THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 9, Issue of February 28, pp. 7247–7256, 2003

Printed in U.S.A.

The DNA Binding Domain of the Gene 2.5 Single-stranded DNA-binding Protein of Bacteriophage T7*

Received for publication, October 16, 2002, and in revised form, December 19, 2002
Published, JBC Papers in Press, December 20, 2002, DOI 10.1074/jbc.M210605200

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Gene 2.5 of bacteriophage T7 encodes a single-stranded DNA-binding protein that is essential for viral survival. Its crystal structure reveals a conserved oligosaccharide/oligonucleotide binding fold predicted to interact with single-stranded DNA. However, there is no experimental evidence to support this hypothesis. Recently, we reported a genetic screen for lethal mutations in gene 2.5 that we are using to identify functional domains of the gene 2.5 protein. This screen uncovered a number of mutations that led to amino acid substitutions in the proposed DNA binding domain. Three variant proteins, gp2.5-Y158C, gp2.5-K152E, and gp2.5-Y111C/Y158C, exhibit a decrease in binding affinity for oligonucleotides. A fourth, gp2.5-K109I, exhibits an altered mode of binding single-stranded DNA. A carboxyl-terminal truncation of gene 2.5 protein, gp2.5-Δ26C, binds single-stranded DNA 10-fold more tightly than the wild-type protein. The three altered proteins defective in single-stranded DNA binding cannot mediate the annealing of homologous DNA, whereas gp2.5-Δ26C mediates the reaction more effectively than does wild-type. Gp2.5-K109I retains this annealing ability, albeit slightly less efficiently. With the exception of gp2.5-Δ26C, all variant proteins form dimers in solution and physically interact with T7 DNA polymerase.

Single-stranded DNA (ssDNA)[1]-binding proteins lack sequence specificity and bind ssDNA with a higher affinity than they bind double-stranded DNA or RNA (1). Primarily, ssDNA-binding proteins function to bind any exposed regions of ssDNA in cells, forming a protective coat around the reactive bases and thus restricting the formation of secondary structures. However, their role is not restricted to extending and protecting DNA in that they also physically and functionally interact with other replication proteins. Bacteriophage T7 encodes its own ssDNA-binding protein, the product of gene 2.5. Gene 2.5 protein is essential for phage survival (2) and plays multiple roles in DNA replication, recombination, and repair (2–12). Gene 2.5 protein interacts directly with both the T7 DNA polymerase (9) and the gene 4 helicase/primase (7), stimulating the activity of each protein. Presumably these interactions explain why coordination of leading and lagging strand synthesis *in vitro* is dependent upon gene 2.5 protein (13). Furthermore, gene 2.5 protein facilitates homologous DNA base pairing,[2] a process that is important during viral recombination (10, 11, 14) and in the repair of double-stranded breaks in the T7 chromosome (12).

Despite functional similarity with other ssDNA-binding proteins, namely the *Escherichia coli* SSB protein and the bacteriophage T4 gene 32 protein, T7 gene 2.5 protein has no sequence homology with these proteins (15, 16). Furthermore, these proteins cannot substitute for gene 2.5 protein *in vivo* (2, 17). The mode of binding of gene 2.5 protein to ssDNA differs from that of *E. coli* SSB and T4 gene 32 protein. Using a fluorescence based study gene 2.5 protein was found to have a binding constant for ssDNA binding of 1.2×10^6 M^{-1} (8) a value that is less than one-tenth the affinity exhibited by *E. coli* SSB (18) and T4 gene 32 protein (19, 20). In addition, gene 2.5 protein binds ssDNA with limited, if any, cooperativity (8). Kim *et al.* (8) reported that gene 2.5 protein bound to ssDNA with a stoichiometry of 7 nucleotides per monomer of protein, although it is not known if gene 2.5 protein binds to ssDNA as a monomer or dimer.

In the absence of DNA, gene 2.5 protein aggregates to form a stable homodimer in solution (8). Dimer formation is postulated to be dependent upon the interactions of its highly acidic carboxyl terminus (21). Its association with the other replication proteins is also facilitated by its carboxyl-terminal amino acids (13, 21). A similar role has also been shown for the acidic carboxyl-terminal tail found in the *E. coli* SSB protein (22) and the bacteriophage T4 gene 32 protein (23, 24).

