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Effect of Single-stranded DNA-binding Proteins on the Helicase and Primase Activities of the Bacteriophage T7 Gene 4 Protein*

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Gene 4 protein (gp4) of bacteriophage T7 provides two essential functions at the T7 replication fork, primase and helicase activities. Previous studies have shown that the single-stranded DNA-binding protein of T7, encoded by gene 2.5, interacts with gp4 and modulates its multiple functions. To further characterize the interactions between gp4 and gene 2.5 protein (gp2.5), we have examined the effect of wild-type and altered gene 2.5 proteins as well as *Escherichia coli* single-stranded DNA-binding (SSB) protein on the ability of gp4 to synthesize primers, hydrolyze dTTP, and unwind duplex DNA. Wild-type gp2.5 and *E. coli* SSB protein stimulate primer synthesis and DNA-unwinding activities of gp4 at low concentrations but do not significantly affect single-stranded DNA-dependent hydrolysis of dTTP. Neither protein inhibits the binding of gp4 to single-stranded DNA. The variant gene 2.5 proteins, gp2.5-F232L and gp2.5-Δ26C, inhibit primase, dTTPase, and helicase activities proportional to their increased affinities for DNA. Interestingly, wild-type gp2.5 stimulates the unwinding activity of gp4 except at very high concentrations, whereas *E. coli* SSB protein is highly inhibitory at relative low concentrations.

The single-stranded DNA (ssDNA)[1]-binding protein of bacteriophage T7, encoded by gene 2.5 of the phage, is essential for T7 DNA replication and hence for growth of the phage (1). Gene 2.5 protein (gp2.5) is one of four proteins that constitute a T7 replisome capable of mediating DNA replication in which leading and lagging strand syntheses are coordinated and the size distribution of Okazaki fragments is maintained via a replication loop formed from the lagging strand (2, 3). In addition to gp2.5 the replisome contains T7 gene 5 DNA polymerase, its processivity factor *Escherichia coli* thioredoxin, and the multifunctional T7 gene 4 helicase-primase or gp4 (4). Gp2.5 is unique among these proteins in that it not only binds to ssDNA but also physically interacts with both T7 DNA polymerase and gp4 (5, 6). Whereas the consequences of the interaction of gp2.5 with ssDNA and with T7 DNA polymerase have been characterized (5, 7), little is known regarding the effect of gp2.5 on the helicase and primase activities of T7 gp4.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; gp4 and gp2.5, gene 4 protein and gene 2.5 protein, respectively; WT, wild type; SSB protein, single-stranded DNA-binding protein; nt, nucleotide.

Gp2.5 belongs to a class of ubiquitous proteins that are not only essential for DNA replication but also play key roles in DNA recombination and repair (8, 9). Gp2.5, for example, is essential for recombination in T7 phage-infected cells and *in vitro* it mediates homologous base pairing (10, 11). Despite a lack of sequence homology, T7 gp2.5 is both structurally and functionally similar to the extensively studied ssDNA-binding protein of *E. coli* (SSB protein) and the gene 32 protein of bacteriophage T4. A recent crystal structure of a truncated gp2.5 (12) revealed that it contains an oligosaccharide/oligonucleotide binding fold conserved among members of the SSB protein family including *E. coli* SSB protein and T4 gene 32 protein.

Although structurally and functionally similar, neither *E. coli* SSB protein nor T4 gene 32 protein can substitute T7 gp2.5 for T7 growth (1, 13). However, the functional interactions of gp2.5 with ssDNA and with T7 DNA polymerase and gp4 that account for this specificity have been difficult to dissect. First to be addressed is the ability of each of the latter two proteins to load onto ssDNA coated with gp2.5. T7 gp2.5 binds to ssDNA with a 10-fold lower affinity ($K_d = 4.6 \times 10^{-6}$ M) than does *E. coli* SSB protein or T4 gene 32 protein (8). Nonetheless, T7 DNA polymerase readily extends a primer on ssDNA coated with gp2.5, *E. coli* SSB protein, or T4 gene 32 protein (5). On the other hand, gp4 cannot mediate strand transfer in the presence of gene 32 protein, presumably because it can not load onto ssDNA coated with gene 32 protein (14). Strand transfer mediated by the gene 4 helicase readily occurs in the presence of gp2.5 (14).

T7 gp2.5, *E. coli* SSB protein, and T4 gene 32 protein all have an acidic C terminus of which a number of studies shown are essential for the protein-protein interactions observed *in vitro* (6, 7, 13, 15–19). A truncated gp2.5, gp2.5-Δ21C, lacking the 21 C-terminal residues cannot support T7 growth, and the purified protein cannot form dimers or physically interact with T7 DNA polymerase or gp4 (6). Whereas the C termini of these proteins are essential for protein-protein interactions, they are not responsible for the specificity observed for T7 growth. Chimeric proteins in which the acidic C termini of either *E. coli* SSB protein or T4 gene 32 protein were substituted for the C terminus of gp2.5 support the growth of T7 phage lacking gene 2.5. Furthermore, they dimerized and physically interacted with gp4 and T7 DNA polymerase. On the other hand, chimeric proteins consisting of SSB protein or gene 32 protein bearing the T7 acidic terminus could not support the growth of T7 lacking gene 2.5 (13).

Using a random mutagenesis procedure, we recently isolated a number of gp2.5s having single amino acid substitutions that could not support T7 growth (20). Whereas studies with these altered proteins led to the identification of sites that affected DNA binding (7, 21) and homologous base pairing (11), none of the altered proteins appeared to be defective in interactions

with gp4 or T7 DNA polymerase. One altered protein, gp2.5-F232L, identified in the study, had a single amino acid substitution in the C-terminal residue (20). Gp2.5-F232L binds more tightly to ssDNA ($K_d = 1.5 \times 10^{-6}$ M) than does WT gp2.5 but not as tightly as gp2.5-Δ21C ($K_d = 1.1 \times 10^{-7}$ M) (7). Gp2.5-F232L stimulates T7 DNA polymerase activity on ssDNA even more so than does WT gp2.5, whereas gp2.5-Δ21C inhibits T7 DNA polymerase activity. This result clearly shows the importance of the C-terminal tail in interactions with the polymerase.

Less well studied are the effects of gp2.5 on the helicase and primase activities of the T7 gp4. The helicase and primase activities of gp4 reside in the C-terminal and N-terminal halves of the 63-kDa protein, respectively (22). The DNA sequence encoding each domain has been independently cloned, and the respective helicase and primase fragments have been overproduced and purified (22). Crystal structures of both the helicase (23, 24) and primase (25) fragments are available. The helicase and primase fragments have helicase and primase activities, respectively, equivalent to that of the wild-type full-length gp4. However, as discussed below, the helicase domain, when covalently attached to the primase domain as in the full-length protein, bestows several desirable properties on the primase domain. The multiple activities catalyzed by the gp4, many of which involve interactions with ssDNA, likewise provide multiple reactions that are potential targets for gp2.5. For this reason, it is important to review briefly the reactions mediated by each domain of the gp4.

