

# The Carboxyl-terminal Domain of Bacteriophage T7 Single-stranded DNA-binding Protein Modulates DNA Binding and Interaction with T7 DNA Polymerase\*

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Gene 2.5 of bacteriophage T7 is an essential gene that encodes a single-stranded DNA-binding protein (gp2.5). Previous studies have demonstrated that the acidic carboxyl terminus of the protein is essential and that it mediates multiple protein-protein interactions. A screen for lethal mutations in gene 2.5 uncovered a variety of essential amino acids, among which was a single amino acid substitution, F232L, at the carboxyl-terminal residue. gp2.5-F232L exhibits a 3-fold increase in binding affinity for single-stranded DNA and a slightly lower affinity for T7 DNA polymerase when compared with wild type gp2.5. gp2.5-F232L stimulates the activity of T7 DNA polymerase and, in contrast to wild-type gp2.5, promotes strand displacement DNA synthesis by T7 DNA polymerase. A carboxyl-terminal truncation of gene 2.5 protein, gp2.5-Δ26C, binds single-stranded DNA 40-fold more tightly than the wild-type protein and cannot physically interact with T7 DNA polymerase. gp2.5-Δ26C is inhibitory for DNA synthesis catalyzed by T7 DNA polymerase on single-stranded DNA, and it does not stimulate strand displacement DNA synthesis at high concentration. The biochemical and genetic data support a model in which the carboxyl-terminal tail modulates DNA binding and mediates essential interactions with T7 DNA polymerase.

Gene 2.5 of bacteriophage T7 encodes a single-stranded DNA (ssDNA)<sup>1</sup>-binding protein (gp2.5) that is essential for viral survival (1). gp2.5 modulates several important reactions in DNA replication, recombination, and repair (1–12). The fundamental reactions at the T7 phage replication fork can be reconstituted with only four proteins (13, 14): T7 gene 5 DNA polymerase, its processivity factor *Escherichia coli* thioredoxin (15, 16), T7 gene 4 helicase/primase (17–19), and T7 gp2.5. gp2.5 physically interacts with T7 DNA polymerase and T7 helicase/primase to stimulate their activities (6, 8). The binding of gp2.5 to ssDNA is critical because it affects both specific DNA-protein and

protein-protein interactions in the replisome (14, 20). In this regard it is essential for coupling leading and lagging strand DNA synthesis *in vitro* (14). gp2.5 is also essential for recombination in T7 phage-infected cells, and in addition to the interactions described above, it also mediates homologous base pairing (11).

Despite a lack of sequence homology, T7 gp2.5 is functionally similar to the extensively studied SSB protein of *E. coli* and the gene 32 protein of bacteriophage T4. Like gp2.5, they are both ssDNA-binding proteins, a class of ubiquitous proteins that are not only essential in DNA replication but also play key roles in DNA recombination and repair (7, 21). Biochemical studies have shown that these proteins, like T7 gp2.5, interact with other proteins at the replication fork. *E. coli* SSB protein interacts with *E. coli* DNA polymerase II, exonuclease I, and other proteins involved in replication (22–24). T4 gene 32 protein physically interacts with at least 10 T4-encoded proteins, including T4 DNA polymerase, that are involved in T4 metabolism (25). The crystal structure of a carboxyl-terminal deleted T7 gp2.5 reveals a conserved oligosaccharide/oligonucleotide binding fold, similar to that of T4 gene 32 protein and *E. coli* SSB protein. The structure also suggests models for DNA binding and dimerization of gp2.5 (26).

Genetic and biochemical experiments suggest that the physical interactions of gp2.5 are specific, as neither *E. coli* SSB protein nor T4 gene 32 protein can functionally replace gene 2.5 protein *in vivo* (1, 20). T7 gene 4 primase-helicase is unable to load onto ssDNA coated with gene 32 protein, a reaction that occurs readily with T7 gp2.5 protein-coated DNA (9). *E. coli* SSB protein, on the other hand, can stimulate T7 DNA polymerase activity, support strand displacement DNA synthesis (8, 27, 28), as well as permit T7 primase-helicase to load onto ssDNA. Moreover, gp2.5 increases the frequency of initiation by T7 primase-helicase, whereas *E. coli* SSB protein does not (6). This specificity for gp2.5 is not surprising as there is little sequence homology between the proteins, and gp2.5 differs from the other proteins significantly in a number of biochemical properties. For instance, the T7 protein binds to ssDNA with a lower affinity than *E. coli* SSB protein or T4 gene 32 protein (7). The oligomeric state of these proteins also differs with gp2.5 existing as a stable dimer in solution (7), whereas *E. coli* SSB protein forms a tetramer (29). T4 gene 32 protein is a monomer that forms multimers at high concentrations (30, 31).

A number of genetic and biochemical studies have focused on the carboxyl-terminal region of gp2.5 (14, 20, 32), an essential domain of the protein. The carboxyl-terminal tail is quite acidic and is required to mediate interactions with the T7 replication proteins described above (32). A truncated gene 2.5 protein,

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<sup>1</sup> The abbreviations used are: ssDNA, single-stranded DNA; wt, wild-type.

gp2.5-Δ21C, which lacks the carboxyl-terminal 21 amino acids, cannot support T7 phage growth (32). Purified gp2.5-Δ21C does not form a dimer and does not interact with T7 DNA polymerase or T7 primase-helicase (32). Unlike the wild-type protein, gp2.5-Δ21C does not support the coordination of leading and lagging strand DNA synthesis *in vitro* (14).

The similar arrangement of domains in *E. coli* SSB protein, T4 gene 32 protein, and T7 gp2.5 suggests that the acidic carboxyl-terminal domains of these three proteins are functionally homologous (20). Interestingly, when the carboxyl-terminal acidic region of either *E. coli* SSB protein or T4 gene 32 protein replace the acidic tail of gp2.5, the chimeric proteins can substitute for T7 gene 2.5 protein to support the growth of phage T7, albeit less efficiently. In contrast, chimeric proteins in which the carboxyl-terminal tail of gp2.5 replaces that of *E. coli* SSB protein or T4 gene 32 protein cannot support growth of T7 phage (20). These results show that although the carboxyl terminus of gp2.5 is essential for protein-protein interaction, it alone cannot account for the specificity of the interaction.

To address further the role of gp2.5 in T7 DNA metabolism, we recently examined mutations in gene 2.5 from a random mutagenic screen of gp2.5 (33). Taken together with the crystal structure, these studies have provided insight into DNA binding and dimerization of the protein (33, 34). In this mutagenic screen, several amino acid changes were identified in the carboxyl-terminal tail. However, except for one mutant, all had multiple amino acid changes that accounted for their lethality. One mutant, however, had a single amino acid substitution, leucine replacing phenylalanine at position 232. This altered gp2.5-F232L could not complement T7 Δ2.5 lacking gene 2.5. In this paper we show that gp2.5-F232L binds more tightly to ssDNA and enables T7 DNA polymerase to catalyze strand displacement DNA synthesis. These studies, taken together with studies on gp2.5-Δ26C, support a role of the carboxyl terminus in modulating ssDNA binding and in interacting with T7 DNA polymerase.