The recently solved crystal structure of a carboxyl-terminal truncation of gene 2.5 protein to a resolution of 1.9 Å (16) revealed a core that consists of a conserved oligosaccharide/oligonucleotide binding fold (OB fold) (Fig. 1), a structure common to other ssDNA-binding proteins (25). As the name suggests this fold is found in proteins that bind either ssDNA such as *E. coli* SSB protein (26, 27), human mitochondrial SSB protein (28, 29), all three subunits of human replication protein A (30–32), and staphlococcal nuclease (33) or oligosaccharides as found in *E. coli* heat liable enterotoxin (34). The OB fold is comprised of a five-stranded anti-parallel β barrel, capped with an α helix. The location of this helix varies among DNA-binding proteins. In gene 2.5 protein this helix is found between the second and third strands (16), whereas in both human replication protein A and *E. coli* SSB protein it connects the third and forth strands (27, 30). In gene 2.5 protein loop extensions from two of the β sheets form a prominent groove on the surface of the fold. This groove most likely represents the ssDNA interface and in Fig. 1 ssDNA is modeled into the crystal structure along this position. Located within this groove

* This work was supported by National Institutes of Health Grant GM54397-39 (to C. C. R.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: ssDNA, single-stranded DNA; OB fold, oligosaccharide/oligonucleotide binding fold; nt, nucleotide; DTT, dithiothreitol; gp, gene product.

[2] S. Tabor and C. C. Richardson, unpublished data.

of gene 2.5 protein are two aromatic residues, tyrosine 111 and tyrosine 158. These aromatic residues, in addition to the adjacent β stands and their connecting loops comprise an evolutionarily conserved trinucleotide binding motif (16) that binds three nucleotides in an orientation analogous with other ssDNA-binding proteins (27, 30). A number of basic residues (Lys^3, Arg^{35}, Lys^{107}, Lys^{109}, Lys^{150}, and Lys^{152}) lie in proximity to this trinucleotide binding motif forming a positively charged cleft, suggesting a role in the interaction with ssDNA (16). However, prior to the current study, there was no direct evidence that implicates these residues in binding ssDNA.

In the current study we have sought experimental evidence to support our hypothesis that the DNA binding domain lies within the OB fold of gene 2.5 protein. A previously reported random mutagenesis screen of gene 2.5 (35) uncovered a number of lethal mutations that lead to alterations in amino acids that were predicted by the crystal structure to interact with ssDNA. Here we show that both the highly conserved aromatic residue Tyr^{158}, one component of the trinucleotide binding motif, and the positively charged Lys^{152}, flanking this motif, are required for the interaction with ssDNA. In addition we provide evidence for the involvement of Lys^{109} in the protein-DNA interaction. Finally, we show that the carboxyl-terminal tail deleted protein, gp2.5-Δ26C, binds DNA with a greater affinity than the wild-type protein.

EXPERIMENTAL PROCEDURES

Materials

Bacterial Strains, Bacteriophage, and Plasmids—E. coli strains BL21(DE3) and HMS 174(DE3), which contain a λ prophage encoding the T7 RNA polymerase gene under the control of the lac promoter (Novagen), were used as host strains for protein overexpression and purification. Wild-type and mutant forms of T7 gene 2.5 are expressed from the pET17b plasmid (Novagen) containing the T7 RNA polymerase promoter. DNA encoding His-tagged gene 2.5 proteins were subcloned into the *Nde*I and *Bam*HI restriction sites of a modified pET19b vector (Novagen). The enterokinase site of this plasmid was replaced with a rhinovirus C protease (PreScission protease, Amersham Biosciences) site located upstream of the start codon. T. Biswas (Harvard Medical School) provided this plasmid. *E. coli* strains HMS 262 and HMS 89 and all T7 bacteriophage are from our laboratory collection. Growth and manipulation of bacteriophage T7 and *E. coli* were performed as described previously (36).