Reactions mediated by the C-terminal helicase domain require the assembly of the protein into a hexamer. In the presence of dTTP, the 63-kDa gp4 assembles as a hexamer on ssDNA (26, 27) with a polarity such that the helicase domain faces the 3′-end of the DNA to which it is bound (26). A major stabilization of the hexamer is dependent on the linker region that connects the helicase and primase domains (23, 24, 28). Once bound to ssDNA, the protein then translocates unidirectionally from 5′ to 3′ on the strand, a reaction coupled to the hydrolysis of dTTP (29, 30). Upon encountering a duplex provided that there is a 3′-ssDNA tail on the strand that it encounters, it will unwind the duplex. Gp2.5 bound to ssDNA and/or to the gp4 could facilitate the loading of the hexamer onto ssDNA. Unlike the DnaB helicase of *E. coli* or the gene 41 helicase of phage T4 (31, 32), the T7 helicase does not have a specific helicase loader. Once loaded onto ssDNA, the presence of gp2.5-coated ssDNA could conceivably facilitate or hinder translocation of the gp4.

The N-terminal primase domain catalyzes the template-directed synthesis of oligoribonucleotides on ssDNA, and these, in turn, are transferred to the T7 DNA polymerase for use as primers to initiate DNA synthesis (33). At the basic primase recognition site, 5′-GTC-3′, the primase catalyzes the synthesis of the dinucleotides pppAC from ATP and CTP (34). Provided that the more extensive recognition sites are present, 5′-GGGTC-3′, 5′-TGGTC-3′, or 5′-GTGTC-3′ and the functional tetraribonucleotide primers, pppACCC, pppACCA, and pppACAC are synthesized (30). A major role in sequence recognition is mediated by a Cys_4 zinc motif located at the extreme N terminus of the primase domain (5, 34, 35). Obviously, the effect of gp2.5-coated DNA on the recognition of the primase sites and the subsequent synthesis of the primer are of considerable importance. The primase fragment lacking the helicase domain catalyzes the template-directed synthesis of oligoribonucleotides equally as well as does the full-length gp4 in the absence of dTTP (36). However, the primase fragment alone has a very low affinity for ssDNA and for its recognition site (37). Consequently, the 63-kDa gp4 has markedly higher primase activity in the presence of dTTP because of the ability of the hexameric helicase to bind tightly to ssDNA (36). On long DNA templates with a scarcity of primase recognition sites, the primase is dependent on translocation by the helicase domain for delivery to these sites (36). As a result, gp2.5 could have an indirect effect on primase activity via its interaction with the helicase domain.

In a recent paper (7), we examined the effect of wild-type gp2.5 and variants of gp2.5 for their ability to bind to ssDNA and to interact with T7 DNA polymerase. Wild-type gp2.5 was found to slightly stimulate DNA synthesis catalyzed by T7 DNA polymerase on ssDNA templates. The ability of T7 DNA polymerase to catalyze synthesis through gp2.5-coated DNA is dependent on the presence of the acidic C-terminal tail in that gp2.5-Δ26C lacking the 26 C-terminal amino acids inhibits synthesis by >10-fold (7). In this communication, we examined the effect of gp2.5 on the multiple activities catalyzed by the helicase and primase domains of gp4. In these studies, we have used two altered gp2.5s, gp2.5-Δ26C and gp2.5-F232L, as well as the wild-type gp2.5. Gp2.5-ΔF232L has a single amino acid substitution in the C-terminal tail that increases its binding to ssDNA yet does not affect its interaction with the T7 replication proteins (7). In addition, we have examined the effect of *E. coli* SSB protein on these reactions to examine the specificity for gp2.5 in the T7 system.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains, Bacteriophages, and Plasmids—*E. coli* BL21(DE3)-(F^- *ompT* $hsdS_B$ ($r_B^-m_B^-$) *gal*$^-$ *dcm* (DE3)) (Novagen) was used as the host strain to express T7 gene 2.5 and to purify wild-type and mutant gp2.5. Wild-type and mutant gene 2.5 are expressed from the pET17b plasmid (Novagen) containing the T7 RNA polymerase promoter. T7 gp2.5-Δ26C was obtained from Edel Hyland (Harvard Medical School). *E. coli* HMS 174 (DE3) was purchased from Invitrogen.

DNA and Oligonucleotides—Oligonucleotides (Table I) used in the assay to measure primase activity (ZHP20, ZHP70, and ZH70) and to prepare helicase substrates (S75 and L95) were purchased from Integrate DNA Technologies. [5′-^{32}P]S75 oligonucleotide was annealed to L95 and used for helicase analysis substrates. M13 ssDNA was purchased from Invitrogen. The oligonucleotides used for cloning of gene 2.5 were described previously (7).

Proteins, Enzymes, and Chemicals—Restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and calf-intestinal phosphatase were purchased from New England Biolabs. *E. coli* SSB protein was purchased from United States Biochemicals Corp. Donald Crampton (Harvard Medical School) supplied T7 gp4, and Seung-Joo Lee (Harvard Medical School) supplied the primase fragment of T7 gp4. All of the chemicals and reagents were from Sigma unless otherwise noted.

Methods

Protein Overexpression and Purification—WT gp2.5 and gp2.5-F232L were overexpressed and purified as described previously (7).

Oligoribonucleotide Synthesis by T7 DNA Primase—The synthesis of oligoribonucleotides catalyzed by gp4 was determined by measuring the incorporation of [α-^{32}P]CMP into oligoribonucleotides using M13 ssDNA or synthetic DNA templates (Table I) containing a primase recognition site (34, 37). The reaction (10 μl) included 4 nM M13 ssDNA or 400 nM oligonucleotide, 0.3 mM each of ATP and [α-^{32}P]CTP (0.1 μCi), 0.5 mM dTTP, 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 50 mM potassium glutamate, and 100 μg/ml bovine serum albumin. The reaction mixtures were preincubated with the indicated amount of ssDNA-binding proteins for 5 min at 37 °C, and the reactions were initiated by the addition of either 80 nM gp4 monomer or 800 nM gp4 primase fragment. After further incubation at 37 °C for 30 min, the reaction was terminated by the addition of 3 μl of sequencing dye (98% formamide, 10 mM EDTA, pH 8.0, 0.1% xylene cyanol FF, and 0.1% bromphenol blue) and loaded onto a 25% denaturing polyacrylamide sequencing gel containing 3 M urea. After electrophoresis, the radioactive oligoribonucleotide products were analyzed using a Fuji BAS 1000 Bioimaging analyzer.

dTTPase Assay—Gp4 catalyzes the ssDNA-dependent hydrolysis of dTTP, a reaction coupled to its translocation on ssDNA (38). The reac-

tion (10 μl) contained 1.2 nM M13 ssDNA, 0.5 mM [α-^{32}P]dTTP (0.1 μCi), 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM potassium glutamate. The reaction mixtures were preincubated with the indicated amount of ssDNA-binding protein for 5 min at 37 °C, and the reactions were initiated by the addition of 80 nM gp4. After further incubation at 37 °C for 30 min, the reactions were terminated by adding EDTA to a final concentration of 25 mM. The reaction mixture was spotted onto a polyethyleneimine cellulose thin layer chromatography (TLC) plate. The TLC plate was developed with a solution containing 1 M formic acid and 0.8 M LiCl. The TLC plate was analyzed using a Fuji BAS 1000 Bioimaging analyzer.