## EXPERIMENTAL PROCEDURES

### Materials

**Bacterial Strains, Bacteriophages, and Plasmids**—*E. coli* BL21(DE3) (F<sup>-</sup> ompT hsdS<sub>B</sub> (r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal<sup>-</sup> 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. DNAs encoding His-tagged gene 2.5 proteins were cloned into the *Nde*I and *Bam*HI restriction sites of modified pET19bPPS vector as described previously (33). Details of the cloning procedure have been described previously (33). T7 gp2.5-Δ26C was obtained from Edel Hyland (Harvard Medical School).

**DNA and Oligonucleotides**—The 70-mer oligonucleotide GACCATA-TCCCTCCACCCTCCCAATATTGACCATCAACCCTTCACCTCACTTCACTCCACTATACCACTC-3' (14), provided by J. Lee (Harvard Medical School), was used in electrophoretic mobility shift assays for assessing binding of gp2.5 to ssDNA. M13mp18(+) and poly(dA)<sub>390</sub> templates used for DNA synthesis, were purchased from Amersham Biosciences. 5'-<sup>32</sup>P-End-labeled primer was annealed to M13mp1-2, a 9950-nucleotide derivative of vector M13mp8 (35), and used for DNA synthesis. Oligonucleotide 5'-GTTTTCCAGTCACGAC-3' and poly(dT)<sub>22</sub> used as primers for DNA synthesis were purchased from Amersham Biosciences. The 34-oligonucleotide TG, 5'-CTAATCAG-GAGGTCATAGCTGTTTCTGTGTGAA-3' that can be partially annealed to M13mp18 (unannealed nucleotides are underlined in the primer sequence), was synthesized by Integrated DNA Technologies. For cloning purposes the following oligonucleotides were purchased from Oligos Etc: T72.5 *Nde*I, 5'-CGTAGGATCCATATGGCTAAGAA-GATTTTCACCTC-3'; and T72.5 *Bam*HI, 5'-CGTAGGATCCACTTA-GAGGTCCTCCGTC-3'. The oligonucleotides pET17b upstream, 5'-CTT-TAAGAAGGAGATATACATATG-3', and pET17b downstream, 5'-GCTAGTTATTGCTCAGCGG-3', used for DNA sequencing were synthesized by the Biopolymer Facility, Harvard Medical School. All radioactive nucleotides were purchased from Amersham Biosciences.

**Proteins, Enzymes, and Chemicals**—Restriction enzymes, polynucle-

otide kinase, T4 DNA ligase, and calf intestinal phosphatase were purchased from New England Biolabs. *E. coli* SSB protein was purchased from U. S. Biochemical Corp. Donald Johnson (Harvard Medical School) supplied T7 DNA polymerase. All chemicals and reagents were from Sigma unless otherwise noted.

### Methods

**Mutagenesis of T7 gp2.5**—pET17b plasmids expressing lethal mutations in gene 2.5 were generated by a random mutagenesis as described previously (33). The plasmid harboring the altered gene 2.5 (694T→C) from which gp2.5-F232L was expressed was isolated from this library as described previously (33).

**Expression and Purification of gp2.5**—wt and gp2.5-F232L were purified as described previously (33) with the following changes. The plasmids pETGp2.5 and pETGp2.5-F232L were transformed into competent *E. coli* BL21(DE3) cells (Novagen). Eight liter cultures were grown in LB with 60 μg/ml ampicillin to an A<sub>595</sub> of 1.0. Cells harboring pETGp2.5 were induced at 37 °C for 4 h after adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Cells harboring pETGp2.5-F232L were induced at 30 °C for 6 h after adding isopropyl-β-D-thiogalactoside to a final concentration of 1 mM. Purified wt gp2.5 and gp2.5-F232L were greater than 99% pure as determined by SDS-PAGE and subsequent staining by Coomassie Blue. Protein concentrations were determined by spectrophotometric absorbance at 260 nm using the extinction coefficient of 2.58 × 10<sup>4</sup> M<sup>-1</sup>cm<sup>-1</sup> calculated according to Gill and Hippel (36).

**Molecular Weight Approximation by Gel Filtration Analysis**—Gel filtration analysis was performed as described previously (7, 33). gp2.5, gp2.5-F232L, and gp2.5-Δ26C were applied to a Superdex 75 column (Amersham Biosciences) and eluted in buffer G (50 mM KPO<sub>4</sub>, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 10% glycerol) at 4 °C. The fractional retention, K<sub>av</sub>, was calculated for each of the standard proteins using the equation K<sub>av</sub> = (V<sub>e</sub> - V<sub>0</sub>)/(V<sub>i</sub> - V<sub>0</sub>). A plot of K<sub>av</sub> value versus log<sub>10</sub> M<sub>r</sub> generated an equation, and from this the molecular weight of each gene 2.5 variant could be approximated based on its peak elution volume.

**Electrophoretic Mobility Shift Assay**—The binding of gp2.5 to ssDNA was performed on a 70-mer oligonucleotide using a modification of an electrophoretic mobility shift assay described previously (33, 34, 37). The oligonucleotide was radioactively labeled at its 5' terminus with <sup>32</sup>P using T4 polynucleotide kinase (New England Biolabs) and [γ-<sup>32</sup>P]ATP. The labeled oligonucleotide was purified using micro Bio-Spin P-30 chromatography columns (Bio-Rad). The reactions (15 μl) for measuring the mobility shift contained 3.3 nM <sup>32</sup>P-labeled 70-mer oligonucleotide and various concentrations (from 0 to 16,000 nM) of gp2.5 diluted in buffer containing 20 mM Tris-Cl, pH 7.5, 10 mM β-mercaptoethanol, and 500 μg/ml bovine serum albumin. The reaction buffer contained 15 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 50 mM KCl, 10% glycerol, 0.01% bromphenol blue. Reactions were performed on ice for 15 min, loaded onto 10% TBE pre-cast gels (Bio-Rad), and run at 300 V for 10 min and 170 V for 40 min at 4 °C using 0.5× Tris-glycine running buffer (12.5 M Tris base, 95 mM glycine, 0.5 mM EDTA). Gels were dried and exposed to a FujiX PhosphorImager plate, and the fraction of DNA bound by gp2.5 was measured using ImageQuant software.

**Binding of gp2.5 ssDNA for Electron Microscopy**—wt and altered gp2.5 were diluted to 50 ng/ml in 20 mM Hepes/NaOH, pH 7.5, 20% glycerol and then mixed with wt M13 ssDNA at 10 ng/ml in a buffer containing 10 mM Hepes, pH 7.5, 50 mM NaCl final concentration. Binding reactions with protein to DNA ratios (g/g) ranging from 40:1 for wt gp2.5 protein to 10:1 for mutant protein were incubated for 15 min at room temperature in a 10-μl total reaction volume.

