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The C-terminal Residues of Bacteriophage T7 Gene 4 Helicase-Primase Coordinate Helicase and DNA Polymerase Activities*

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The gene 4 protein of bacteriophage T7 plays a central role in DNA replication by providing both helicase and primase activities. The C-terminal helicase domain is not only responsible for DNA-dependent dTTP hydrolysis, translocation, and DNA unwinding, but it also interacts with T7 DNA polymerase to coordinate helicase and polymerase activities. The C-terminal 17 residues of gene 4 protein are critical for its interaction with the T7 DNA polymerase/thioredoxin complex. This C terminus is highly acidic; replacement of these residues with uncharged residues leads to a loss of interaction with T7 DNA polymerase/thioredoxin and an increase in oligomerization of the gene 4 protein. Such an alteration on the C terminus results in a reduced efficiency in strand displacement DNA synthesis catalyzed by gene 4 protein and T7 DNA polymerase/thioredoxin. Replacement of the C-terminal amino acid, phenylalanine, with non-aromatic residues also leads to a loss of interaction of gene 4 protein with T7 DNA polymerase/thioredoxin. However, neither of these modifications of the C terminus affects helicase and primase activities. A chimeric gene 4 protein containing the acidic C terminus of the T7 gene 2.5 single-stranded DNA-binding protein is more active in strand displacement synthesis. Gene 4 hexamers containing even one subunit of a defective C terminus are defective in their interaction with T7 DNA polymerase.

The replisome of bacteriophage T7 consists of only four proteins: three phage-encoded proteins (gene 2.5 ssDNA[2]-binding protein, gene 4 helicase-primase, and gene 5 DNA polymerase) and one host protein *Escherichia coli* thioredoxin (trx) as a processivity factor. Nonetheless, this simple replisome mediates a coordinated synthesis of leading and lagging strands using a double-strand template (1). The limited number of components suggests that T7 has evolved a replisome in which individual protein functions in several capacities via multiple interactions with the other components of the replisome. Such an example is the coexistence of both helicase and primase activities in a single polypeptide, the T7 gene 4 protein. This tandem organization of two essential enzyme domains assures that the primase can immediately access ssDNA template generated as the tethered helicase domain unwinds dsDNA (2). *E. coli* thioredoxin binds to a unique loop in T7 gene 5 DNA polymerase with a strong binding affinity of a dissociation constant in the nanomolar range (3). Another equally important interaction occurs between T7 DNA polymerase and the helicase domain of gene 4 protein. Although T7 DNA polymerase/thioredoxin (T7 DNA pol/trx) complex catalyzes processive DNA synthesis on ssDNA templates (3), it cannot polymerize nucleotides through duplex DNA (strand displacement synthesis) (4). Consequently, the helicase domain of gene 4 protein is required for unwinding of duplex DNA to expose ssDNA template. To coordinate helicase unwinding of the DNA with the polymerization of nucleotides by T7 DNA pol/trx, an interaction of the two proteins is essential. Likewise, gene 2.5 ssDNA-binding protein must interact with both the T7 DNA pol/trx and gene 4 protein in order for them to function on gene 2.5 protein-coated DNA. In the accompanying article (19), we explored this interaction of gene 2.5 protein.

Because each protein component of the replisome contributes its own unique function to the replication process, there is no reason to anticipate any sequence or structural homology among the proteins. In the accompanying article (19), we described the interactions of the C-terminal region of the gene 2.5 ssDNA-binding protein with other components of the replisome. These interactions were dependent not only on the acidic nature of the C terminus but also on the presence of phenylalanine as the C-terminal residue. Interestingly, the gene 4 helicase-primase also has a C terminus rich in acidic amino acids and a phenylalanine as the C-terminal residue. The C-terminal region of the gene 4 protein did not diffract in the crystal structure (5, 6) and taken together with the inability of a full-length gene 2.5 protein to crystallize it raises the possibility that the C terminus is quite flexible. Such a speculation was supported by studies in which deletion of the C termini of these proteins abolished their ability to interact with DNA polymerase (7, 8). Recently, we demonstrated that the thioredoxin-binding domain of T7 DNA polymerase contacts the acidic tails of both gene 4 and gene 2.5 proteins, an interaction that is also enhanced by the binding of thioredoxin (9).

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[2] The abbreviations used are: ssDNA, single-stranded DNA; DTT, dithiothreitol; dsDNA, double-stranded DNA; SPR, surface plasmon resonance; nt, nucleotide; trx, thioredoxin.

In a previous study, we showed that deletion of the 17 C-terminal residues of gene 4 protein results in an inability to physically interact with T7 DNA polymerase. The absence of this interaction prevents the helicase and T7 DNA pol/trx from mediating strand displacement DNA synthesis (8). Whereas this earlier study clearly showed that the helicase domain of gene 4 protein interacts with polymerase via its C-terminal during leading strand DNA synthesis, it did not address the properties of the C terminus important for this interaction. Furthermore, the finding in the accompanying article (19) that the C-terminal phenylalanine of gene 2.5 protein is equally as important as the acidic nature of the C terminus both *in vivo* and *in vitro* warranted an examination of the role of this same residue in the gene 4 protein. Thus, a comparison of the properties of the C termini of the two proteins could hopefully elucidate the important role of these two almost identical segments in these two essential proteins. To investigate mechanistic details of the interaction with T7 DNA pol/trx at the C terminus of gene 4 protein, we constructed a variety of alterations on the C-terminal region and their effects on the protein function were examined.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were obtained from Integrated DNA Technology. Restriction endonucleases and Deep Vent® polymerase were purchased from New England Biolabs. T4 polynucleotide kinase, T4 DNA ligase, radiolabeled nucleotides, and high molecular weight protein markers were purchased from Amersham Biosciences. Agarose and exonuclease-free T7 DNA polymerase were from USB Corp. T7 DNA polymerase/*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). Sensor chips and coupling reagents for surface resonance plasmon assay were from Biacore (Uppsala, Sweden).

Construction of Plasmids and Purification of Gene 4 Proteins—Plasmids that express gene 4 containing alterations in the sequence encoding the C terminus were constructed as described previously (10). After the coding region for gene 4 of the plasmids was confirmed by DNA sequence analysis, proteins were overexpressed in *E. coli* HMS 174(DE3) by IPTG induction and then purified following a standard procedure (11). 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—All assays (DNA unwinding, template-directed oligoribonucleotide synthesis, oligomerization, strand displacement DNA synthesis, RNA-primed DNA synthesis, and gel mobility shift assays) were carried out at 37 °C and used a reaction buffer containing 40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, and 50 mM potassium glutamate plus additional components described in each assay. Except as noted below, all assays were carried out as described previously (12).