DNA Unwinding Assay—The assay for helicase activity measures the release of a radioactively labeled oligonucleotide partially annealed to a complementary ssDNA (39). A helicase substrate was prepared by annealing a 5′-end-labeled 75-mer oligonucleotide S75 (Table I) to a 95-mer oligonucleotide L95 (Table I) in 0.1 M NaCl. The helicase reaction (10 μl) contained 60 nM DNA substrate, 1 mM dTTP, 40 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, and 50 mM potassium glutamate. The reaction mixtures were preincubated with the indicated amount of ssDNA-binding protein for 5 min at 37 °C, and the reactions were initiated by the addition of 80 nM gp4. After further incubation for 10 min at 37 °C, the reaction was terminated by adding EDTA to a final concentration of 25 mM. The reaction mixture was loaded onto a 10% denaturing polyacrylamide gel. After electrophoresis, the radioactive oligonucleotide disassociated from the partial duplex was measured using a Fuji BAS 1000 Bioimaging analyzer. In a control experiment in which 60 nM each of ssDNA 75 and 95-mer were incubated, we found that there is significant annealing (~30%) of the DNA strands. Therefore, it is likely that some reannealing of ssDNA strands arising during the helicase reaction occurs, thus leading to an underestimation of DNA unwinding.

RESULTS

Effect of Gp2.5 and E. coli SSB Protein on Primer Synthesis—An earlier study using a gp4 preparation enriched for the 63-kDa species showed that gp2.5-stimulated gp4 catalyzed oligoribonucleotide synthesis on M13 ssDNA 3.5-fold (40). Subsequent studies confirmed this result and also revealed that gp2.5-Δ21C, lacking its C-terminal 21 amino acids, severely inhibited oligoribonucleotide synthesis (13). In that study, *E. coli* SSB protein stimulated oligoribonucleotide synthesis ~2-fold, whereas T4 gp32 protein inhibited synthesis by >10-fold (13). To further characterize these interactions, we examined the ability of WT gp2.5 and *E. coli* SSB protein to affect gp4-catalyzed primer synthesis at various concentrations. Primase activity was assayed using M13 ssDNA coated with either WT gp2.5 or *E. coli* SSB protein. Consistent with the previous studies, both gp2.5 and *E. coli* SSB protein stimulated oligoribonucleotide synthesis ~2–3 fold (Fig. 1*A*). Our earlier studies using gel shift analysis have shown that 1 μM gp2.5 is sufficient to coat 3.3 nM 70-mer ssDNA (21). In this experiment containing 4 nM M13 ssDNA, we used a gp2.5 concentration of 20 μM, which is sufficient to coat 20 nM M13 ssDNA based on the assumption that one gp2.5 molecule coats 6–7 nucleotides on ssDNA (8). *E. coli* SSB protein has approximately a 10-fold higher affinity for ssDNA relative to gp2.5 (8). Interestingly, maximal stimulation occurred with the lowest amount of gp2.5 (2 μM), an amount sufficient to coat 50% of the ssDNA. *E. coli* SSB protein stimulated oligoribonucleotide synthesis at an even lower concentration of 0.2 μM (Fig. 1*A*).

To eliminate the effect of secondary structure in the M13 DNA substrate, we used a 70-mer oligonucleotide, ZH70, containing only one primase site as a template (Table I) to analyze primer synthesis. For the assay, an increasing concentration of ssDNA-binding protein was present to coat the ssDNA in the reaction. Consistent with the results obtained with M13 ssDNA, both WT gp2.5 and *E. coli* SSB protein stimulated the primase activity of gp4 at low concentrations (Fig. 1*B*).

Effect of Gp2.5 and E. coli SSB Protein on Binding of Gp4 to ssDNA—The first step of primer synthesis is the loading of gp4 onto the ssDNA template. To determine whether ssDNA-binding proteins affect the ability of gp4 to load onto ssDNA template, we used a non-hydrolyzable analog of dTTP, β,γ-Me-dTTP (41). Previous studies have shown that β,γ-Me-dTTP promotes the binding of gp4 to ssDNA (K_d = 10 nM)[2] but does not allow for translocation on ssDNA (41). Initially, gp4 was loaded onto either a 20- or 70-nt oligonucleotide in a 1-min incubation in the presence of β,γ-Me-dTTP. gp2.5 or *E. coli* SSB protein then was added in excess of the oligonucleotide. After a 30-min incubation, oligoribonucleotide synthesis was assessed. The amount of oligoribonucleotide synthesized was essentially identical on the 20- and 70-mer template (Fig. 2*A*). The presence or absence of either gp2.5 or *E. coli* SSB protein had no effect on oligoribonucleotide synthesis on either template other than the slight stimulation observed in Fig. 1. As would be expected, the amounts of primer synthesis after 30 min (*lanes 2* and *6*) were much higher than observed for the 1-min reactions (*lanes 1* and *5*) (Fig. 2*A*). These results demonstrate that neither the presence of WT gp2.5 nor *E. coli* SSB protein inhibits the binding of gp4 to the ssDNA and the synthesis of oligoribonucleotide.

To assess the ability of gp4 to load and perform primer

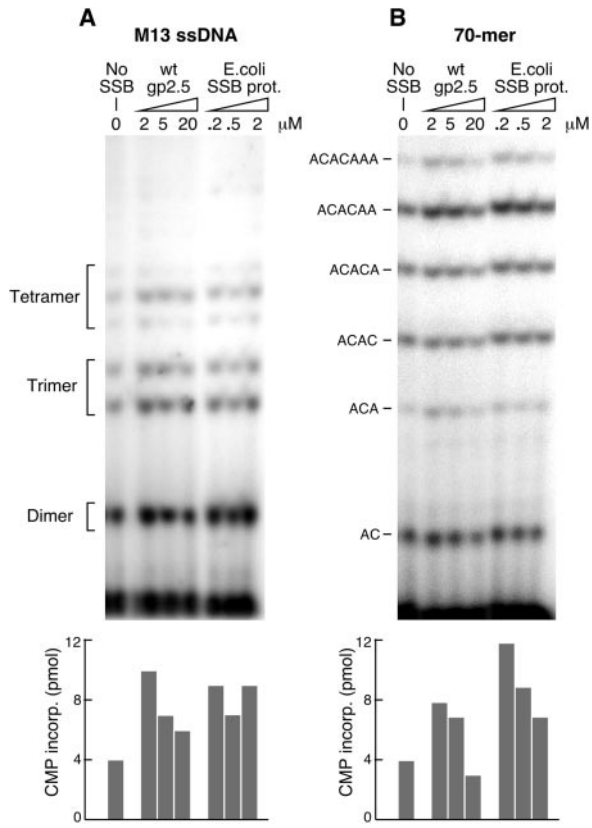

FIG. 1. **Effect of gp2.5 and *E. coli* SSB protein on gp4-catalyzed oligoribonucleotide synthesis.** Oligoribonucleotide synthesis catalyzed by T7 gp4 was determined after electrophoretic separation of the products as described under "Experimental Procedures." The reaction mixture contained 4 nM M13 ssDNA or 400 nM oligonucleotide template, 0.3 mM each of ATP and [α-^{32}P]CTP (0.1 μCi), 0.5 mM dTTP, 80 nM (monomeric concentration) gp4, and the indicated concentration of ssDNA-binding proteins. After a 5-min preincubation at 37 °C with ssDNA-binding protein and ssDNA, the reactions were initiated by the addition of 80 nM gp4. *A*, M13 ssDNA as template for primer synthesis. *B*, 70-mer oligonucleotide as template. The sites of oligoribonucleotides are indicated. After electrophoresis, the radioactive oligoribonucleotide products were analyzed quantitatively using a Fuji BAS 1000 Bioimaging analyzer. The amount of oligoribonucleotide synthesized is presented in the graph below the autoradiographs.