**Electron Microscopy**—gp2.5 bound to ssDNA was fixed with an equal volume of 1.2% glutaraldehyde for 5 min at room temperature. Sample volume was increased to 50 μl with a buffer containing spermidine (38) and quickly applied to a mesh copper grid coated with a thin carbon film, glow-charged shortly before sample application. Following adsorption of the samples to the EM support for 1–2 min, the grids were subjected to a dehydration procedure in which the water content of the washes was gently replaced by a serial increase in ethanol concentration to 100% and then air-dried. The samples were then rotary shadowcast with tungsten at 10<sup>-7</sup> torr and examined in a Philips CM 12 TEM instrument at 40 kV. Micrographs, taken at ×46,000, were scanned using a Nikon LS-4500AF film scanner, and panels were arranged using Adobe Photoshop.

**Expression and Purification of Gene 2.5 Histidine Fusion Proteins**—pET19bPPS2.5, pET19bPPS-F232L, and pET19bPPS-Δ26C were transformed into *E. coli* BL21 (DE3) competent cells. One-liter cultures of

each were grown in LB media containing ampicillin, and the cells induced at 37 °C were harvested as described previously (33). The cell pellet was resuspended in 25 ml of buffer B (50 mM Tris-Cl, pH 7.5, 500 mM NaCl, 70 mM imidazole). Following three freeze-thaw cycles, the cells were lysed by incubation for 1 h at 4 °C with lysozyme at a final concentration of 0.5 mg/ml. The cell debris was collected by centrifugation at  $8,000 \times g$  for 30 min at 4 °C, and the supernatant was filtered through a 0.22- $\mu$ m bottle top filter. The resulting filtrate was introduced onto a nickel-nitrilotriacetic acid-agarose column (Qiagen) with a bed volume of 5 ml. The resin was washed with 10 column volumes of buffer B, and the protein was eluted in 8 ml of buffer B containing 500 mM imidazole. Each protein was then dialyzed against buffer S and stored at -20 °C.

**Surface Plasmon Resonance Analysis of T7 DNA Polymerase-gp2.5 Interaction**—The interaction of gp2.5 with T7 DNA polymerase was examined using surface plasmon resonance as described previously (33).

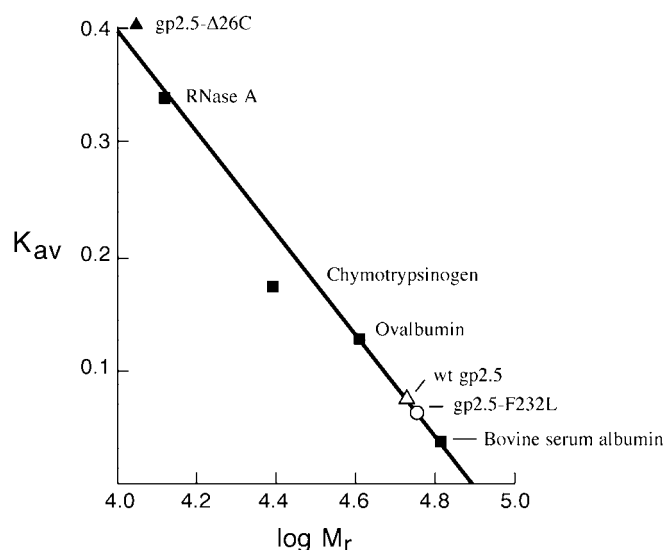
**DNA Synthesis Catalyzed by T7 DNA Polymerase**—The assay for T7 DNA polymerase was a modification of one described previously (14, 15, 20). The reaction mixture contained 50 mM Tris-Cl, pH 7.5, 10 mM MgCl<sub>2</sub>, 50 mM potassium glutamate, 100  $\mu$ g/ $\mu$ l bovine serum albumin, and the indicated ssDNA-binding proteins. For the assay of stimulation of DNA synthesis by ssDNA-binding proteins, poly(dA)<sub>390</sub>-(dT)<sub>22</sub> was used as a primer-template. A final concentration of 10 nM T7 DNA polymerase was added in the reaction. The reactions were carried out at 25 °C as described previously (15). For the assay of stimulation of strand displacement activity of T7 DNA polymerase by ssDNA-binding proteins, primed M13 ssDNA was used as template. A final concentration of 100 nM T7 DNA polymerase was added in the reaction. The reaction was carried out at 37 °C. The reaction mixtures were preincubated for 5 min, and the reactions were initiated by the addition of T7 DNA polymerase. Five- $\mu$ l aliquots were withdrawn at the indicated times and the reactions quenched by adding EDTA to a final concentration of 25 mM. The reaction mixture was transferred to Whatman DE81 filter, dried at room temperature for 30 min, and then washed with 0.3 M ammonium formate, pH 8.0, four times and once with 95% ethanol. The filters were dried, and the radioactivity retained on the filters was determined by scintillation counting.

**Alkaline-Agarose Gel Electrophoresis**—Alkaline-agarose gels were prepared as described (39). Ten  $\mu$ l of DNA synthesis reaction samples were added to 5  $\mu$ l of alkaline loading buffer containing 0.25% bromophenol, 0.25% xylene cyanol FF, 30% glycerol, 50 mM NaOH, and 1 mM EDTA. The sample was loaded onto the gel and electrophoresed at 25 V for 14–18 h at room temperature. The gel was dried and exposed to a FujiX PhosphorImager plate, and the fraction of DNA bound by gp2.5 protein was measured using ImageQuant software.

## RESULTS

**gp2.5-F232L Is a Dimer**—wt gp2.5 is a homodimer in solution with a molecular weight of 51,124 (7). gp2.5- $\Delta$ 21C, lacking the 21 carboxyl-terminal residues, on the other hand, is a monomer in solution (32). To ascertain whether gp2.5-F232L is a monomer or dimer, we estimated its molecular weight by gel filtration analysis. wt gp2.5 and gp2.5-F232L eluted from a Superdex 75 column at almost the same volume, whereas gp2.5- $\Delta$ 26C eluted in a considerably larger volume. By using a standard curve derived from the elution volume of four commercially available proteins standards, the molecular weight of gp2.5-F232L was estimated to be 58,000 (Fig. 1), consistent with the protein being a dimer. The value is nearly identical to that of 57,000 estimated for the wt gp2.5. As shown previously (33), gp2.5- $\Delta$ 26C elutes at a volume consistent with a monomer. These results show that the single amino acid change does not disrupt dimer formation, and we conclude that the protein is likely to be properly folded.