Strand Displacement DNA Synthesis—Either M13 dsDNA containing a 5′-non-complementary ssDNA region or a minicircle containing a replication fork was used as a template. The M13 template was prepared by annealing a 66-mer primer (5′-T_{36}AATTC GTAAT CATGG TCATA GCTGT TTCCT-3′) to M13 ssDNA in 10 mM NaCl and then extending its 3′-end with T7 DNA pol/trx in the presence of 0.3 mM each of dATP, dGTP, dCTP, and dGTP at 37 °C for 30 min. The preparation of the minicircular template was described previously (1). The reaction contained either the M13 DNA template (10 nM) or the minicircle template (60 nM), 0.3 mM of all four dNTPs (0.1 μCi of [α-^{32}P]dGTP or dTTP), 20 nM T7 DNA pol/trx, and the indicated amounts of gene 4 protein. After incubation at 37 °C for 30 min for the M13 template reaction or 10 min for the minicircle template reaction, the reaction was terminated by the addition of EDTA to a final concentration of 20 mM. Aliquots of the reaction mixture were spotted on DE-81 membrane and washed three times with 0.3 M ammonium formate (pH 8.0). The amount of DNA synthesis was determined by measuring incorporation of radioactive dNMP into DNA retained on the membrane. The reaction products were also analyzed by electrophoresis through 0.6% alkaline agarose gel.

Gel Mobility Shift Assay—In the presence of ssDNA and a non-hydrolyzable dTTP analog, T7 DNA pol/trx and gene 4 protein form a stable complex that can be distinguished from oligomeric gene 4 protein by its migration on a non-denaturing gel (8). The reaction contained 2.4 μM gene 4 protein, 1.8 μM exonuclease-free T7 DNA polymerase, 100 nM 5′-^{32}P-radiolabeled 24-mer ssDNA (CGCCA GGGTT TTCCC AGTCA CGAC), and 0.5 mM β,γ-methylene dTTP. After incubation at 37 °C for 10 min, the reaction mixture was loaded on a 5% non-denaturing gel, and electrophoresis was performed with a buffer containing 25 mM Tris-HCl (pH 7.5), 190 mM glycine, and 10 mM $Mg(OAc)_2$. After electrophoresis at room temperature for 3 h, the gel was dried for autoradiography.

Surface Plasmon Resonance Assay—SPR analysis was performed by using a Biacore-3000 instrument (Biacore, Uppsala, Sweden). Various gene 4 proteins were coupled via primary amine groups to the carboxymethyl-5 (CM-5) chip at a concentration of 100 μg/ml in NaOAc (pH 5.0) as described previously (9). A control flow cell, which was activated and blocked in the absence of protein, was used to subtract the bulk refractive index and nonspecific interactions. Binding studies were performed at room temperature at a flow rate of 40 μl/min in running buffer containing 20 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 5 mM DTT, 250 mM potassium glutamate, and 0.005% Tween 20 (v/v). The surface of the chip was regenerated by two injections of running buffer containing 1 M NaCl. The observed dissociation binding constant, K_D, was calculated using the average response units under steady-state conditions as described previously (9). Data were fitted using the steady-state model provided by BIAEVAL 3.0.2 computational software (Biacore).

RESULTS

A previous study showed that the C terminus of the gene 4 protein is critical for its interaction with the T7 DNA pol/trx complex during leading strand DNA synthesis (8). The deletion of the C-terminal 17 amino acid residues does not affect the activities of the protein with regards to primer synthesis, dTTP hydrolysis, and DNA unwinding. However, the deletion of the

gp4	
wt	– PSSYSGEEESHSESTDWSNDTDF
Δ17	– PSSYSG
uncharged	– PSSYSGQQQSHSQSTNWSNNTNF
F566D	– PSSYSGEEESHSESTDWSNDTDD
F566L	– PSSYSGEEESHSESTDWSNDTDL
F566Y	– PSSYSGEEESHSESTDWSNDTDY
c-term gp2.5	– PSSYSGWDEDDEESEEADEDGDF

FIGURE 1. **Alterations of the C-terminal region of T7 gene 4 protein.** Alignment of the C-terminal amino acid sequences of gene 4 proteins used in this study. The sequence of the C terminus of wild-type gene 4 protein with the acidic residues indicated. Δ17 is a truncated gene 4 protein lacking the C-terminal 17 residues. uncharged is a genetically modified gene 4 protein in which the acidic aspartic and glutamic acid residues were replaced with asparagines and glutamines, respectively. F556D, F556L, and F566Y are gene 4 proteins in which the C-terminal phenylalanine has been replaced with aspartic acid, leucine, or tyrosine. *c-term gp2.5* is a chimeric gene 4 protein in which the 17 C-terminal residues have been replaced with the 17 C-terminal residues of T7 gene 2.5 protein (*underlined*).