[2] D. Crampton and C. C. Richardson, unpublished data.

TABLE I
Oligonucleotides (Oligo) and their sequences

Oligo	Sequence (5′→3′)	Underlined sequence
S75[a]	CGCCGGGTACCGAGCTCGAATTCACTGGCCGTCGTTTTACAACGTCGTGACATGCTTTTTTTTTT TTTTTTTTTT	Annealed nucleotides
L95[a]	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCATGTCACGACGTTGTAAAACGAC GGCCAGTGAATTCGAGCTCGGTACCCGGCG	Annealed nucleotides
ZH70	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGTGTCCCCCCCCCCCCCCCC	Pimase site
ZHP20	GTGTCTTTTTTTTTTTTTTT	Primase site
ZHP70	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCGTGTCTTTTTTTTTTTTTT	Primase site

[a] S75 and L95 oligonucleotides were annealed to generate the DNA substrate for measuring helicase activity.

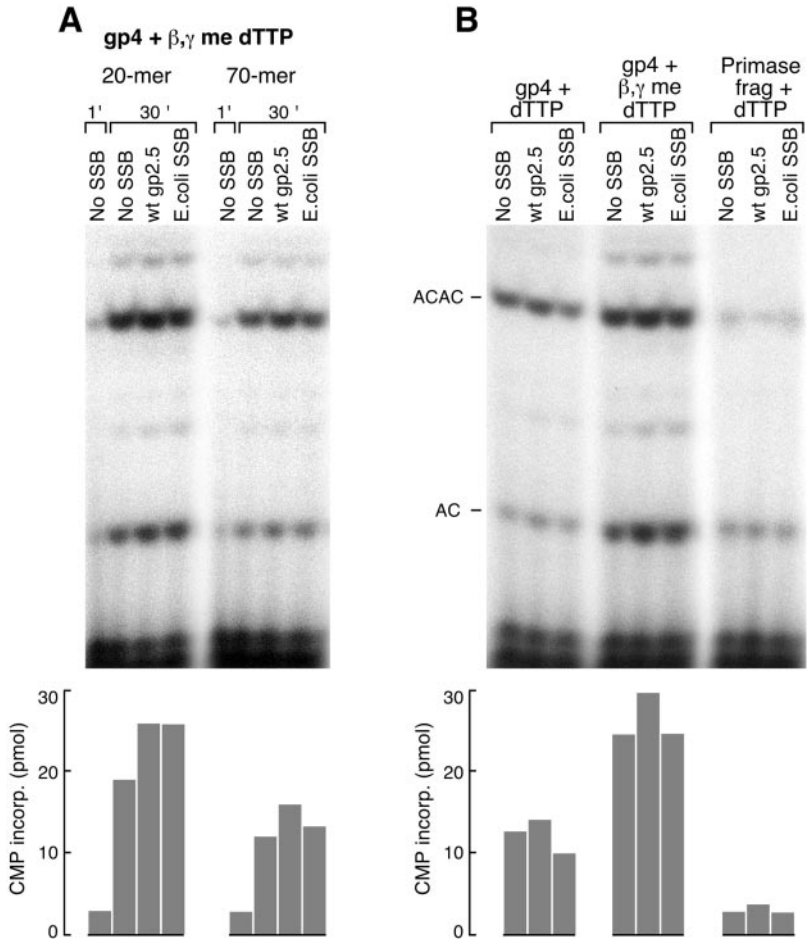

FIG. 2. **Effect of ssDNA-binding protein on gp4 for ssDNA.** Oligoribonucleotide synthesis catalyzed by gp4 and gene 4 primase fragment was determined by measuring the amount of radioactively labeled oligoribonucleotide product after electrophoretic separation of the products as described under "Experimental Procedures." The reaction mixture contained 400 nM oligonucleotide, 0.3 mM each of ATP and [α-^{32}P]CTP (0.1 μCi), 80 nM (monomeric concentration) gp4 or 400 nM primase fragment, and the indicated amount of ssDNA-binding protein. *A*, autoradiograph of products of primer synthesis using 20 or 70-mer templates and nonhydrolyzable β,γ-Me-dTTP. After a 1-min preincubation of gp4 with the template, either WT gp2.5 (40 μM) or *E. coli* SSB protein (20 μM) was added. After an additional 30 min, the reaction products were analyzed by electrophoretic separation. *B*, autoradiograph of the production of primer synthesis on the 20-mer template in the presence of either β,γ-Me-dTTP or dTTP. After preincubation with 40 μM gp2.5 or *E. coli* SSB protein for 5 min at 37 °C, primer synthesis reactions were initiated by the addition of either gp4 or primase fragment. The products were separated as described above, and the radioactive oligoribonucleotide products were analyzed quantitatively using a Fuji BAS 1000 Bioimaging analyzer. The amount of oligoribonucleotide synthesized is presented in the graph below the autoradiographs.

synthesis on a short template, the 20-mer template was coated with gp2.5 or *E. coli* SSB protein. As shown in Fig. 2*B* (*lanes 1–3*), WT gp4 in the presence of dTTP can load equally well onto the 20-mer template precoated with either gp2.5 or *E. coli* SSB protein. The efficiency of gp4 in the presence of dTTP is ~700 nM.[2] To determine whether translocation of gp4 is necessary for this effect, we substituted the non-hydrolyzable analog β,γ-Me-dTTP for dTTP (lane 4–6). In the presence of the non-hydrolyzable analog, there is a 2–3-fold increase in primer synthesis (Fig. 2*B*). The primase fragment of gp4 lacking the helicase domain is considerably less efficient for primer synthesis (Fig. 2*B*, *lanes 7–9*). The lower efficiency derives from its inability to bind tightly to ssDNA via hexamer formation (36). These results demonstrate that gp4 and the primase fragment have no difficulty loading onto the 20-mer template in the presence of either WT gp2.5 or *E. coli* SSB protein.

Effect of Gp2.5 and E. coli SSB Protein on dTTPase Activity—The unidirectional 5′→3′-translocation of gp4 on ssDNA is fueled by the hydrolysis of dTTP (41). The translocation on ssDNA is essential for helicase activity, and it also enables the primase domain to access distal primase recognition sites (30). To examine the effect of gp2.5 and *E. coli* SSB protein on ssDNA-dependent dTTPase activity of gp4, we measured dTTPase activity at increasing concentrations of the two ssDNA-binding proteins with 1.2 nM M13 ssDNA (Fig. 3, *A* and *B*). In this experiment, the amount of gp2.5 (15 μM) we used is sufficient to coat 15 nM M13 ssDNA (8). Gp2.5 stimulates the dTTPase activity ~25% at concentrations greater than 3 μM (Fig. 3, *A* and *B*). *E. coli* SSB protein, on the other hand, leads to a slight (15%) inhibition when present at 2 μM or higher (Fig. 3, *A* and *B*). Interestingly, the amount of gp2.5 and *E. coli* SSB protein required to coat the M13 ssDNA is 1.2 and 1 μM, respectively. Thus, the relatively small effects of the ssDNA-binding protein on dTTPase activity most probably arises when the ssDNA is fully coated with the ssDNA-binding proteins (Fig. 3).