DNA and Oligonucleotides—M13 mGP1-2 is a 9950-nucleotide derivative of vector M13 mp8 containing an insert of phage T7 DNA (37) and was provided by S. Tabor (Harvard Medical School). BCMP 206, a 25-mer oligonucleotide with the sequence 5′-TAACGCCAGGGTTTTCCCAGTCACG-3′, was synthesized by the Biopolymer Facility at Harvard Medical School. The 70-mer and 38-mer oligonucleotides used in the electrophoretic mobility shift assay for assessing ssDNA binding had the sequences 5′-GACCATATCCTCCACCCTCCCCAATATTGACCATCAACCCTTCAC CTCACTTCACTCCACTATACCACTC-3′ and 5′-CCTTTAAGTTCAAATGCTGCGC GTCTTTCCAAGACAAG-3′, respectively. These oligonucleotides were both synthesized and gel purified by Oligos *Etc*. Inc. For cloning purposes the following oligonucleotides were purchased from Oligos *Etc*. Inc. T7 2.5 *Bam*HI 5′-CGTAGGATCCACTTAGAAGTCTCCGTC-3′ and T7 2.5 *Nde*I 5′-CGTAGGATCCATATGGCTAAGAAGATTTTCACCTC-3′. The oligonucleotides, pET17b upstream, 5′-CTTTAAGAAGGAGATATACATATG-3′ and pET17b downstream, 5′-GCTTCCTTTCGGGCTTTG-3′, used to sequence the Y111C/Y158C construct were obtained from Integrated DNA Technologies. All radioactive nucleotides were purchased from Amersham Biosciences.

Proteins, Enzymes, and Chemicals—All restriction enzymes as well as T4 polynucleotide kinase, T4 DNA ligase, and calf intestinal phosphatase were purchased from New England Biolabs. S. Tabor provided *E. coli* SSB protein and T7 DNA polymerase-Δ28. D. Johnson and J. Lee (Harvard Medical School) supplied wild-type T7 DNA polymerase. T7 gp2.5-Δ26C and His-tagged gp2.5-Δ26C were obtained from E. Toth (Harvard Medical School) and J. Stattel (Harvard Medical School), respectively. Gene 4 protein was kindly provided by D. Crampton (Harvard Medical School). All chemicals and reagents were from Sigma unless otherwise noted.

Methods

Mutagenesis of T7 Gene 2.5—pET17(b) plasmids expressing mutated gene 2.5, which lead to the alterations gp2.5-Y158C, gp2.5-K109I, and gp2.5-K152E, were isolated from a genetic screen for lethal mutants of gene 2.5 as previously described (35). To express the variants gp2.5-Y111C/Y158C and gp2.5-Y111C separate vectors were constructed using Stratagene's QuikChange™ site-directed mutagenesis kit. Initially both pET17(b) expressing wild-type gene 2.5 and pET17(b) expressing gp2.5-Y158C were amplified with oligonucleotides 5′-CCTTTAAGTTCAAATGCT*G*CGCGTCTTTCCAAGACAAG-3′ and 5′-CTTGTCTTGGAAAGACGCG*C*AGCATTTGAACTTAAAGG-3′ (sequence changes from wild-type T7 gene 2.5 have been underlined) using the polymerase chain reaction. The resulting constructs were used to transform XL1-Blue super-competent *E. coli* cells (Stratagene). The oligonucleotides were synthesized and gel purified by Oligos *Etc.* Inc.