**ssDNA Binding Properties of gp2.5-F232L**—In a separate report we have described a gel shift assay to assess the binding of gp2.5 to ssDNA (34). In the present study, we used this method to compare the ssDNA binding ability of gp2.5-F232L to wt gp2.5 and gp2.5- $\Delta$ 26C. In the experiment shown in Fig. 2, a fixed amount (3.3 nM) of a <sup>32</sup>P-labeled 70-mer oligonucleotide was incubated with increasing amounts of wt gp2.5, gp2.5-F232L, or gp2.5- $\Delta$ 26C in the presence of 15 mM MgCl<sub>2</sub>. The



**FIG. 1. Determination of the molecular weight of gp2.5-F232L by gel filtration.** Gel filtration was carried out using a Superdex 75 column as described under "Experimental Procedures." The protein standards, ovalbumin (43 kDa), chymotrypsinogen (25 kDa), bovine serum albumin (67 kDa), and ribonuclease A (13.7 kDa), were used to calibrate the column. As controls, wt gp2.5 and gp2.5- $\Delta$ 26C were also included. Standard curves were generated by plotting  $K_{av}$  versus  $\log_{10} M_r$  for known molecular weight standards. The elution volumes of blue dextran and xylene cyanol determined the void volume and total volume of the column, respectively. wt gp2.5, gp2.5- $\Delta$ 26C, and gp2.5-F232L were applied to the column in three independent experiments. The  $K_{av}$  for each protein was calculated based on their elution volumes. Their positions on the curve are noted with a dash.

oligonucleotide and oligonucleotide-protein complexes were then resolved by electrophoresis on a native polyacrylamide gel. Consistent with the results reported earlier (34), two species of oligonucleotide-protein complexes are observed with wt gp2.5 (Fig. 2A), a rapidly migrating complex and a slower migrating complex. By using the Langmuir isotherm to calculate the dissociation constant, the  $K_d$  for wt gp2.5 is  $4.6 \times 10^{-6}$  M. gp2.5-F232L, on the other hand, binds more tightly to the oligonucleotide, and only the faster migrating oligonucleotide-protein complex is observed (Fig. 2C). An oligonucleotide-protein complex was observed with gp2.5-F232L at 1300 nM, whereas complex formation required 2700 nM with wt gp2.5. The  $K_d$  calculated for gp2.5-F232L is  $1.5 \times 10^{-6}$  M, ~3-fold lower than that calculated for the wild-type protein.

gp2.5- $\Delta$ 21C, lacking the carboxyl-terminal tail, binds to M13 ssDNA essentially as well as does wt gp2.5 as measured by nitrocellulose binding (32). We have compared the binding of gp2.5- $\Delta$ 26C to the 70-mer oligonucleotide using the more quantitative gel shift assay (Fig. 2B). With gp2.5- $\Delta$ 26C only the slower DNA-protein complex is observed with a  $K_d$  of  $1.1 \times 10^{-7}$  M. Thus, elimination of the acidic terminal tail increases the affinity of the truncated protein for the oligonucleotide as compared with the wild-type protein, which is consistent with the result described previously (34).

Under strand annealing and replication conditions, which include magnesium, wt gp2.5, gp2.5- $\Delta$ 26C and gp2.5-F232L, all generate highly compact structures when viewed by electron microscopy (7, 40). The binding of the three proteins to M13 ssDNA was examined in the absence of magnesium. Whereas wt gp2.5 did not show significant binding to the ssDNA circles at a protein:DNA ratio of 10:1 (not shown), gp2.5-F232L coated much of the ssDNA and extended it (Fig. 3), although less so than gp2.5- $\Delta$ 26C (not shown).

**Interaction of gp2.5-F232L with T7 DNA Polymerase**—Earlier studies using both affinity chromatography, fluorescence

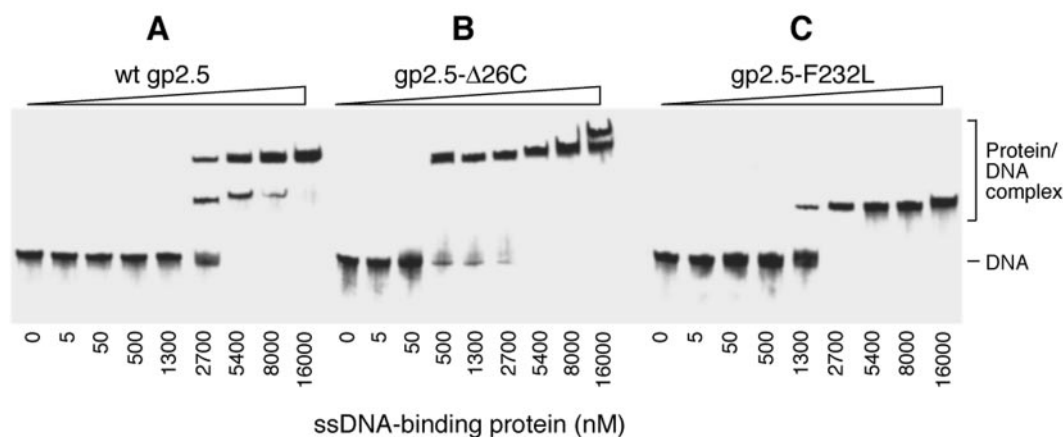


FIG. 2. **Binding of gene 2.5 proteins to ssDNA.** An electrophoretic mobility shift assay was employed to assess the binding of gene 2.5 proteins to a 70-mer oligonucleotides. 3.3 nM  $5'$ - $^{32}$ P-labeled 70-mer oligonucleotide was incubated for 10 min on ice with increasing concentrations (0, 5, 50, 500, 1300, 2700, 5300, 8000, and 16,000 nM) of wt gp2.5 (A), gp2.5-F232L (B), and gp2.5- $\Delta$ 26C (C). Reaction products were resolved on a 10% non-denaturing polyacrylamide gel. The amount of radioactivity in each band was measured as described under "Experimental Procedures."

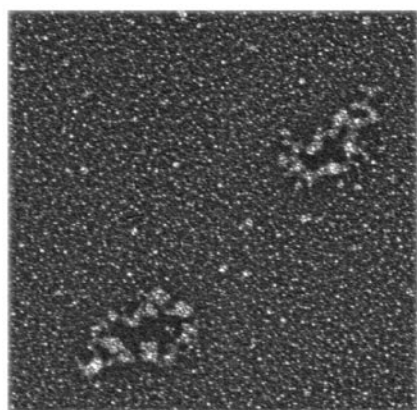


FIG. 3. **Electron microscopic analysis of gp2.5-F232L binding to single-stranded DNA.** gp2.5-F232L was incubated with M13 ssDNA at a weight ratio of 10:1. The sample was fixed with glutaraldehyde and further prepared for EM as described under "Experimental Procedures."