Effect of Gp2.5 and E. coli SSB Protein on Unwinding Activity—To determine whether the unwinding ability of gp4 is affected by the presence of ssDNA-binding proteins, helicase activity was measured in the presence and absence of gp2.5 and *E. coli* SSB protein. The DNA substrate used in the unwinding assay is shown in Fig. 4*A*. The DNA substrate consists of a

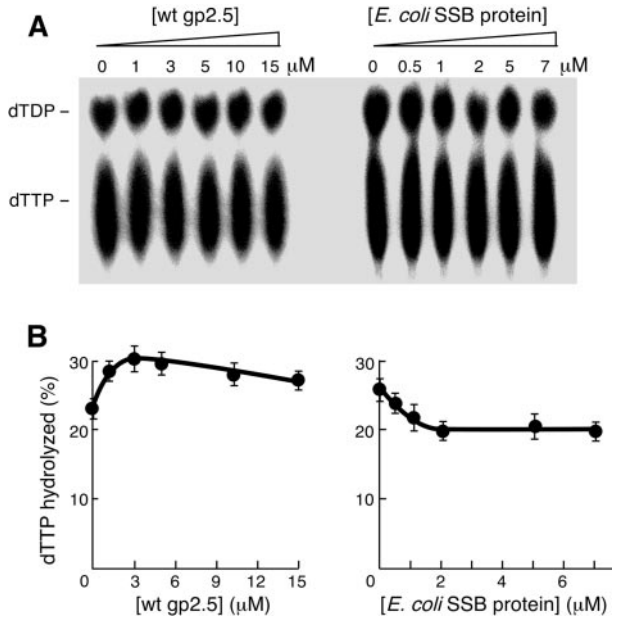

FIG. 3. **Effect of gp2.5 and *E. coli* SSB protein on ssDNA-dependent hydrolysis of dTTP.** Gp4 catalyzes the ssDNA-dependent hydrolysis of dTTP to dTDP and P_i. Each reaction contained 1.2 nM M13 ssDNA, 0.5 mM [α-^{32}P]dTTP (0.1 μCi), 80 nM (monomeric concentration) gp4, and the indicated concentration of either gp2.5 or *E. coli* SSB protein. After incubation at 37 °C for 30 min, the [^{32}P]dTDP product of the reaction was analyzed by TLC as described under "Experimental Procedures." The TLC plate was exposed to x-ray film (*A*), and radioactive product was quantitatively analyzed by using a Fuji BAS 1000 Bioimaging analyzer. The dTDP produced is plotted as a function of the concentration (examined as monomer) of the ssDNA-binding protein (*B*). The data represent an average of three independent experiments, and the *error bars* represent the range of values obtained in those experiments.

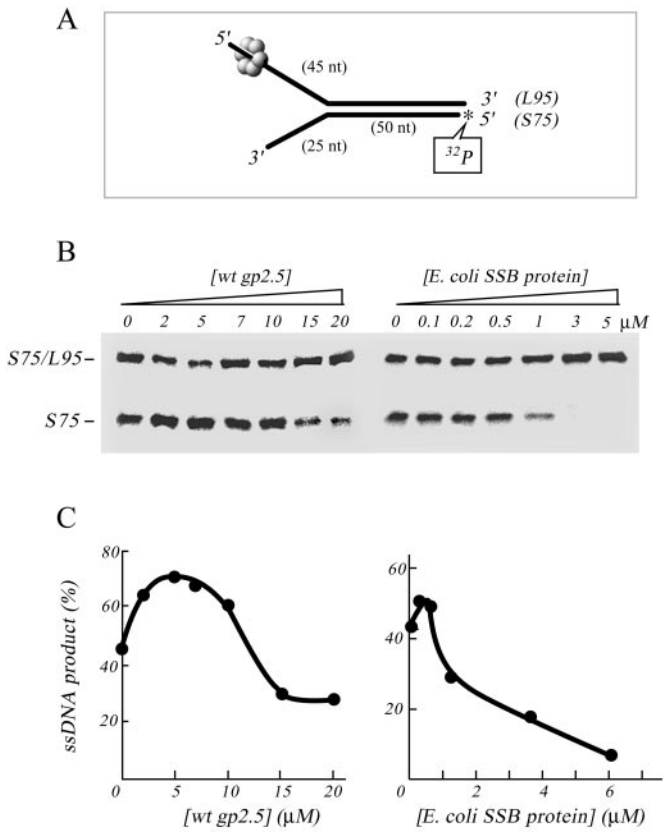

FIG. 4. **Effect of gp2.5 and *E. coli* SSB protein on the unwinding activity of gp4.** *A*, schematic illustration of partially annealed DNA substrate used in the unwinding assay. A 5′-^{32}P-radiolabeled 75-mer oligonucleotide, S75, was annealed to a 95-mer oligonucleotide, L95 (Table I), to generate a helicase DNA substrate. The substrate contains a 50-nt double-stranded sDNA region with a 45-nt 5′-ssDNA tail arising from L95 and a 25-nt 3′-ssDNA tail arising from S75. *B*, denaturing polyacrylamide gel analysis of products of unwinding activity. The reaction contained 60 nM helicase DNA substrate, varying amounts of either gp2.5 or *E. coli* SSB proteins, 1 mM dTTP, and 80 nM gp4. Reaction products were separated as described under "Experimental Procedures." *C*, the radioactive 75-mer released from the partial duplex substrate by the helicase action was measured using a Fuji BAS 1000 Bioimaging analyzer. The amount of radioactively labeled S75 oligomer released from the duplex in the reactions shown in *B* is plotted as a function of the concentration of the two ssDNA-binding proteins. As described under "Experimental Procedures," this assay underestimates the amount of unwinding due to reannealing of the separated strands.

partial duplex (50 nt) DNA with a 5′- and a 3′-single-stranded tail (45 and 25 nt, respectively). The 5′-tail provides a site for gp4 to assemble as a hexamer, and the 3′-tail prevents gp4 from translocating over the duplex DNA (42). After the substrate was coated with one of the ssDNA-binding proteins, gp4 was added to initiate the unwinding reaction. Gp2.5 stimulates the unwinding activity ~1.7-fold at 5 μM (Fig. 4, *B* and *C*). The stimulative effect is manifest between 3 and 10 μM, after which it has a slight inhibitory effect. In contrast, *E. coli* SSB protein has only a slight (10%) stimulatory effect on unwinding activity at a very low concentration (1 μM). More striking is the inhibitory effect at the high concentration of SSB protein, resulting in a 90% inhibition at 6 μM (Fig. 4, *B* and *C*). Neither ssDNA-binding protein has any unwinding activity alone (<1%) (data not shown).

