modulation by DNA contacts. Among the protein-protein interactions, two are unequivocal. The processivity factor, *E. coli* thioredoxin, binds to a unique segment in the thumb sub-domain of T7 gene 5 protein with a K_{obs} of 5 nM such that dissociation is rarely observed under physiological conditions (29). Even more striking is the association of the T7 helicase with the T7 DNA primase in that the two activities reside within two distinct domains of a single polypeptide, the gene 4 protein (3, 4). In other replication systems, there is also a clear requirement for helicase/primase interactions but the association involves proteins encoded by separate genes (30–32).

Two apparent advantages of the association of primase and helicase derive from the translocation and DNA unwinding activities of the helicase. Translocation provides a mechanism by which the primase can be transported to primase recognition sites on DNA and the unwinding of the duplex DNA generates the ssDNA required by the primase to access its recognition site. These two advantages are intimately related in the case of the T7 gene 4 helicase-primase since the covalent association assures both translocation of the primase and the immediate access to the ssDNA template generated behind the translocating helicase at the replication fork. Aside from the replication fork, primase activity on ssDNA is stimulated greatly by the helicase activity in that the hexameric helicase in the presence of NTP binds tightly to ssDNA whereas the primase has a low affinity for ssDNA including its recognition site (33). Not surprisingly, alterations in the helicase domain of gene 4 protein that affect translocation or helicase activity have drastic effects on primase activity (17, 34). It is less clear if the helicase domain of gene 4 protein derives benefits from its fusion to the primase. However, it has been proposed that gene 4 protein might load onto ssDNA via its primase domain (35).

The fusion of helicase to primase is not unique to bacteriophage T7. A BLAST (36) search for proteins that share high homology with the T7 gene 4 protein (E-value $<10^{-6}$) identifies 37 proteins, predominately replicative helicases. Among these, proteins from 10 bacteriophages and 2 bacteria have a covalent attachment of primase to helicase in which all the conserved motifs of primase and helicase are conserved. Inspection of the linker regions of these proteins reveals a resemblance to the linker region found in T7 gene 4 protein. In particular, at the positions corresponding to Asn^{256}, Ala^{257}, and Pro^{262}-Asp^{263}-Gly^{264} in the T7 gene 4 protein, 8–10 proteins out of a total of 12 have residues identical to the T7 protein. The stringency for Ala^{257} and Asp^{263} is clearly relevant to the present study. Other helicases, such as Twinkle (37), POM, and a number of mitochondrial and bacterial helicases that show homology to the T7 gene 4 protein, do contain an N-terminal extension of the polypeptide. Although some of the extensions of these proteins have a TOPRIM sequence found in DNA primases, there is no homology to any portion of the linker found in the fused primase-helicases.

An obvious and important role of the linker region is to tether the primase to the helicase in order to achieve the advantages of the primase-helicase association discussed above. On the other hand, the linker region appears to play additional roles in maintaining the structure and function of the gene 4 protein. Characterization of a series of truncated gene 4 proteins generated by proteolytic digestion suggested that a wide range of N-terminal of the helicase, which includes the entire linker (residues 246–271), is necessary for both DNA unwinding and dTTP hydrolysis activity (6). Requirement of the linker region for helicase activity was more clearly demon-

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strated by comparison of C-terminal fragments of gene 4 protein, one of which does not contain the linker region (8). Deletion of the linker decreases oligomerization, which leads formation of functional hexamer. Unless hexamers are assembled, the protein cannot bind to ssDNA and consequently does not display ssDNA-dependent hydrolysis of dTTP or translocation on ssDNA.

Both translocation of the gene 4 protein and unwinding of duplex DNA are dependent on the hydrolysis of dTTP (13, 18). In the crystal structures of the gene 4 protein (9, 10), dTTP is bound at the interface of adjacent subunits of the hexamer, contributing to the stability of the hexamer via contacts with each of the two subunits. These contacts and the asymmetry of the hexamer in one crystal structure (9) led to a model by which the hydrolysis of dTTP is coupled to conformational changes in the hexamer and, in turn, movement along the DNA. The key role of the linker region in oligomerization, its proximity to the nucleotide binding site, and its likely involvement in conformational changes arising from nucleotide hydrolysis suggest that it plays a critical role in movement of the protein as well as in its assembly. A recently solved crystal structure of the gene 4 protein positioned the linker region within a heptamer, in which the portion of the linker examined in this study poses a flexible swivel conformation so that it allows movements of the primase toward the helicase domain (38). This observation suggests that not only subunit packing within the hexameric structure but also transition of conformations are modulated by the linker region.