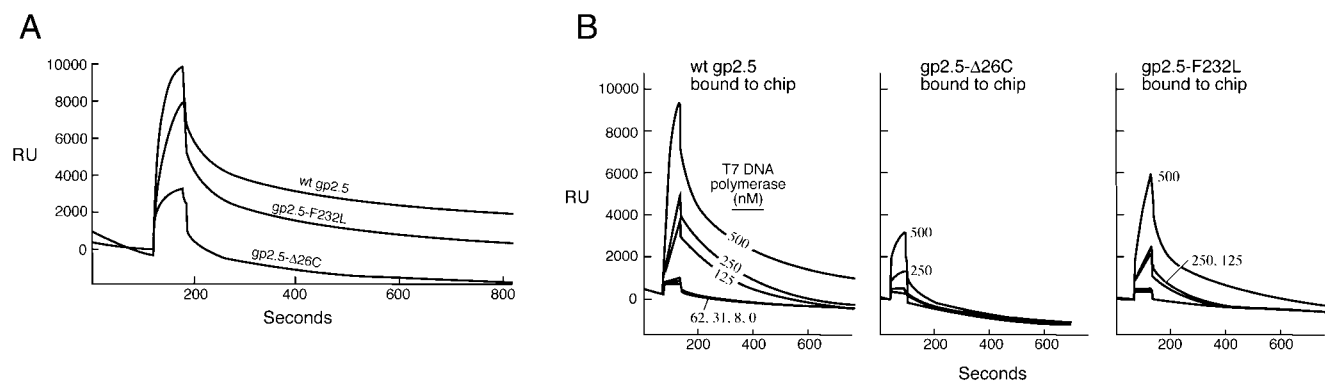
emission anisotropy, and surface plasmon resonance (8, 33) showed that gp2.5 physically interacts with T7 DNA polymerase. The interaction is dependent on the acidic carboxyl terminus in that gp2.5- $\Delta$ 21C cannot interact with T7 DNA polymerase (32). We have examined the ability of gp2.5-F232L to interact with T7 DNA polymerase using surface plasmon resonance as described previously (33). Histidine-tagged gp2.5 variants were immobilized on the chip surface and then T7 DNA polymerase passed over the bound gp2.5. The dissociation of the polymerase from the bound gp2.5 was monitored over a 10-min period. Experiments demonstrating the binding of T7 DNA polymerase to histidine-tagged wt gp2.5, gp2.5-F232L, and gp2.5- $\Delta$ 26C are depicted in Fig. 4A. As demonstrated previously, wt gp2.5 binds the polymerase, whereas gp2.5- $\Delta$ 26C does not. A binding curve similar to that found with wt gp2.5 was observed when gp2.5-F232L was bound to the chip (Fig 4A). Therefore, gp2.5-F232L retains the ability to bind to T7 DNA polymerase. A more quantitative evaluation of the binding of each of the three gp2.5s to T7 DNA polymerase is shown in Fig. 4B. In this experiment the concentration of T7 DNA polymerase was varied from 0 to 500 nM. The dissociation constant of wt gp2.5 was calculated to be  $9.08 \times 10^{-6}$  M which is in agreement with the value reported previously (8, 33). gp2.5- $\Delta$ 26C, however, had a higher dissociation constant, outside the detection limit of this technique, again consistent with previous studies showing that the carboxyl-terminal tail is

required for gp2.5-T7 DNA polymerase interaction (32). The binding of gp2.5-F232L, on the other hand, had a dissociation constant of  $3.95 \times 10^{-5}$  M, a value close to that of the wild-type protein.

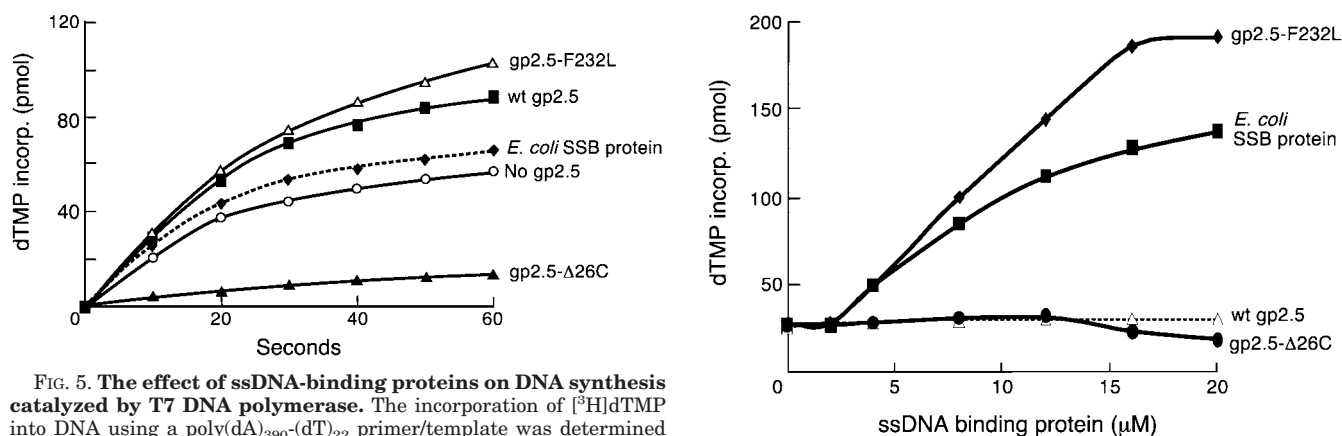
**Ability of ssDNA-binding Proteins to Stimulate T7 DNA Polymerase**—Because alteration of the terminal residue of gp2.5 did not adversely affect the ability of the protein to interact physically with T7 DNA polymerase, we examined its ability to stimulate DNA synthesis catalyzed by T7 DNA polymerase. In an earlier experiment we had found that wt gp2.5 and *E. coli* SSB protein stimulate DNA synthesis catalyzed by T7 DNA polymerase (8). By contrast, gp2.5- $\Delta$ 21C did not stimulate T7 DNA polymerase (32). Consequently, we compared gp2.5-F232L with wt gp2.5, gp2.5- $\Delta$ 26C, and *E. coli* SSB protein. In order to eliminate the effect of secondary structure in the DNA, we have used poly(dA)<sub>390</sub> annealed to a 22-mer oligonucleotide primer. In each reaction sufficient ssDNA-binding protein was present to coat all of the ssDNA in the reaction.

As expected, wt gp2.5 and *E. coli* SSB protein gave a small but significant stimulation of DNA synthesis (Fig. 5). gp2.5- $\Delta$ 26C not only failed to stimulate DNA synthesis but was strikingly inhibitory. gp2.5-F232L stimulated T7 DNA polymerase even more so than did wt gp2.5. These results clearly demonstrate that an interaction between gp2.5 and T7 DNA polymerase is essential for DNA synthesis on a gp2.5-coated template. Due to the absence of secondary structure in this template, it is most likely that an interaction with the carboxyl-terminal tail is required for T7 DNA polymerase to pass through gp2.5-coated DNA.

**gp2.5-F232L Stimulates Strand Displacement DNA Synthesis Catalyzed by T7 DNA Polymerase**—wt T7 DNA polymerase, although extremely processive on ssDNA templates, is unable to initiate strand displacement synthesis when it encounters a duplex region (41). However, T7 DNA polymerase lacking its 3'-5' proofreading exonuclease activity does mediate strand displacement synthesis (41), and *E. coli* SSB protein can bestow strand displacement synthesis on wild-type DNA polymerase (28). In the experiment shown in Fig. 6, we have examined the ability of gp2.5-F232L and gp2.5- $\Delta$ 26C to mediate strand displacement synthesis catalyzed by T7 DNA polymerase. In the experiment, M13 ssDNA annealed to a 24-mer oligonucleotide primer was incubated with T7 DNA polymerase and each of the four ssDNA-binding proteins in amounts sufficient to coat all of the ssDNA. In the 30-min reaction all of the available template was converted to duplex circular DNA in the absence of gp2.5 or in the presence of wt gp2.5. As expected,



**FIG. 4. Interaction between gene 2.5 proteins and T7 DNA polymerase.** The interaction between gene 2.5 protein and T7 DNA polymerase was monitored using surface plasmon resonance on a BIAcore 3000 (33). The surface of the chip was activated by saturating the nitrilotriacetic acid sites with running buffer (100 mM Hepes-NaOH, pH 7.5, 50  $\mu$ M EDTA, 0.1 mM dithiothreitol, 50 mM NaCl) containing 0.5 mM  $\text{NiCl}_2$ . In all graphs time (seconds) is plotted on the x axis; response units (RU) are plotted on the y axis. Five nmol of histidine-tagged gene 2.5 proteins were immobilized on the chip surface. Following a period of stabilization, T7 DNA polymerase was passed over the chip and then allowed to dissociate for 10 min. *A*, an overlay plot depicting the interaction of 500 nM T7 DNA polymerase with wt gp2.5, gp2.5- $\Delta$ 26C, and gp2.5-F232L. *B*, an overlay plot depicting the interaction of various concentrations (0–500 nM) of T7 DNA polymerase with wt gp2.5, gp2.5- $\Delta$ 26C, and gp2.5-F232L.