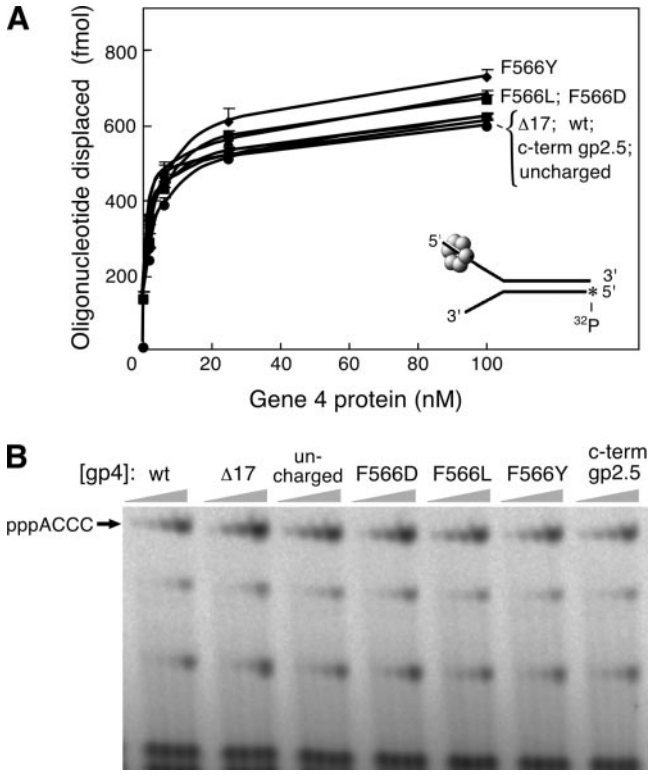

FIGURE 2. **Helicase and primase activity of gene 4 proteins.** *A,* DNA unwinding. Displacement of a 5′-^{32}P-labeled 45-mer oligonucleotide that is partially annealed to a 65-mer oligonucleotide by gene 4 protein was measured. The DNA substrate (100 nM) was incubated with the indicated amounts of each gene 4 protein (1.6, 6.3, 25, and 100 nM in monomeric concentration) for 5 min at 37 °C in the presence of 0.5 mM dTTP. The reaction products were separated on a non-denaturing PAGE and the amount of the radioactively labeled 45-mer displaced from the 65-mer was determined by phosphorimage analysis. Error bars were derived from two independent experiments. *B,* template-directed oligoribonucleotide synthesis. A 15-mer (5′-GGGTCA$_{10}$-3′) containing a primase recognition sequence 5′-GGGTC-3′ was incubated with various concentrations (15, 44, 133, and 400 nM) of gene 4 proteins in the presence of 0.1 mM [α-^{32}P]CTP and ATP. After incubation for 20 min at 37 °C, the reaction products were detected on 25% denaturing polyacrylamide gels. The major reaction product, pppACCC is indicated on the *left side* of the gel.

C-terminal region abolished a proper interaction with T7 DNA pol/trx, a loss that resulted in a failure of the proteins to carry out strand displacement DNA synthesis. The C terminus of gene 4 protein is unique in two respects. One is its high content of acidic residues; 8 of the 17 residues are negatively charged. The other unique feature is the C-terminal aromatic residue, phenylalanine. In general, neither of these two features is conserved in other replicative helicases. Interestingly, another protein involved in T7 replication, gene 2.5 ssDNA-binding protein has both features (19). Although the early study clearly demonstrated the importance of the C-terminal of gene 4 protein in strand displacement DNA synthesis, it is not known what features of the C-terminal region are responsible for its critical interaction with T7 DNA polymerase. Considering the known interaction of both gene 2.5 protein and gene 4 protein with T7 DNA pol/trx (9), it is clearly important to understand how this motif common to both gene 4 and gene 2.5 proteins mediates this interaction. To achieve this end, we introduced a variety of alterations in the C terminus of the gene 4 protein (Fig. 1). First, we replaced all 7 acidic residues, aspartic acids and glutamic acids in the 17 residue C terminus with asparagines and glutamines, respectively (gp4-uncharged in Fig. 1). Second, the C-terminal phenylalanine was replaced with aspartic acid, leucine, or tyrosine (gp4-F566D, -L, and -Y in Fig. 1). These alterations allow us to investigate separately the effect of either the acidic characteristics or the last aromatic residue on function. We also created a chimeric gene 4 protein in which the C-terminal 17 residues were replaced with the corresponding region from gene 2.5 protein (gp4-c-term gp2.5 in Fig. 1). The chimeric protein maintains the last phenylalanine residue but has five additional acidic residues. The altered proteins were purified from *E. coli* cells overexpressing the altered gene 4 in plasmids (11).

Activities of the Gene 4 Proteins

DNA Unwinding—The effect of the C-terminal alterations on the helicase activity of the gene 4 protein was examined using a mini-replication fork as DNA substrate. To unwind the substrate, a functional gene 4 protein first assembles as a hexamer on the ssDNA region of the DNA substrate, translocates in the 5′ to 3′ direction, and then unwinds the duplex DNA. The radioactively labeled displaced strand can be measured by gel electrophoresis. This assay can thus detect a defect in one of those activities of gene 4 helicase. Results of the assay shown in Fig. 2*A* indicate that all altered proteins unwound the substrate as efficiently as did the wild-type protein. We also separately examined the ability of the proteins to hydrolyze dTTP in the presence of ssDNA. No differences between the wild-type protein and the altered proteins were observed (data not shown).

Primer Synthesis—The primase domain of gene 4 protein catalyzes the synthesis of oligoribonucleotides in a template-directed reaction (13). The tetraribonucleotides generated at specific recognition sequences can then be used as primers by T7 DNA pol/trx. The ability of the altered gene 4 proteins to produce oligoribonucleotides was examined by incubating a 15-mer ssDNA template containing a primase recognition sequence, 5′-GGGTC-3′, [α-^{32}P]CTP and ATP, with the gene 4 proteins. The radioactively labeled tetraribonucleotide products can then be measured on a denaturing polyacrylamide gel.

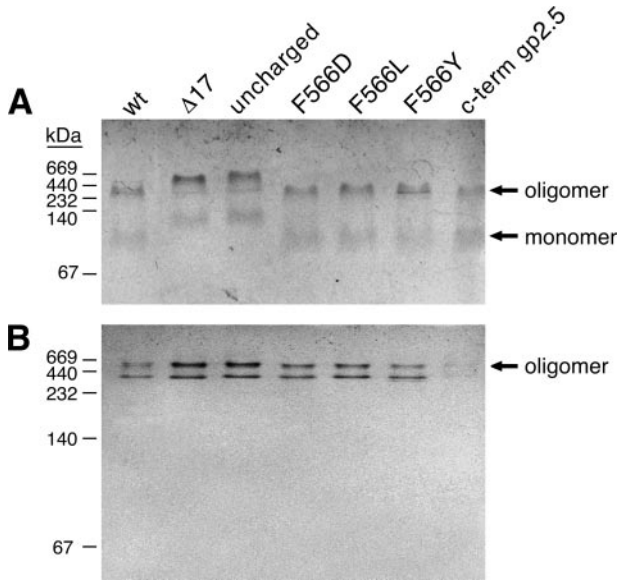

FIGURE 3. **Oligomerization of gene 4 protein.** Gene 4 proteins were incubated with 0.1 mM β,γ-methylene dTTP and 0.1 μM 45-mer oligonucleotide for 20 min at 37 °C. The reaction mixtures were loaded onto 10% non-denaturing polyacrylamide gels and run at room temperature for 6 h (*A*) or at 4 °C overnight (*B*). The resulting forms of the protein were visualized by staining with Coomassie Blue. Migrating positions corresponding to oligomers and monomers of gene 4 protein are indicated.

As shown in Fig. 2*B*, all of the altered gene 4 proteins synthesized oligoribonucleotides as well as did the wild-type protein.