Effect of Variants of Gp2.5 on dTTPase Activity of Gp4—Previous studies have shown that the C-terminal acidic tail of gp2.5 is critical for its interaction with itself and the other T7 replication proteins. T7 gp2.5-Δ26C or gp2.5-Δ21C, lacking the C-terminal 26 or 21 amino acids, respectively, do not form dimers or physically interact with T7 DNA polymerase or T7 gp4 (8, 20). These variant gp2.5s have a 10-fold greater affinity for ssDNA relative to the WT gp2.5 (7, 8, 21). They also inhibit DNA synthesis catalyzed by T7 DNA polymerase on M13 ssDNA, and gp2.5-Δ21C has been shown to inhibit the primase activity of gp4 (13). Another genetically altered gp2.5, gp2.5-F232L, has a single amino change in the C-terminal residue (7, 20). This lethal mutation gives rise to a gp2.5 that binds ~3-fold tighter to ssDNA relative to WT gp2.5 (7). However, in contrast to the C-terminal deletion mutations, gp2.5-F232L dimerizes and maintains its interactions with T7 DNA polymerase (7). In fact, gp2.5-F232L stimulates T7 DNA polymerase activity on ssDNA templates (7). We have used these genetically altered gp2.5s to characterize further the interactions of gp2.5 with gp4.

In the experiment shown in Fig. 5, we have compared the effects of gp2.5-Δ26C and gp2.5-F232L on the ssDNA-dependent dTTPase activity of gp4. Wild-type gp2.5 is included for comparison. In contrast to the slight stimulation observed with wild-type gp2.5, both of the variant gp2.5s inhibited dTTPase activity (Fig. 5). Gp2.5-Δ26C is clearly the most inhibitory. Inhibition is observed with the lowest concentration (0.5 μM) of the protein, and the activity is essentially abolished at concentrations greater than 1 μM (Fig. 5*C*). The amount of gp2.5 sufficient to coat the M13 ssDNA is 1.2 μM. Gp2.5-F232L inhibits dTTPase activity at concentrations greater than 3 μM, and the activity is reduced by 50% at the highest concentration of 15 μM (Fig. 5*B*). However, there is a slight stimulation of activity at the low concentrations, resembling the pattern observed with wild-type gp2.5.

Effect of Variants of Gp2.5 on DNA Unwinding by Gp4—The effect of gp2.5-Δ26C and gp2.5-F232L on the unwinding activity of gp4 was examined using the DNA substrate and unwind-

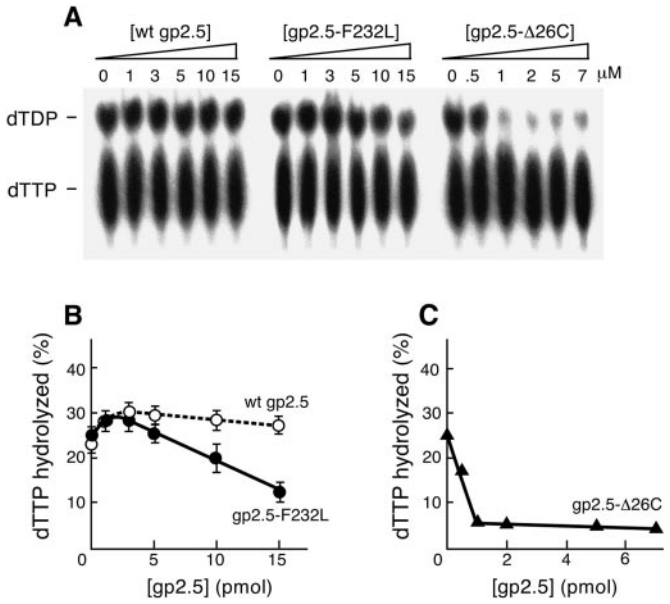

FIG. 5. **Gp4 catalyzes the ssDNA-dependent hydrolysis of dTTP in the presence of variants of gp2.5.** ssDNA-dependent hydrolysis of dTTP was measured in a reaction containing 1.2 nM M13 ssDNA, 0.5 mM [α-^{32}P]dTTP (0.1 μCi), 80 nM gp4, and the indicated concentration of gp2.5. The reactions were performed, and the radioactive product [^{32}P]dTDP of the reaction was analyzed by TLC as described under "Experimental Procedures." The TLC plate was exposed to x-ray film (*A*), and the radioactive product was quantitatively analyzed using a Fuji BAS 1000 Bioimaging analyzer (*B*). *C*, the percentage of dTTP hydrolysis is plotted as the function of the concentration of the different ssDNA-binding proteins. The data represent an average of three independent experiments, and the error bars represent the range of values obtained in those experiments.

ing assay outlined in Fig. 4. As noted before, wild-type gp2.5 displayed a stimulation of unwinding activity at concentrations up to 10 μM. Not surprisingly, in view of its inhibition of dTTPase activity and hence translocation, gp2.5-Δ26C strongly inhibited unwinding catalyzed by gp4 (Fig. 6, *A* and *B*). Gp2.5-F232L on the other hand behaved in a manner similar to wild-type gp2.5 with the exception that the range of concentration providing stimulation was more narrow (Fig. 6, *A* and *B*).

Effect of Variants of Gp2.5 on Primer Synthesis Catalyzed by Gp4—As in the earlier experiments with gp2.5 and *E. coli* SSB protein, we examined the effect of gp2.5-Δ26C and gp2.5-F232L on oligoribonucleotide synthesis catalyzed by gp4 on a 70-mer oligonucleotide containing a primase recognition site (Fig. 7). As found earlier in this study, wild-type gp2.5 had a slight effect on primase activity, providing an initial slight stimulation and at higher concentrations a 30% inhibition (Fig. 7*A*). Consistent with previous results (13), gp2.5-Δ26C is strikingly inhibitory to primase activity, whereas gp2.5-F232L resembles wild-type gp2.5 with a somewhat greater inhibition observed at the higher concentrations (Fig. 7*A*). Oligoribonucleotide synthesis catalyzed by gp4 first requires the recognition of a primase recognition sequence and subsequently the polymerization of nucleotides (30, 34). The recognition of the basic recognition sequence 5′-GTC-3′ is mediated by the Cys_4 zinc motif of the primase domain (5, 34, 35). Efficient recognition of these sites on ssDNA requires the helicase activity of gp4, which not only translocates the primase domain to the site but also tethers the primase domain to the DNA via the tight binding of the hexameric helicase to the ssDNA (36). Because the variant gp2.5s inhibit the translocation of gp4 on ssDNA as shown above, at least a portion of inhibition of primase activity observed in Fig. 7*A* is probably the result of the inability of the helicase domain to properly position the primase domain at its recognition site. To dissect these parameters, we have used the primase fragment of gp4 lacking the helicase domain. As shown in Fig. 7*B*, neither gp2.5-Δ26C nor gp2.5-F232L had any affect on oligonucleotide synthesis catalyzed by the primase fragment. It is important to note that oligoribonucleotide synthesis catalyzed by the primase fragment is considerably less efficient than that observed when it is covalently attached to the helicase domain, necessitating a 10-fold higher concentration of primase fragment relative to that used for the full-length protein in Fig. 7, *panel A*. Nonetheless, the inherent activity of the primase is unchanged by the presence of these variant gp2.5s.