**FIG. 5. The effect of ssDNA-binding proteins on DNA synthesis catalyzed by T7 DNA polymerase.** The incorporation of [ $^3\text{H}$ ]dTMP into DNA using a poly(dA) $_{390}$ -(dT) $_{22}$  primer/template was determined as described under "Experimental Procedures." The incorporation of dTMP (picomoles) is plotted as a function of reaction time (seconds) for each ssDNA-binding protein. 20  $\mu$ M each of wt gp2.5, gp2.5- $\Delta$ 26C, gp2.5-F232L, or *E. coli* SSB protein were each preincubated with 70 nM poly(dA) $_{390}$ -(dT) $_{22}$  DNA primer/template at 25  $^\circ\text{C}$  for 5 min. DNA synthesis reactions were initiated by adding T7 DNA polymerase to a final concentration of 10 nM. 5- $\mu$ l aliquots were removed at the indicated time points; the reactions were stopped by addition of EDTA, and the incorporation of [ $^3\text{H}$ ]dTMP into DNA was measured.

gp2.5- $\Delta$ 26C was inhibitory at higher concentrations. Surprisingly, gp2.5-F232L provides for extensive DNA synthesis, exceeding the amount of template present in the reaction mixture by 5-fold. A similar result is observed with *E. coli* SSB protein in support of its known role in promoting strand displacement synthesis (27).

To confirm that the extensive DNA synthesis observed with gp2.5-F232L and *E. coli* SSB protein was a result of strand displacement synthesis, we examined the products on a denaturing alkaline-agarose gel (Fig. 7). In the case of wt gp2.5, all of the radioactivity migrated to the position of full-length M13 DNA (Fig. 7A). The product observed with gp2.5- $\Delta$ 26C was heterogeneous ranging from full-length down to considerably shorter fragments (Fig. 7D). gp2.5-F232L on the other hand, like *E. coli* SSB protein, supported the formation of very long DNA products, most of which exceeded the resolving power of the 0.6% agarose gel (Fig. 7, B and C). We conclude that both gp2.5-F232L and *E. coli* SSB protein enable T7 DNA polymerase to catalyze extensive strand displacement synthesis.

To estimate the number of DNA molecules on which strand displacement was occurring in the presence of gp2.5-F232L and *E. coli* SSB protein, we examined the fate of 5'- $^{32}\text{P}$ -labeled

**FIG. 6. Ability of ssDNA-binding proteins to promote strand displacement synthesis catalyzed by T7 DNA polymerase.** The incorporation of [ $^3\text{H}$ ]dTMP into primed M13 ssDNA was determined as described under "Experimental Procedures." The reaction was carried out at 37  $^\circ\text{C}$  for 30 min. Each reaction (10  $\mu$ l) contained 4 nM primed M13 ssDNA and the indicated amount (0–20  $\mu$ M) of wt gp2.5, gp2.5-F232L, gp2.5- $\Delta$ 26C, or *E. coli* SSB protein. The reaction was preincubated at 37  $^\circ\text{C}$  for 5 min after the ssDNA-binding protein was added. A final concentration of 100 nM T7 DNA polymerase was added to initiate the reaction. The total incorporation of dTMP into DNA for a 5- $\mu$ l reaction mixture is plotted as a function of the concentration of each ssDNA-binding protein.

primers on M13 DNA (Fig. 8). Strand displacement synthesis was observed with both gp2.5-F232L and *E. coli* SSB protein but not with wt gp2.5. Strand displacement synthesis was initiated somewhat earlier with gp2.5-F232L protein, but the number of molecules undergoing strand displacement synthesis was eventually greater with *E. coli* SSB protein.

*Role of ssDNA Binding Protein Is not Limited to Initiation of Strand Displacement Synthesis*—In one model, a major role of gp2.5-F232L and *E. coli* SSB protein in strand displacement synthesis is simply to facilitate the partial denaturation of the duplex region at the 5' terminus of the strand to be displaced. If so, then strand displacement synthesis should be facilitated on a primer-template in which the 5' terminus of the primer is not homologous to the template as depicted in the inset to Fig. 9. We therefore examined the ability of T7 DNA polymerase to catalyze strand displacement synthesis both alone and in the presence of ssDNA-binding protein (Fig. 9). No strand displacement synthesis occurred with T7 DNA polymerase alone or in the presence of wt gp2.5. However, both gp2.5-F232L and

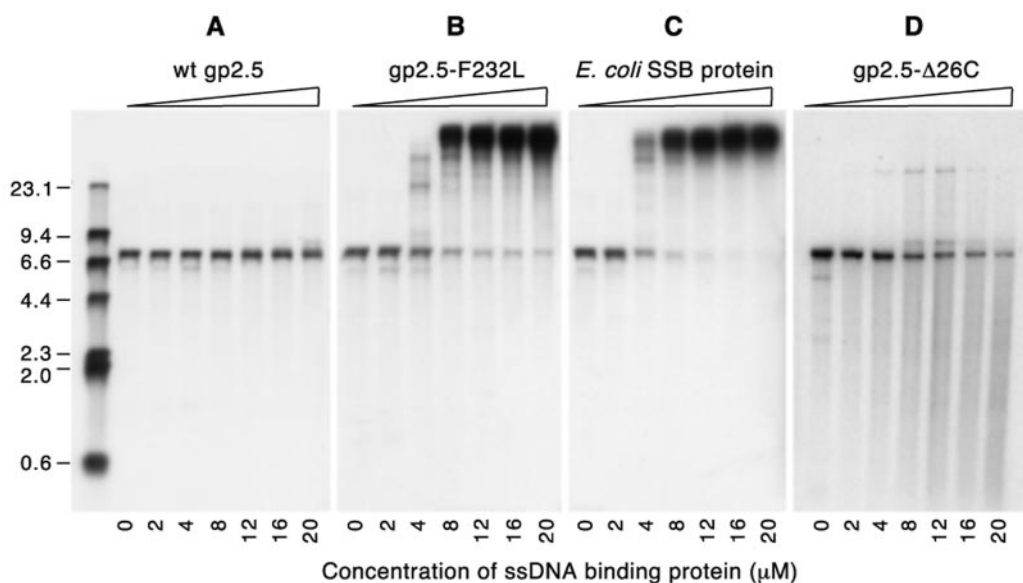


FIG. 7. Alkaline gel analysis of the products of DNA synthesis catalyzed by T7 DNA polymerase in the presence of ssDNA-binding proteins. The incorporation of [ $^{32}$ P]dGMP on an M13 ssDNA template was determined as described under "Experimental Procedures." DNA synthesis by 100 nM T7 DNA polymerase on a 4 nM primed M13mp18 template in the presence of increasing levels (0–20  $\mu$ M) of either wt gp2.5, gp2.5-F232L, gp2.5-Δ26C, or *E. coli* SSB protein was carried out as described above. The radioactively labeled products were denatured and separated by electrophoresis through a 0.6% alkaline agarose gel at 25 V for 14–18 h. The gel was dried, and the bands were visualized by autoradiography as described under "Experimental Procedures." A, wt gp2.5; B, gp2.5-F232L; C, *E. coli* SSB protein; D, gp2.5-Δ26C.