Oligomerization of Gene 4 Protein—As discussed above, the functional form of the gene 4 protein is a hexamer. The presence of β,γ-methylene dTTP, ssDNA and Mg^{2+}facilitates the oligomerization of the gene 4 protein and the oligomerization can be detected by electrophoresis of the proteins on non-denaturing polyacrylamide gels. At room temperature wild-type gene 4 protein is present as monomers as well as oligomers (Fig. 3*A*). Gp4-Δ17, lacking the C terminus, gives rise to a greater abundance of the oligomeric species. Likewise, eliminating the negative charges in the C terminus as in gp4-uncharged also leads to a greater degree of oligomerization. The precise oligomeric state of these gene 4 proteins is not known but molecular weight species larger than hexamers and heptamers have been visualized (12). Eliminating or altering the C-terminal phenylalanine has no effect. On the other hand, the chimeric protein with the C terminus of gene 2.5 protein clearly oligomerizes to a lesser extent than does the wild-type protein. The chimeric protein has five additional acidic residues and thus further strengthens the argument that increasing the negative charge in this region decreases oligomerization. At 4 °C the formation of oligomers is enhanced and this enhancement is seen with the wild-type protein where all the monomers are now present in two high molecular weight species (Fig. 3*B*). The general pattern of oligomerization seen at room temperature with each of the altered proteins is again apparent but the decreased oligomerization with the chimeric protein is more apparent.

Gene 4 Protein Interactions with T7 DNA Pol/trx

Enzymatic Interactions of Gene 4 Protein with T7 DNA Pol/trx—Gene 4 protein supplies two vital activities at the T7 replication fork. For leading strand synthesis to occur, the helicase domain of gene 4 protein, assembled on the lagging strand as a hexamer, must unwind the duplex DNA to expose a ssDNA template for the leading strand DNA polymerase. To coordinate these activities, a physical interaction must occur between the polymerase and helicase domain of gene 4 protein. As ssDNA is extruded behind the translocating helicase, the primase domain of gene 4 protein must synthesize tetraribonucleotides and hold them in position until the lagging strand DNA polymerase can use them as primers. Tetraribonucleotides themselves cannot serve as primers for T7 DNA pol/trx unless they are bound to the gene 4 protein. We have examined those two interactions of the gene 4 protein with polymerases.

In the first assay, the interaction of gene 4 protein with T7 DNA pol/trx during strand displacement DNA synthesis was examined. A primer-template consisting of a circular M13 dsDNA molecule in which one of the two strands bears a 36-nt ssDNA tail resembles a replication fork (see *inset* to Fig. 4*A*). The 5′-ssDNA provides a site for the assembly of the hexameric gene 4 protein. Upon the addition of T7 DNA pol/trx and dNTPs, the two proteins act together to mediate strand displacement synthesis. As shown in Fig. 4*A*, there is essentially no strand displacement synthesis in the absence of gene 4 protein but upon the addition of increasing amounts of wild-type gene 4 protein, there is a dramatic stimulation of DNA synthesis. The products of DNA synthesis obtained with wild-type gene 4 protein represent many circumnavigations of the protein complex around the 7,249-nt M13 DNA as measured by electrophoresis of the products through an alkaline agarose gel where the larger products of synthesis exceed the resolving power of the gel (*lane 2* in Fig. 4*B*). Gene 4 protein lacking the C terminus (gp4-Δ17), as previously shown (8), is unable to interact with T7 DNA pol/trx to mediate strand displacement synthesis (Fig. 4, *A* and *B*). Retention of the C terminus but elimination of the acidic residues (gp4-uncharged) is likewise detrimental to this interaction.

Most interestingly, replacement of the C-terminal phenylalanine with a non-aromatic residue (gp4-F566L or gp4-F566D) reduces the ability of the altered gene 4 protein to promote strand displacement synthesis greater than 2- or 5-fold, respectively. The absence of high molecular products with these two altered proteins (*lanes 5* and *6* in Fig. 4*B*) dramatically attests to this defect. Substitution of another aromatic residue, tyrosine, for the C-terminal phenylalanine is well tolerated (Fig. 4, *A* and *B*). This requirement for a C-terminal aromatic residue mimics the same requirement found in gene 2.5 protein for its interaction with T7 DNA pol/trx (19).

Surprisingly, the chimeric gene 4 protein containing the C terminus of gene 2.5 protein (gp4-c-term gp2.5) has enhanced strand displacement activity, perhaps due to its higher content of acidic residues (Fig. 4*A*, *lane 8* in Fig. 4*B*). We have also used the 70-nt minicircle primer-template previously described (1) to examine the ability of the altered gene 4 proteins to mediate leading strand DNA synthesis (Fig. 4*C*). Essentially the same results were observed as found for the M13 DNA primer-template just discussed.

In the second assay, we tested the ability of various gene 4 proteins to synthesize and deliver oligoribonucleotides to T7 DNA pol/trx for RNA-primed DNA synthesis on M13