DISCUSSION

The gene 4 helicase-primase of bacteriophage T7 has multiple functions at the T7 replication fork. The primase domain catalyzes the synthesis of oligoribonucleotides on the lagging strand that, in turn, are transferred to T7 DNA polymerase for use as primers (43–45). The helicase domain is responsible for the 5′ to 3′-unidirectional translocation of the protein on ssDNA as well as the unwinding of duplex DNA to generate single-stranded template for the leading strand DNA polymerase (24, 39, 46, 47). Previous studies have shown that gp2.5 is essential for the coordination of leading and lagging strand DNA synthesis at a replication fork (2, 3). Because gp2.5 is known to physically interact with two other major constituents of the T7 replisome, T7 DNA polymerase (5) and gp4 (6), as well as bind to their ssDNA substrates, it is important to understand the consequences of these interactions on their biochemical properties.

In a recent study (7), we demonstrated that interactions of gp2.5 with T7 DNA polymerase are important for its ability to catalyze DNA synthesis on gp2.5-coated ssDNA. In the present study, we have examined the effect of gp2.5 on both the primase and helicase activities of gp4. These studies have been facilitated by a comparison of wild-type gp2.5 with genetically altered gp2.5 proteins and with *E. coli* SSB protein. *E. coli* SSB protein cannot substitute for T7 gp2.5 during phage T7 growth, and it has a 10-fold higher affinity for ssDNA relative to gp2.5 (1). The two altered gp2.5 proteins, gp2.5-Δ26C and gp2.5-F232L, have defects in their acidic C-terminal tails, a subdomain that is essential for the protein-protein interactions described above, and similar to *E. coli* SSB protein, they display a higher affinity for ssDNA compared with wild-type gp2.5 (7).

The ability of gp4 to assemble onto ssDNA and to translocate along the DNA strand can be quantitatively assessed by measuring the ssDNA-dependent hydrolysis of dTTP (39). Using this assay, we find that the binding of either gp2.5 or *E. coli* SSB protein to ssDNA has little effect on the ability of gp4 to translocate on ssDNA. In contrast, gp2.5-Δ26C lacking the C-terminal tail is very inhibitory for translocation. Gp2.5-F232L also showed a significant inhibition at relatively higher concentrations. Because *E. coli* SSB protein has the highest affinity for ssDNA yet does not inhibit translocation, it seems probable that the inhibitory properties of gp2.5-Δ26C derive from its inability to interact with gp4. Our earlier studies with chimeric ssDNA-binding proteins showed that this subdomain could be interchanged among the T4, T7, and *E. coli* ssDNA-binding proteins (13). In any case, it seems unlikely that gp4 frequently encounters gp2.5-coated ssDNA at the replication fork because it is closely associated with T7 DNA polymerase as it invades duplex DNA with the ssDNA extruded behind gp4.

The effect of the ssDNA-binding proteins on unwinding of duplex DNA mimics their effects on translocation, albeit they are more striking. *E. coli* SSB protein and gp2.5-Δ26C are very inhibitory at relatively low concentrations. Surprisingly, both wild-type gp2.5 and gp2.5-F232L are inhibitory at high concentrations, a finding for which we have no explanation unless it

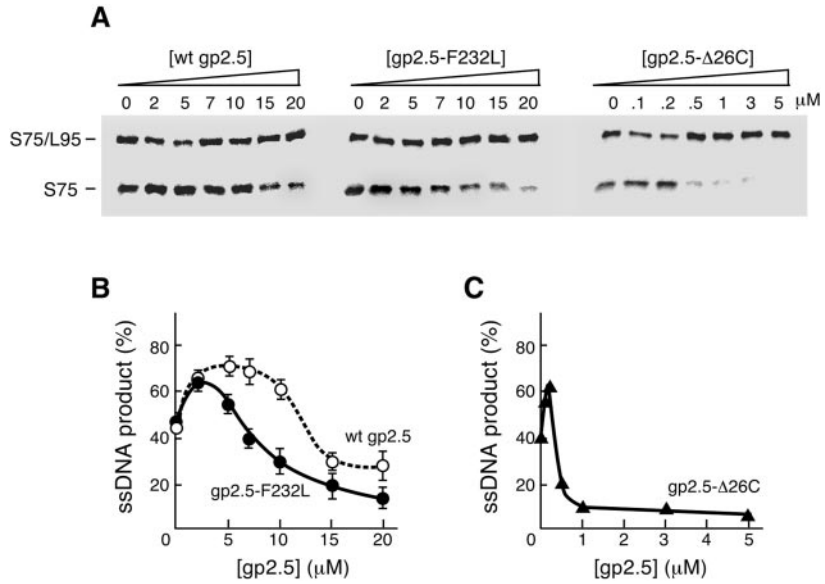

FIG. 6. **Effect of variants of gp2.5 on the unwinding activity of gp4.** DNA unwinding catalyzed by gp4 was measured using the DNA substrate and assay described in Fig. 4. The reaction contained 60 nM DNA substrate, 1 mM dTTP, 80 nM gp4, and the indicated concentration of gp2.5. The reaction products were separated as described under "Experimental Procedures." *A*, radioactive oligonucleotide separated from the partial duplex DNA substrate by gp4 was measured using a Fuji BAS 1000 Bioimaging analyzer. The percentage of the duplex DNA unwinding in *panel A* is plotted as a function of the concentration of the ssDNA-binding protein (*B* and *C*). The data represent an average of three independent experiments, and error bars represent the range of values obtained in those experiments. As described under "Experimental Procedures," this assay underestimates the amount of unwinding due to reannealing of the separated strands.