Expression and Purification of Gene 2.5 Proteins—Ten liters of *E. coli* BL21(DE3) cells expressing wild-type gene 2.5 protein from pET17b were grown to an A_{595} of 1.0 in a Bioflo 2000 fermentor, in the presence of 6 μg/ml ampicillin. They were then induced with isopropyl-β-D-galactoside at a concentration of 1 mM. After 4 h the cells were harvested by centrifugation at 2,000 rpm for 40 min, resuspended in 250 ml of lysis buffer (50 mM Tris-Cl, pH 7.5, 10% sucrose, 0.1 mM EDTA), and frozen on dry ice. Prior to purification the cells were thawed at 4 °C overnight in the presence of 50 mM β-mercaptoethanol. Cell lysis was accomplished by the addition of lysozyme at a final concentration of 0.5 mg/ml (diluted in lysis buffer) followed by incubation at 4 °C for 45 min. Lysed cells were heated to 20 °C in a 37 °C water bath, then chilled on ice and centrifuged at 100,000 × *g* for 45 min at 4 °C. To precipitate T7 gene 2.5 protein, polyethylenimine, at a final concentration of 0.1%, was added to the supernatant and the solution incubated at 4 °C for 1 h. The suspension was centrifuged at 21,000 × *g* for 15 min and the resulting pellet was resuspended in 90 ml of buffer A (50 mM Tris-Cl pH 7.5, 0.1 mM EDTA, 1 mM DTT, 10% glycerol) containing 1 M NaCl. This suspension was rotated at 4 °C for 1 h then centrifuged at 21,000 × *g* for 15 min. The resulting supernatant (~70 ml) was diluted with buffer A to 175 ml and 84 g of $(NH_4)_2SO_3$ was added slowly over a 1-h time period. The suspension was stirred for another hour at 4 °C followed by centrifugation at 21,000 × *g* for 15 min. The protein pellet was resuspended in 75 ml of buffer A and filtered through a 0.22-μm bottle top filter. This suspension was loaded onto a POROUS HQ column (PE Biosystems) and T7 gene 2.5 protein eluted in a 50 mM to 1 M NaCl gradient with most of the protein eluting at ~550 mM NaCl. The eluted protein was precipitated by the addition of $(NH_4)_2SO_3$ to 85% saturation and the protein was collected by centrifugation at 21,000 × *g* for 15 min. The resulting pellet was resuspended in 500 μl of buffer G (50 mM potassium phosphate buffer, pH 7.0, 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, 10% glycerol) and loaded onto a Superose 12 size exclusion column (Amersham Biosciences). T7 gene 2.5 protein eluted from the column in ~15 ml of buffer G and was then dialyzed into buffer S (50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 1 mM DTT, 50% glycerol). The protein was over 99% pure as determined by SDS-polyacrylamide electrophoresis and subsequent staining by Coomassie Blue. Gp2.5-Y158C, gp2.5-K152E, and gp2.5-K109I were purified as described for wild-type. It was necessary to express mutant gp2.5-Y111C/Y158C in *E. coli* strain HMS174 (DE3) pLys-S (Novagen) and it was purified from a 4-liter culture. Protein concentrations were determined by spectrophotometric absorbance at 280 nm using the extinction coefficient of the protein calculated according to Gill and von Hippel (38).

Expression and Purification of Gene 2.5 Histidine Fusion Proteins—E. coli BL21(DE3) competent cells were transformed with pET19b 2.5PPS, pET19b 2.5PPS-Y158C, pET19b 2.5PPS-K109I, pET19b 2.5PPS-K152E, pET19b 2.5PPS-Y111C/Y158C, and pET19b 2.5PPSΔ26C. One-liter cultures were grown of each in LB media containing ampicillin. They were induced and harvested as described previously and the pelleted cells were resuspended in 20 ml of buffer B (50 mM Tris-Cl, pH 7.5, 500 mM NaCl, and 70 mM imidazole). Following one freeze-thaw cycle the cells were lysed in the presence of 0.5 mg/ml lysozyme by incubating 4 h at 4 °C. To degrade the *E. coli* DNA, 125 units of Benzonase nuclease (Novagen) was added to the suspension and the suspension was heated to 20 °C in a 37 °C water bath. The cell debris was collected by centrifugation at 8,000 × *g* for 40 min at 4 °C. The cleared lysates were introduced onto a nickel-nitrilotriacetic-agarose column (Qiagen) with a bed volume of 5 ml. The resin was washed with 20 column volumes of buffer B and the protein was eluted in 20 ml of buffer B containing 500 mM imidazole. Each protein was then dialyzed against buffer S and stored at −20 °C.

In Vivo Complementation Assays—Each variant protein was ana-

lyzed for its ability to support bacteriophage T7 growth *in vivo*. Electrocompetent *E. coli* HMS 262 cells were transformed with pETGP2.5 plasmids containing each individual mutation. Cells expressing wild-type and mutant gene 2.5 proteins were infected with T7 bacteriophage lacking gene 2.5, and overlaid in soft agar onto LB plates. The number of plaques formed on each plate was counted after a 4-h incubation at 37 °C. The total amount of plaques produced by cells expressing wild-type gene 2.5 protein was represented as a plating efficiency of one. Therefore, plating efficiencies of the variant proteins could be determined by comparison with wild-type. Those mutations that could not substitute for wild-type gene 2.5 protein *in vivo* were examined for their ability to inhibit the growth of wild-type bacteriophage T7. *E. coli* HMS 89 cells expressing each variant protein from the pET17(b) vector were infected with wild-type bacteriophage T7 using the standard protocol. The formation of plaques was examined after an overnight incubation at 37 °C.