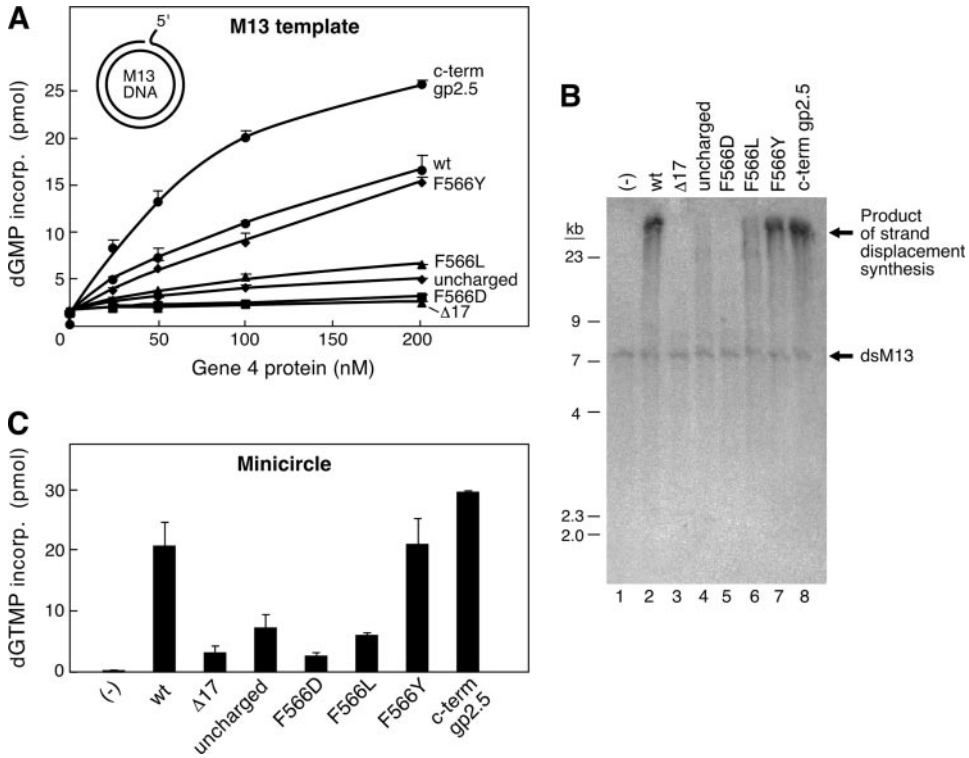

FIGURE 4. **Strand displacement DNA synthesis by T7 DNA polymerase/thioredoxin and gene 4 proteins.** On a circular duplex DNA containing a 5′-protruding tail, T7 DNA polymerase/thioredoxin is able to catalyze strand displacement DNA synthesis in the presence of gene 4 protein. *A,* using dsM13 DNA containing a 5′-ssDNA tail (see *inset*), the efficiency of the strand displacement DNA synthesis mediated by various gene 4 proteins was determined. The M13 primer-template was prepared as described under "Experimental Procedures." The reaction contained the dsM13 template (10 nM), 0.3 mM of dATP, dCTP, dTTP, [α-^{32}P]dGTP, 20 nM T7 DNA polymerase/thioredoxin, and the indicated amount of gene 4 proteins (25, 50, 100, and 200 nM). After incubation for 30 min at 37 °C, reactions were terminated by the addition of EDTA and the amount of [α-^{32}P]dGMP incorporated into DNA was measured as described under "Experimental Procedures." Error bars were derived from two independent experiments. *B,* products of the reaction described in panel *A* (200 nM gene 4 protein) were analyzed by electrophoresis on 0.6% alkaline agarose gel. The products corresponding to full-length dsM13 DNA and the product of strand displacement synthesis are indicated on the *right side* of the gel. *C,* similar reaction to *panel A* was carried out using 60 nM minicircle template containing a replication fork as a template DNA. The reaction mixture was incubated at 37 °C for 10 min. *Error bars* were derived from three independent experiments.

ssDNA. Oligoribonucleotides are synthesized from specific recognition sites on the template DNA by the primase domain of gene 4 protein and proper delivery of the primers to T7 DNA pol/trx allows synthesis of dsDNA. Essentially, all the gene 4 proteins demonstrated the same level of DNA synthesis proportional to the amount of gene 4 protein as measured by incorporation of dNMP into dsDNA (Fig. 5). The results indicate that the alterations at the C-terminal of gene 4 protein do not affect the interaction between the primase domain of gene 4 protein and DNA polymerase. Because this RNA-primed assay requires activity of gene 4 protein to both synthesize primer and translocate on a long ssDNA template to search for primase recognition sites, these results also confirmed that all of the altered proteins have similar ability to synthesize oligoribonucleotide (Fig. 2*B*) and translocate on ssDNA (Fig. 2*A*).

Physical Associations of Gene 4 Protein with T7 DNA Pol/trx—Inasmuch as all the altered gene 4 proteins unwind duplex DNA as efficiently as does the wild-type protein (Fig. 2*A*), their inability to function with T7 DNA pol/trx to mediate strand displacement synthesis most likely arises from a defect in a proper interaction between the two proteins. Therefore, we have examined complex formation using two independent techniques: gel mobility shift assay and surface plasmon resonance.

It has been shown previously that the interaction between the two proteins can be detected by gel mobility shift assay (8). In the presence of a non-hydrolyzable dTTP, binding of gene 4 protein to ssDNA is significantly enhanced and thus a stable DNA-gene 4 protein complex can be detected by non-denaturing gel electrophoresis. Addition of T7 DNA pol/trx to the reaction leads to form a stable ternary complex between the DNA-gene 4 protein complex and T7 DNA pol/trx, distinct from the binary complex. The ability of the altered gene 4 proteins to form such a ternary complex with DNA and T7 DNA pol/trx is shown in Fig. 6*A*. Consistent with earlier results (8), T7 DNA pol/trx alone did not form a stable complex with ssDNA (*lane 2* in Fig. 6*A*). Gene 4 protein alone forms a binary complex with ssDNA (*lane 3*) and upon the addition of T7 DNA pol/trx there is a further shift to the slower migrating ternary complex (*lane 4*), consistent with earlier studies. We have used Western blot analysis to confirm that the slower migrating band seen in *lane 4* of Fig. 6*A* contains both gene 4 protein and DNA polymerase (data not shown). Gp4-Δ17, gp4-uncharged, and gp4-F566D were unable to form a stable ternary complex with DNA and T7 DNA pol/trx (*lanes 5–10*) whereas gp4-F566L, gp4-F566Y, and gp4-c-term gp2.5 did (*lanes 11–16*).

Measurements of the interaction of gene 4 proteins using surface plasmon resonance confirm the results obtained with the gel shift assays (Fig. 6*B*). In this assay the gene 4 proteins were immobilized via their amine groups to the Biacore CM-5 chip and T7 DNA pol/trx was injected over the chip at varying concentrations. Using steady state analysis, as described under "Experimental Procedures," we calculated the K_D for the various protein interactions. Both the wild-type gene 4 protein and gp4-F566Y interact with a K_D of 575 and 760 nM, respectively. Gp4-c-term gp2.5 forms a considerably more stable complex with a K_D of 450 nM. Gp4-F566L binds weakly to T7 DNA pol/trx, and no binding was observed with gp4-Δ17. We do not know why the gel shift assay reveals relatively stable ternary complex with T7 DNA pol/trx and gp4-F566L unless the presence of ssDNA in some way compensates for the clearly weaker interaction of