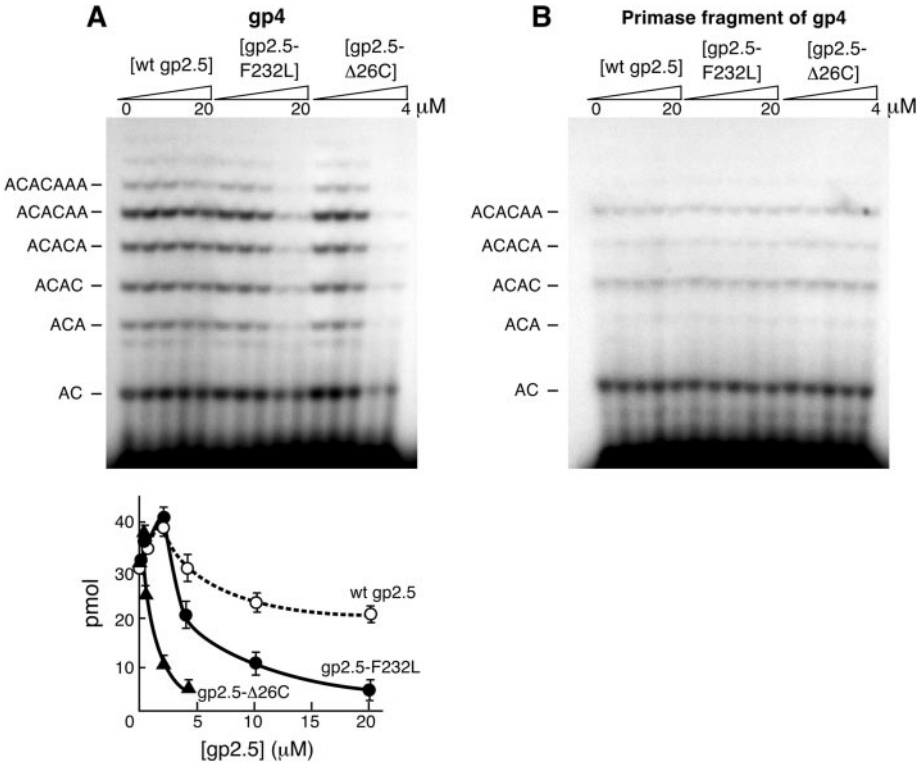

FIG. 7. **Effect of variants of gp2.5 on primase activity.** Oligoribonucleotide synthesis catalyzed by gp4 or the primase fragment was performed as previously described using a 70-mer oligonucleotide containing a primase recognition site. The reaction mixture contained 400 nM synthetic 70-mer template ZH70, 0.3 mM each of ATP and [α-^{32}P]CTP (0.1 μCi), 80 nM monomeric concentration gp4 (*A*) or 800 nM primase fragment (*B*), and different concentrations of WT gp2.5 or gp2.5-F232L (0, 0.5, 2, 10, and 20 μM) or gp2.5-Δ26C (0, 0.1, 0.4, 2, and 4 μM). After a 5-min preincubation at 37 °C of ssDNA-binding protein with ssDNA, the reactions were initiated by the addition of gp4 or primase fragment. After incubation for 30 min, primase activity was determined by measuring the amount of radioactively labeled oligoribonucleotides synthesized. Quantitative analysis of the products synthesized by gp4 shown in *panel A* was carried out with a Fuji BAS 1000 Bioimaging analyzer. The [α-^{32}P]CMP incorporated into oligoribonucleotide (pmol) is plotted as the function of the concentration of gp2.5. The sites of oligoribonucleotides synthesized are indicated. The data represent an average of three independent experiments, and the error bars represent the range of values obtained in those experiments.

derives from the ability of gp2.5 to mediate homologous base pairing, thus leading to reannealing of the DNA strands after their separation by helicase action.

Evaluation of the effect of gp2.5 on primase activity is more difficult to assess. Primase activity is stimulated by the binding of gp4 via its helicase domain to ssDNA (36). In addition, the ability of gp4 to translocate on ssDNA, again via its helicase domain, enables it to reach distal primase recognition sites on a DNA strand. Hence, any effect of gp2.5 on the translocation activity of gp2.5 will be manifest in primase activity as well. However, this problem can be circumvented by the use of a primase fragment containing only the primase domain of gp4 or the use of very short oligonucleotides so that translocation is not a prerequisite to primer synthesis. However, the latter DNA template does not eliminate the tighter binding of the full-length gp4 to ssDNA via hexamer formation, a reaction that stimulates primase activity significantly (36). We find that gp4 can load onto either gp2.5 or *E. coli* SSB protein-coated ssDNA without difficulty, and once loaded onto ssDNA, earlier studies have shown that primase sites can be accessed far distal to the binding site for helicase domain. Taking all of these parameters into consideration, our results show that gp2.5 or *E. coli* SSB protein stimulates primer synthesis at low concentrations. Both gp2.5-Δ26C and gp2.5-F232L are both quite inhibitory to DNA primase activity, a result that can be explained by their inhibition of translocation of gp4 along ssDNA. This latter interpretation is supported by the inhibition of the DNA-dependent dTTPase activity by these binding proteins (Fig. 5).

Previously, we have shown that gp2.5-Δ26C ($K_d = 1.1 \times 10^{-7}$ M) has a 40-fold better affinity for ssDNA than WT protein ($K_d = 4.6 \times 10^{-6}$ M) and gp2.5-F232L ($K_d = 1.5 \times 10^{-6}$ M) has

an affinity for ssDNA that is between those of WT gp2.5 and gp2.5-Δ26C (7). In this study, we found that inhibition of primase activity by gp2.5-Δ26C begins at a concentration of 0.1 μM, whereas gp2.5-F232L inhibits primer synthesis at a concentration of approximately 1 μM. WT gp2.5 does not inhibit primer synthesis until the concentration reaches 3 μM (Fig. 7). Thus, the variant gp2.5s inhibit gp4 primase activity proportional to their increased affinities for DNA. Similarly, altered gp2.5s inhibit dTTPase and helicase activities of gp4 and the inhibitions increase with increasing concentrations of gp2.5s (Figs. 5 and 6). This property appears to be correlated with the DNA binding activity of the gp2.5 protein as well.

It was shown previously (7, 20) that gp2.5-F232L does not support the growth of T7Δ2.5 phage lacking gene 2.5. At the time, it seemed unlikely that the 3-fold increase in the affinity of this altered protein for ssDNA and its slightly reduced interaction with T7 DNA polymerase could explain its inability to support T7 growth. Our finding (7) that gp2.5-F232L could promote strand displacement synthesis catalyzed by T7 DNA polymerase raised the possibility that such a reaction could displace Okazaki fragments on the lagging strand or interfere with the multiple recombination events that occur during phage infection. The observation reported here that gp2.5-F232L inhibits the primase activity of gp4 provides an alternative explanation for its lethal phenotype.

Based on an earlier observation that gp4 helicase-mediated strand transfer occurs in the presence of gp2.5 but not T4 gp32, it was suggested that T7 gp4 is unable to load onto ssDNA coated with T4 gp32 (14). Our current results show that gp4 can load onto ssDNA coated with *E. coli* SSB protein and with gp2.5 proteins that have altered DNA binding properties, suggesting that this parameter may not be important. It is important to consider that *E. coli* SSB protein is most probably present in the cell during T7 DNA replication and therefore the T7 replication may have evolved mechanisms to ignore or to utilize some of the properties of *E. coli* SSB protein. Unfortunately, the earlier studies on strand transfer activity did not differentiate among loading, translocation, or unwinding of duplex DNA.

The existence of relatively large amounts of *E. coli* SSB protein in T7 phage-infected cells as discussed above poses several questions. Why can SSB protein not substitute for gp2.5 in T7 DNA replication, and why does it not interfere with T7 DNA replication? *E. coli* SSB protein stimulates T7 DNA polymerase activity on ssDNA even more so than does gp2.5 (7, 8, 13), and it affects gene 4 primase activity similar to T7 gp2.5. Most probably, gp2.5 has assumed additional responsibilities in the phage-infected cell, responsibilities not mediated by *E. coli* SSB protein. For example, gp2.5 mediates homologous base pairing, and this activity most probably is essential for concatamer formation and recombination events in phage-infected cells (5). On the other hand, *E. coli* SSB protein has some properties that could prove detrimental to T7 DNA replication. *E. coli* SSB protein enables T7 DNA polymerase to catalyze strand-displacement synthesis (48, 49), and as shown in the present study, it inhibits unwinding of duplex DNA catalyzed by gp4. The significantly tighter binding of *E. coli* SSB protein to ssDNA suggests that SSB protein would preferentially bind to ssDNA relative to gp2.5. One possibility is that specific interactions between gp2.5 and the T7 DNA replication proteins allow the replication machinery to essentially ignore or bypass *E. coli* SSB protein-coated regions of DNA.

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