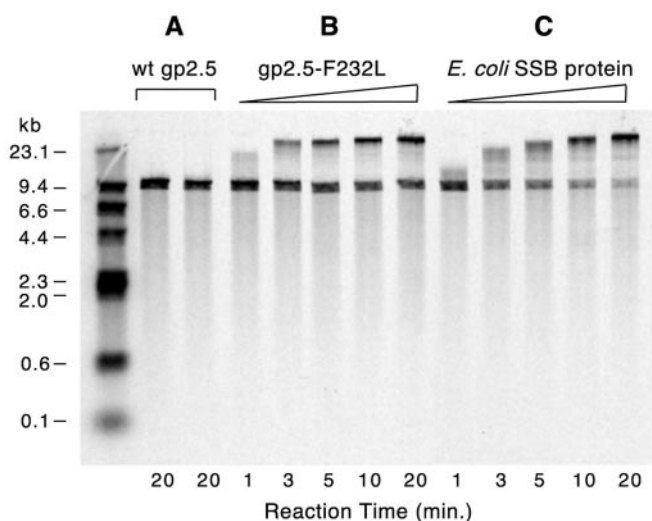


FIG. 8. Alkaline gel analysis of the labeled products of extensive DNA synthesis catalyzed by T7 DNA polymerase using a radioactively labeled primer. The primer-template was the 5'- $^{33}$ P-end-labeled 22-mer oligonucleotide annealed to M13mp18 ssDNA. 8  $\mu$ M of each ssDNA-binding protein was added to the reaction mixture for DNA synthesis containing 4 nM primed template. The reactions were carried out as described under "Experimental Procedures." After preincubation at 37  $^{\circ}$ C for 5 min, the reactions were initiated by adding 100 nM T7 DNA polymerase. For each reaction, 5- $\mu$ l aliquots were removed at 1, 3, 5, 10, and 20 min, respectively, and mixed with 5  $\mu$ l of 50 mM EDTA to stop the reaction. The reaction products were loaded onto a 0.6% alkaline agarose gel, and electrophoresis was carried out at 25 V for 14–18 h. The gel was dried, and the bands were visualized by autoradiography.

*E. coli* SSB protein promote strand displacement synthesis to the same extent as found with the primer-template lacking a single-stranded 5'-tail. Thus, gp2.5-F232L or *E. coli* SSB protein is needed in both initiating and maintaining strand displacement DNA synthesis.

#### DISCUSSION

Protein-protein interactions are essential for the establishment and maintenance of a functional replisome (42). Recent

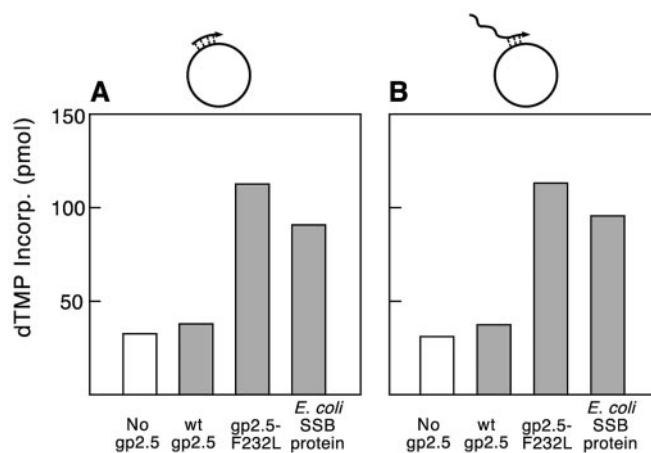


FIG. 9. Strand displacement synthesis on a primer-template with 5'-ssDNA tail. DNA synthesis catalyzed by T7 DNA polymerase in the absence and presence of ssDNA-binding protein was measured using the two DNA primer-templates depicted in the top. A, the primer is fully homologous to the M13 DNA template. B, the 5' terminus of the primer is not homologous, resulting in a 5' ssDNA tail. The reaction mixture (10  $\mu$ l) contained 4 nM primed M13 ssDNA, 12  $\mu$ M of wt gp2.5, gp2.5-F232L, gp2.5-Δ26C, or *E. coli* SSB protein. The reactions were initiated by adding 100 nM T7 DNA polymerase. The DNA synthesis reactions were performed at 37  $^{\circ}$ C for 30 min, and the incorporation of [ $^3$ H]dTMP into DNA was measured as described under "Experimental Procedures."

studies (42, 43) in several replication systems have identified the importance of the carboxyl-terminal domain of a number of replication proteins in modulating their activities. In the T7 replication system the acidic carboxyl-terminal domain of gene 4 helicase-primase is essential for its interaction with T7 DNA polymerase (44), and the carboxyl-terminal domain of T7 gp2.5 is involved in interaction with T7 DNA polymerase and gp4 helicase-primase (32).

In a separate report (33) we described a random mutagenesis screen that identified numerous lethal mutations in gene 2.5. Surprisingly, only one single alteration mapped to the carboxyl terminus of gene 2.5 (33). The mutation leads to the substitu-

tion of leucine for phenylalanine at the terminal amino acid of the protein. Interestingly, the terminal amino acid of *E. coli* SSB protein is also a phenylalanine (45), and this residue is conserved between bacteriophage T7, bacteriophage T3, and bacteriophage  $\phi$ YeO3-12 (46–48). Its location and its conservation suggest that the residue has a critical role in protein-protein interactions.

We have previously characterized gp2.5 lacking either 21 (32) or 26 amino acids (33, 34) at their carboxyl terminus, and we have constructed chimeric proteins in which the acidic carboxyl terminus of *E. coli* SSB protein or T4 gene 32 protein replaced that of gene 2.5 protein (20). These studies showed that the carboxyl terminus domain of gene 2.5 protein is essential for protein-protein interactions but that it alone cannot account for the specificity of all its essential function *in vivo*. In this study we have biochemically characterized gp2.5-F232L and shown that the variant protein had significantly different properties than gp2.5- $\Delta$ 26C lacking the acidic carboxyl terminus. Whereas gp2.5- $\Delta$ 26C inhibits T7 DNA polymerase activity, gp2.5-F232L can stimulate T7 DNA polymerase activity and, most significantly, can promote strand displacement DNA synthesis catalyzed by T7 DNA polymerase. A comparison of the properties of wt gp2.5, gp2.5- $\Delta$ 26C, and gp2.5-F232L supports a model in which the carboxyl-terminal tail modulates DNA binding and also allows for recognition by T7 DNA polymerase.