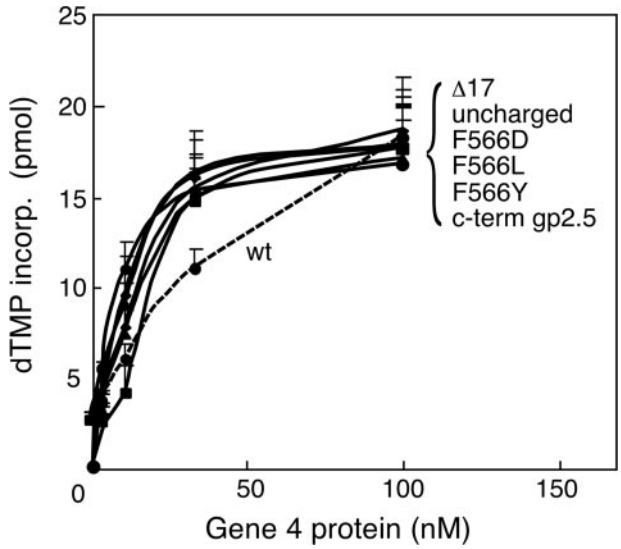

FIGURE 5. **RNA-primed DNA synthesis.** Oligoribonucleotides required for the initiation of DNA synthesis by T7 DNA polymerase/thioredoxin on M13 ssDNA are generated by the primase activity of gene 4 protein. The reaction containing ATP, CTP, dATP, dCTP, dGTP, [α-^{32}P]dTTP, M13 ssDNA, T7 DNA polymerase/thioredoxin, and the indicated amount of gene 4 proteins (3.7, 11, 33 and 100 nM) were incubated for 10 min at 37 °C. The reaction products were spotted on DE-81 membrane, and the filter was washed extensively with ammonium formate. The amount of DNA synthesis was determined by measuring the amount of radioactive dTMP incorporated into DNA retained on the filter. *Error bars* were derived from two independent experiments.

the two proteins observed by surface plasmon resonance. This altered protein did support some, albeit low, strand displacement synthesis (Fig. 4) consistent with weak binding between the two proteins.

Effect of C-terminal-altered Subunit within the Gene 4 Proteins Hexamer on Strand Displacement Synthesis

Because gene 4 protein functions as hexamer, a defect in the C terminus of one subunit might affect the ability of adjacent subunits to interact with the polymerase. To examine the effect of a defective subunit on the function of oligomeric gene 4 protein, we measured efficiency of strand displacement DNA synthesis mediated by a mixture of wild-type gene 4 protein and the altered gene 4 proteins described above. We have shown previously that subunits arising from one hexamer can exchange randomly with those in another (14). In the experiment shown in Fig. 7*A*, we have screened all of the altered proteins for their ability to affect strand displacement synthesis when they are present at an equimolar concentration with the wild-type gene 4 protein. Both the incorporation of nucleotides and the distribution of the products on an alkaline agarose gel are presented. For comparison, the reactions were carried out without (*lane 1*) or with single species of gene 4 protein (*lanes 2–6*). Upon addition to wild-type gene 4 protein, gp4-Δ17, gp4-uncharged, and gp4-F566D all led to a reduction in strand displacement synthesis (*lanes 7–9* in Fig. 7*A*). The addition of gp4-F566L to the wild-type protein did not result in reduction in synthesis as did the addition of the other altered proteins (*lane 10*). However, synthesis with the mixture containing 2-fold more protein was not significantly different from that observed with the wild-type protein alone, suggesting that gp4-F566L does not provide an additive effect on the activity of gene 4 protein. No combination of altered proteins in mixtures resulted in synergistic stimulation in the synthesis possibly by complementing defects each other (*lanes 11–16*). The effect of the altered subunit is proportional to the amount of altered gene 4 protein added to wild-type protein (Fig. 7*B*). Mixtures of gene 4 proteins lacking the acidic residues in the C terminus but maintaining the C-terminal phenylalanine (gp4-uncharged) and gene 4 protein lacking the C-terminal phenylalanine but retaining the acidic residues (gp4-F566D) showed that the two defects could not be corrected by assembling subunits of each into a hexamer (Fig. 7*C*).

DISCUSSION

The essential nature of the acidic C termini of the T7 gene 4 helicase-primase and the gene 2.5 ssDNA-binding protein has been recognized from earlier studies (7, 8). Because the obvious distinguishing feature of these C-terminal segments was the acidic amino acids, we generally assumed that the negative charge density was critical to their essential role. In this report as well as in the accompanying article (19), we have examined in detail the role of the acidic residues as well as other aspects of the C-terminal regions. Here we show that indeed the acidic nature of the C terminus of gene 4 protein is important in its physical interaction with T7 DNA pol/trx and to our surprise the C-terminal phenylalanine is equally important. The consequence of this defect is manifest in the inability of gene 4 proteins lacking C-terminal acidic residues or the C-terminal phenylalanine to function with T7 DNA pol/trx to mediate strand displacement synthesis. Otherwise, the two activities of the gene 4 protein, helicase and primase, are similar to that found in the wild-type gene 4 protein. The only other alteration observed was a tendency for the more highly charged C-terminal segments that we constructed to interfere with oligomerization of the protein.

All DNA polymerases require a 3-hydroxyl-terminated primer and a ssDNA template for DNA synthesis. At a replication fork the duplex DNA must be unwound in order to provide a ssDNA for leading strand DNA synthesis. A few DNA polymerases can accomplish the unwinding of the duplex without accessory proteins. Both Phi29 and T5 DNA polymerases contain unique domains not found in polymerase superfamilies (15, 16), and they alone can generate the ssDNA template for leading strand DNA synthesis. Deletion of these non-conserved regions results in loss of the ability of the polymerases to catalyze strand displacement DNA synthesis (16, 17). However, the T7 DNA pol/trx complex, like most other replicative DNA polymerases, requires a DNA helicase to catalyze the unwinding of the duplex DNA. The T7 gene 4 helicase, despite being a distinct protein, must physically interact with the T7 DNA pol/trx that constitutes the functional T7 DNA polymerase. That interaction does involve a unique segment in the thumb subdomain of the T7 DNA polymerase to which the processivity factor, thioredoxin also binds (9).