With these considerations, it is not surprising that the single amino acid changes, identified in this and an earlier study (17), which lie within the linker region, severely affect helicase activity. The most striking finding is that amino acid substitutions at position 257 or 263 lead to altered gene 4 proteins that cannot support the growth of T7 phage lacking gene 4. Studies with the purified altered gene 4 proteins reveal that substitutions at either position 257 or 263 render the proteins unable to unwind duplex DNA either alone or in conjunction with DNA synthesis catalyzed by T7 DNA polymerase, the consequence of which is to halt all DNA synthesis on duplex DNA. The defect in unwinding duplex DNA appears to arise from an inability of the altered proteins to translocate unidirectionally along ssDNA. The inability of the altered proteins to translocate along ssDNA is most clearly manifest in the inability of its functional primase domain to access primase recognition sites on large DNA molecules. On long ssDNA with a few primase recognition sites, the primase domain of the gene 4 protein is dependent on translocation mediated by the helicase domain to efficiently reach those recognition sites. The concomitant loss of unwinding activity and translocation activity is not surprising in that it is likely that translocation itself is the major driving force for unwinding of the duplex (12).

The potential defects responsible for loss of translocation include the inability to oligomerize or hydrolyze dTTP. However, neither of these parameters appears to explain the loss of translocation and DNA unwinding activities. Amino acid substitutions at either position 257 or 263 result in a decrease in dTTP hydrolysis but only 2–3-fold. All the proteins form hexamers and higher molecular weight species in the presence of Mg^{2+}, similar to the wild-type protein. However, oligomerization of gene 4 proteins altered at either position 257 or 263 is affected under conditions where even the wild-type gene 4 protein does not readily oligomerize (in the absence of Mg^{2+}). The more dramatic effect is seen with substitutions at position 257. For example, Gp4-A275V oligomerizes to an even greater extent than does the wild-type gene 4 protein with a relatively large amount of the protein having apparent molecular weights greater than that of a hexamer. Substitutions at position 263, on the other hand, appear to decrease oligomer formation. With substitutions at either of these two critical positions, however, the ability to bind to ssDNA remains little changed.

Whereas the alterations within the linker region do affect oligomerization and to a limited extent dTTP hydrolysis, it seems likely that the basic defect in these altered proteins involves the coupling of dTTP to the conformational changes necessary for translocation on ssDNA. Although the mechanism by which these two reactions are coupled is largely unknown, the crystal structure of the gene 4 protein with bound nucleotide suggested a model for how cooperative dTTP binding and hydrolysis are controlled around the hexameric protein, and in turn coupled to conformational changes in the protein (10). In one scenario, the coordination between nucleotide binding, hydrolysis, and conformational changes would be uncoupled in the case of the altered gene 4 proteins examined in this study. The consequence of this uncoupling would be the retention of ssDNA binding and dTTP hydrolysis without the proper conformational change in the protein required for movement along the DNA strand. Interestingly, the presence of one or more subunits of the altered gene 4 proteins within a hetero-oligomer containing wild-type gene 4 subunits did not affect unwinding activity, suggesting that the linker region is sufficiently flexible enough to confine the uncoupling within the altered protein subunit without disrupting the wild-type subunit. The failure to assume the alternative conformation that gives rise to the deviation from the 6-fold symmetry of the hexamer observed in the crystal structure (10) could be the consequence of either no severe conformational change or the formation of an alternative, non-functional conformation.

A number of earlier studies provide indirect evidence that the linker region is flexible and hence a candidate for playing a critical role in the switching of the hexamer from one conformation to another during translocation on DNA. The linker region has the potential for flexibility in that it is exposed to the solvent and susceptible to digestion with proteases (6, 17, 38). Attempts to crystallize a truncated polypeptide containing the primase domain and the linker region (residues 1–271) have failed whereas a smaller primase fragment (residues 1–255) lacking a portion of the linker region was successfully crystallized (11). The crystal structure of this latter primase fragment reveals the C-terminal region of the polypeptide to be packed tightly within the catalytic core (11). The inability to crystallize the primase fragment with the linker region may arise from the flexible linker region hindering the packing of the C-terminal region into a stable crystal array. A crystal structure of the helicase domain containing the linker region (residues 241–566) provides more direct evidence for the flexibility of the linker region in that a portion of the linker region (residues 241–260) is disordered (10). The contribution of the linker region to the stability of the hexamer is unclear. The major interaction between subunits appears to be helix A (residues 272–281) adjacent to the linker region, which interacts with a loop formed by helix D1-D3 (residues 345–388) of the neighboring subunit (9). Disruption of this interaction by substituting helix D2D3 for a short polar peptide destabilizes the hexamer, resulting in loss of DNA unwinding activity (27). These points, taken together, make it likely that the linker domain modulates the conformation of the hexamer and perhaps also serves to coordinate helicase activity with primase activity. At present, there is no evidence to support a role of the linker region in affecting primase activity. However, a model in which the linker region can control primase activity by affecting the conformation of the catalytic domain to which it is closely associated, is attractive.

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