*E. coli* SSB protein has been shown previously to stimulate T7 DNA polymerase on ssDNA templates (8), and its ability to support strand displacement synthesis (27) even at the high concentrations used in this study is not surprising in this light. We have postulated that T7 DNA polymerase encounters significant *E. coli* SSB protein-coated DNA during DNA replication in phage-infected cells. The ability to interact functionally with SSB protein would thus be essential for normal T7 DNA synthesis. We have also observed a slight difference between gp2.5-F232L and *E. coli* SSB protein in allowing for strand displacement synthesis. gp2.5-F232L promotes strand displacement synthesis more rapidly than does *E. coli* SSB protein. *E. coli* SSB protein, on the other hand, drives more M13 templates into strand displacement DNA synthesis. However, the molecular basis for the difference is not known.

All the studies described above point to the pivotal role of the acidic carboxyl-terminal tail of gp2.5. Unfortunately, this domain of gp2.5 was not present in the protein used to determine the crystal structure, and attempts to obtain a co-crystal with ssDNA were unsuccessful (26). Nonetheless, the location of the existing carboxyl terminus and the likelihood that this domain is highly flexible led to a model in which the acidic tail of a monomer of gp2.5 mimics ssDNA and competes with ssDNA for the proposed basic DNA-binding site of a second monomer of gp2.5 (26). Recent *in vitro* mutagenesis studies (34) have provided strong evidence that the site proposed for DNA binding is correct. This model is supported by the considerably higher affinity of gp2.5- $\Delta$ 26C (40-fold) and gp2.5-F232L (3-fold) for ssDNA reported in this study. The higher affinity of gp2.5-F232L for ssDNA is thus compatible with the model on the basis that the single amino acid change in the tail decreases its affinity for the DNA-binding site of the protein. It has been proposed that the interaction of the carboxyl tail of the T4 gene 32 ssDNA-binding protein with its DNA-binding site modulates its affinity for ssDNA (49).

Gene 2.5 protein is a dimer in solution (7) and in the crystal structure (26). Based on the dimer interface observed in the crystal, we altered amino acids in the interface and found that the stability of dimers was diminished (33). The carboxyl terminus, however, is also involved in dimer stabilization because

gp2.5- $\Delta$ 21C does not form dimers in solution (32). The above model also provides an explanation for this requirement of the carboxyl terminus for dimer stabilization (26). The carboxyl-terminal tail would stabilize the dimer by a domain swapping interaction (50) across the dimer interface. Although the phenylalanine to leucine change at position 232 increases the affinity of the protein for ssDNA, we find that gp2.5-F232L retains its ability to dimerize. However, we have not determined binding affinities for either wt gp2.5 or gp2.5-F232L.

In addition to the carboxyl tail interacting with another gp2.5 monomer to form dimers, it also physically and functionally interacts with T7 DNA polymerase and the T7 gene 4 helicase/primase (8). gp2.5- $\Delta$ 21C does not interact with these two replication proteins (32). Again, however, we find that gp2.5-F232L maintains its ability to physically interact with T7 DNA polymerase albeit slightly less strongly than does wt gp2.5. From these considerations it is obvious that interactions of the carboxyl-terminal tail of the DNA-binding site of another gp2.5 monomer could modulate the binding of the protein to T7 DNA polymerase or gene 4 protein. Only upon binding to ssDNA with the concomitant release of the carboxyl-terminal tail could an interaction with these proteins occur. Indeed, it has been proposed that the sequestration of the acidic tail within a dimer may prevent other protein interactions until DNA binding occurs (26).

A surprising finding was the ability of gp2.5-F232L to enable T7 DNA polymerase to catalyze strand displacement synthesis. Although the T7 gene 5 protein-thioredoxin complex (T7 DNA polymerase) catalyzes the polymerization of nucleotides in a highly processive manner on ssDNA templates, it is unable to continue synthesis when it encounters a duplex region (41). In contrast, *E. coli* DNA polymerase will continue polymerization through the duplex region with resulting displacement of the strand annealed to the template (41). The inability of T7 DNA polymerase to catalyze strand displacement synthesis resides within its extremely high 3'–5' exonuclease activity, because T7 DNA polymerase lacking the exonuclease activity catalyzes strand displacement synthesis (41, 51). The exonuclease activity presumably enables the highly processive polymerase to idle at nicks with a turnover of nucleotides. gp2.5-F232L enables the wild-type T7 DNA polymerase to catalyze strand displacement synthesis, whereas wt gp2.5 protein does not. We postulate that the tighter binding of gp2.5-F232L to ssDNA enables it to facilitate unraveling of the duplex region of the DNA, exposing an ssDNA template for the polymerase. In support of this model is our finding that T7 gp2.5- $\Delta$ 26C and *E. coli* SSB protein, both of which bind even more tightly to ssDNA, allow for strand displacement synthesis.

A striking difference between gp2.5-F232L and gp2.5- $\Delta$ 26C is the inhibition of strand displacement synthesis by gp2.5- $\Delta$ 26C observed at higher concentrations of the protein. We propose that both gp2.5-F232L and gp2.5- $\Delta$ 26C, at sufficiently high concentrations, unwind the duplex sufficiently for them to bind to the template strand ahead of the polymerase. gp2.5- $\Delta$ 26C, lacking the carboxyl-terminal tail, is unable to interact with T7 DNA polymerase, and consequently the polymerase is unable to displace the bound protein. gp2.5-F232L, with an essentially intact carboxyl terminus, is able to mediate an interaction with the polymerase, and synthesis can proceed. In support of this model is the slight but significant stimulation of T7 DNA polymerase observed with gp2.5-F232L on ssDNA templates but the striking inhibition of DNA synthesis by gp2.5- $\Delta$ 26C. Thus these studies provide the first definitive information on the role of interaction between T7 DNA polymerase and T7 gp2.5 via the carboxyl terminus of the latter protein.

As a member of the bacteriophage T7 replisome, gene 2.5 protein is essential for T7 DNA replication and phage growth. In this study we have shown that the carboxyl-terminal domain of gp2.5 protein regulates the interaction of the protein with ssDNA and with T7 DNA polymerase. Why can gp2.5-F232L not support the growth of T7  $\Delta$ 2.5 phage? It seems unlikely that the increase in its affinity for ssDNA and its slightly reduced interaction with T7 DNA polymerase explains its inability to support phage growth. More likely, its ability to promote strand displacement DNA synthesis is responsible for the lethal phenotype. Strand displacement synthesis could occur on the lagging strand when a growing Okazaki fragment encounters a completed Okazaki fragment or during the multiple recombination events that occur during phage infection. Unexplained, however, is the mechanism by which strand displacement synthesis by *E. coli* SSB protein is circumvented.

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