The physical interaction of T7 DNA pol/trx with the T7 gene 4 helicase probably arises from the necessity of coordinating the activities of the two proteins. In the absence of a physical contact between the proteins, it is likely that the helicase would occasionally outpace the polymerase, thus

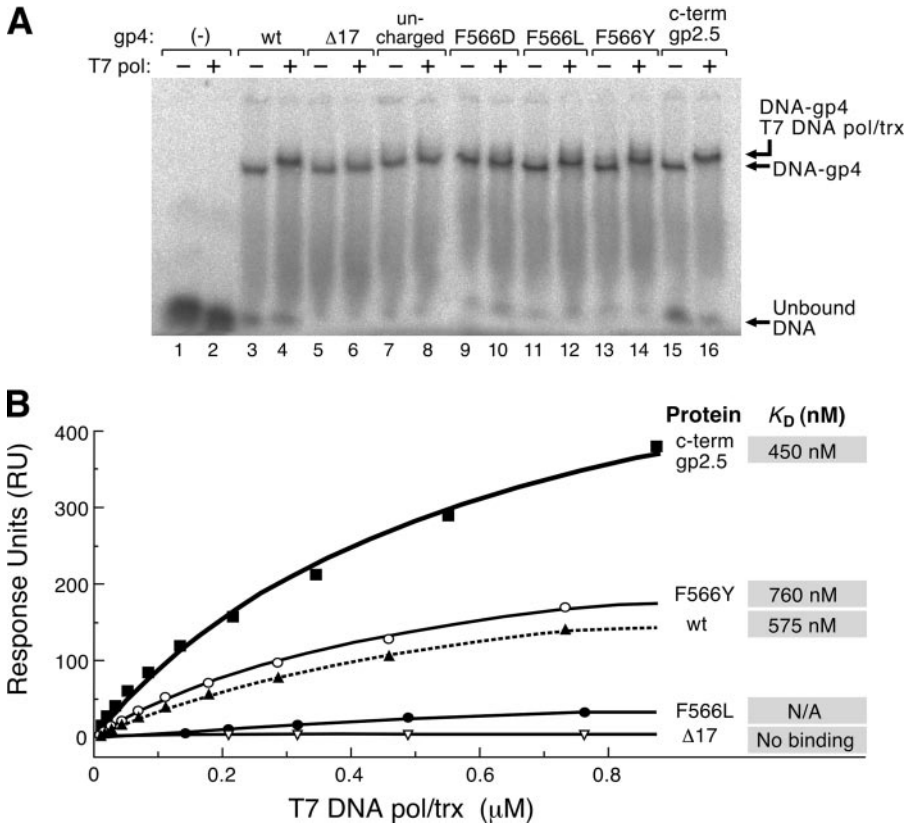

FIGURE 6. **Complex formation between gene 4 proteins and T7 DNA polymerase/thioredoxin.** *A*, detection of T7 DNA polymerase/thioredoxin-gene 4 protein complex by gel mobility shift assay. T7 DNA polymerase/thioredoxin forms a stable complex with gene 4 protein in the presence of ssDNA and a non-hydrolyzable analog of dTTP. Reactions containing 2.4 μM of indicated gene 4 protein, 1.8 μM exonuclease free T7 DNA polymerase, 100 nM 5′-^{32}P-labeled 24-mer ssDNA, and 0.5 mM β,γ-methylene dTTP were incubated at 37 °C for 10 min. Reaction mixtures were analyzed on 5% a non-denaturing gels. Migrating positions corresponding to unbound DNA, DNA-gene 4 protein binary complex and DNA-gene 4 protein-T7 DNA polymerase/thioredoxin complex are indicated. *B*, determination of K_D for gene 4 protein-T7 DNA polymerase/thioredoxin complex. Gene 4 proteins were immobilized via their amine groups to Biacore CM-5 chips and T7 DNA polymerase/thioredoxin was flowed over the chip. The amounts of immobilized gene 4 protein for wild-type, gp4C-Δ17, gp4-c-term gp2.5, gp4-F566Y, and gp4-F566L were 1320, 4000, 1850, 1600, and 1400 RU, respectively. Data points represent the average RU upon the binding of various concentrations of T7 DNA polymerase/thioredoxin to the indicated gene 4 protein under a steady state conditions. The *solid line* represents the theoretical curve calculated from steady-state fit model provided by BIAEVAL-3.0.2 computation software. Representative data from three independent experiments are shown.

leaving the latter stalled at duplex regions as the DNA re-anneals behind the helicase. In addition, strong binding of the hexameric helicase domain of gene 4 protein to ssDNA provides a mechanism for assembling the replisome at the replication fork in that both the leading and lagging strand DNA polymerases can bind to one or more of the subunits of the gene 4 protein. In any case, gene 4 proteins deficient in their binding to T7 DNA pol/trx as a result of alterations in the C terminus are unable to function with the T7 DNA pol/trx to mediate strand displacement synthesis.

We have recently shown that a major interaction of the gene 4 protein with T7 DNA pol/trx involves two small basic loops that arise when thioredoxin binds to the T7 DNA polymerase at the thioredoxin binding domain (9). T7 DNA polymerases lacking one or both of these loops are defective in interacting with gene 4 protein. Thus, the acidic nature of the C terminus of gene 4 protein seemed the most likely explanation as to the loss of binding of gene 4 proteins lacking this segment to these basic loops in the T7 DNA pol/trx. Our results show that indeed the acidic nature of the C terminus plays a critical role in the interaction of gene 4 protein with T7 DNA pol/trx. Replacing the acidic residues with uncharged residues did not affect the catalytic properties of either the helicase or primase domains of the gene 4 protein. However, the ability of the altered protein to bind to T7 DNA pol/trx or to mediate strand displacement synthesis was dramatically diminished.

Unanticipated was the finding that the C-terminal phenylalanine of gene 4 protein is also essential for these interactions of the gene 4 protein with T7 DNA pol/trx. The aromaticity of the C-terminal residue appears to be important in that tyrosine could replace phenylalanine without any loss of the interaction with T7 DNA pol/trx. The molecular basis for the requirement for a C-terminal aromatic residue in the gene 4 protein, or for the gene 2.5 protein (19) is not known. Unfortunately, the crystal structure of gene 4 protein or of the helicase domain alone is not helpful in delineating the role of the C-terminal residue because the C-terminal residues did not diffract in the crystal structure (5, 6, 18). This observation suggests that the C-terminal residues form a flexible motif, a motif that could thus contact either the gene 4 protein itself or the T7 DNA pol/trx. A similar argument was made for the C terminus of the gene 2.5 protein in that the protein would not crystallize unless the C terminus was deleted. Hopefully, suppressor mutation analysis and cross-linking studies can identify the points of contact. Comparison of amino acid sequences shows the aromatic residue at the C terminus is conserved within T7-like fused helicase-primase. However, it is not a common characteristic for replicative helicases (glutamic acid in *E. coli* Dna B, phenylalanine in T4 gp41, alanine in *Bacillus subtilis* Dna *C*, etc.).

In addition to its role in interacting with T7 DNA pol/trx, the C terminus affects the oligomerization of gene 4 protein. Either removal or neutralization of the acidic region resulted in a more stable oligomeric form. The presence or absence of the C-terminal phenylalanine had no effect on oligomerization. Most likely, removing the acidic residues reduces the repulsion arising from negatively charged C termini on adjacent subunits. In support of this model, substituting the more acidic C terminus of gene 2.5 protein for that of the gene 4 protein has the opposite effect of decreasing oligomerization of the gene 4 protein. We have also observed that the presence of a positively charged

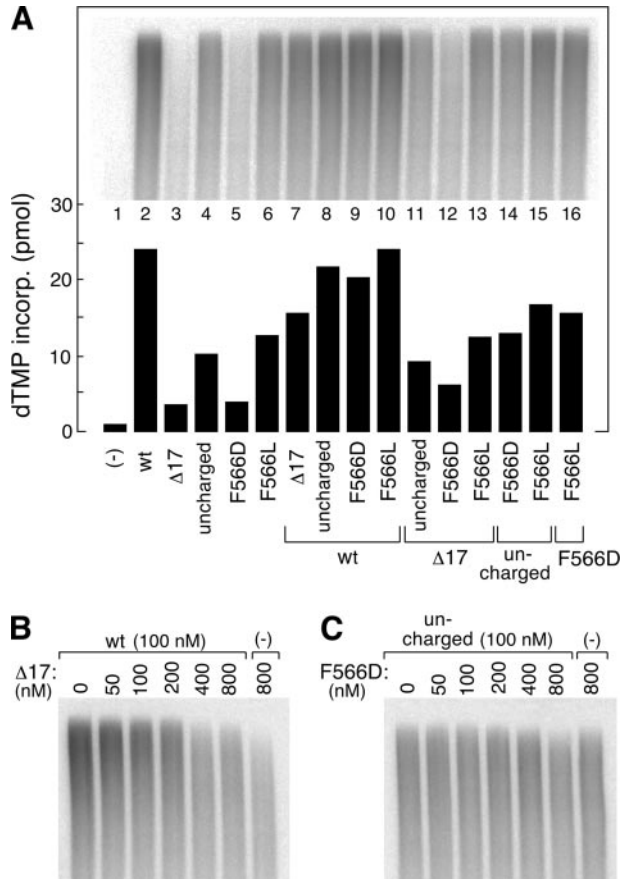

FIGURE 7. **Effect of C-terminal altered subunit within the gene 4 proteins hexamer on strand displacement synthesis.** *A*, the ability of gene 4 protein hexamers containing one or more altered subunits to mediate strand displacement DNA synthesis was compared. Reactions were similar to those described in Fig. 4*A*. No gene 4 protein was present in *lane 1*. *Lanes 2–6* contained single species of indicated gene 4 protein at the concentration of 100 nM. *Lanes 7–16* contained 100 nM gene 4 protein indicated *under the bracket* plus equimolar amounts of other altered gene 4 proteins. Reaction products were analyzed on 0.6% alkaline agarose gel (*top section*). The total amount of dTMP incorporated into DNA is presented in the *bottom section*. Representative data from four independent experiments are shown. *B*, effect of gp4-Δ17 on the ability of wild-type gene 4 protein to mediate strand displacement DNA synthesis. A constant amount of wild-type protein (100 nM) was mixed with the increasing amounts of gp4-Δ17. *C*, effect of gp4-F566D on the ability of gp4-uncharged to mediate strand displacement DNA synthesis. A constant amount of gp4-uncharged (100 nM) was mixed with the increasing amounts of gp4-F566D.

histidine tag at the C terminus of gene 4 protein reduces the ability of the protein to hydrolyze dTTP and to unwind dsDNA.[3] This latter effect is interesting in that the simple elimination of acidic residues did not affect enzyme activity. The entire C-terminal face of the gene 4 protein is acidic (5) and thus the addition of a basic motif may have a more dramatic effect. Different from an organized interaction with basic loop(s) from T7 DNA poly/trx, a random interaction of the poly-histidine tail with the C-terminal surface of gene 4 protein might impair function of the helicase. Interestingly, the stability of the oligomer of gene 4 protein inversely correlates with its ability to form a stable T7 DNA pol/trx complex and to mediate strand displacement synthesis.

The gene 4 protein plays a pivotal role at the T7 replication fork. We have proposed that the gene 4 protein is the keystone in that it coordinates leading and lagging strand synthesis by providing binding sites for both the leading and lagging strand DNA polymerases (9). In this model, the acidic C termini of subunits of gene 4 protein each can contact the two small basic loops located in the thioredoxin domain of the thumb subdomain T7 DNA pol/trx. Although stoichiometry between gene 4 protein and T7 DNA pol/trx is not known, it is not likely that all six subunits in gene 4 protein each possess a DNA polymerase partner. Rather, it seems likely that T7 DNA pol/trx would have to switch from subunit to subunit as the proteins simultaneously unwind and replicate the primer-template. In such a model all six subunits must be capable of binding T7 DNA pol/trx such that the polymerase can be passed from one subunit to an adjacent subunit. Our results using heterooligomers composed of one or more altered subunits support such a model. The presence of the defective altered C terminus in the hexameric gene 4 protein might interrupt switching of T7 DNA pol/trx from subunit to subunit of gene 4 protein, resulting in reduced efficiency in strand displacement synthesis.

The similarity of the C termini of gene 4 protein and gene 2.5 ssDNA-binding protein is striking. Both have a high content of acidic residues and both have a C-terminal phenylalanine. Not surprisingly, we find that the C terminus of gene 2.5 protein can functionally replace the C terminus of gene 4 protein. In a recent study, we demonstrated that both proteins bind to the two basic loops in the thioredoxin binding domain (TBD) of T7 DNA polymerase, an interaction that is enhanced when thioredoxin is also bound to the TBD (9). Having the same features at the C termini, both gene 4 and gene 2.5 proteins might compete for binding to the two basic loops in T7 DNA pol/trx. However, because unwinding of dsDNA by gene 4 helicase is the first step during elongation of daughter DNA strands by DNA polymerase, the C terminus of gene 4 protein has to interact with the polymerase prior to gene 2.5 protein. As suggested by higher contents of acidic residues in the C-terminal region, gene 2.5 protein might have lower binding affinity to DNA than gene 4 protein, but higher to the basic loop(s) in T7 DNA pol/trx. Such differences in the binding affinity provide basis for distinctive participation of the two proteins in each step during coordinated DNA synthesis.

[3] S.-J. Lee and C. C. Richardson, unpublished results.

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