Molecular Weight Approximation by Gel Filtration Analysis—The molecular weight of the gene 2.5 mutant proteins in solution was approximated by gel filtration analysis on a Superdex 75 column (Amersham Biosciences) (8). Gel filtration was performed at 4 °C in buffer G at a flow rate of 0.6 ml/min. The column was initially calibrated using the following protein standards: ovalbumin (43 kDa), ribonuclease A (13.7 kDa), chymotrypsinogen (25 kDa), and bovine serum albumin (67 kDa) (Amersham Biosciences). The elution volumes of blue dextran and xylene cyanol determined the void volume (V_o) and total volume (V_t) of the column, respectively. Five hundred μl of 0.2 μg/μl gene 2.5 variants, diluted in buffer S, were applied to the column and their elution was monitored through spectrophotometric absorbance at 280 nm. The fractional retention, K_{av}, was calculated for each of the standard proteins using the equation: $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_e is the peak elution volume of each protein. The molecular weight of each gene 2.5 variant was approximated using a standard curve generated by plotting the K_{av} value *versus* $\log_{10} M_r$.

Surface Plasmon Resonance—The interaction of gene 2.5 protein with T7 DNA polymerase was examined using surface plasmon resonance (39). Initially the sensor-chip NTA (BIAcore) was activated by passing 10 μl of running buffer (100 mM Hepes-NaOH, pH 7.5, 50 μM EDTA, 0.1 mM DTT, 100 mM NaCl) containing 0.5 mM $NiCl_2$ over its surface at a rate of 10 μl/min. Histidine-tagged wild-type gp2.5, gp2.5-Y158C, gp2.5-K109I, gp2.5-K152E, gp2.5-Y111C/Y158C, and gp2.5-Δ26C were diluted in running buffer supplemented with 500 nM bovine serum albumin. To immobilize gene 2.5 proteins, 10 μl of the protein solution was injected onto individual lanes on the surface of the chip at a rate of 10 μl/min. Running buffer was passed over the chip for 2 min. At this time an increase in resonance units of ~7,000 on the surface of the chip was noted, thus establishing a baseline. Ten μl of various concentrations (up to 500 nM) of T7 DNA polymerase were injected onto the chip over 1 min, followed by 10 min of buffer. The association and dissociation of T7 DNA polymerase with gene 2.5 protein was monitored during the experiment by noting the change in resonance unit value. All proteins were removed and the chip was regenerated by passing 20 μl of running buffer containing 350 mM EDTA over its surface.

Affinity Chromatography—The ability of wild type and altered gene 2.5 proteins to physically interact with the 63-kDa form of gene 4 protein was assessed by affinity chromatography as described previously (9). Briefly, all proteins were dialyzed against 100 mM Hepes/NaOH, pH 7.5, 0.1 mM DTT, 0.5 mM EDTA, and 10% glycerol. Affi-Gel 15 (Bio-Rad) was prepared according to the manufacturers instructions. Gene 2.5 protein affinity columns were made by binding 1 mg of wild-type or altered gene 2.5 protein to 500 μl of Affi-Gel 15. Gene 4 protein (100 μg) was passed over the column. The column was washed with 5 ml of the dialysis buffer, then eluted in a step gradient (0–250 mM NaCl). One-ml fractions were collected, and the amount of protein eluted from the columns was monitored by absorbance at 280 nm.

Electrophoretic Mobility Shift Assay—The ssDNA binding ability of purified gene 2.5 proteins was assessed on both a 38- and 70-base length oligonucleotide using an electrophoretic mobility shift assay (40). The oligonucleotides were 5′-end labeled with [γ-^{33}P]ATP using polynucleotide kinase at 37 °C for 2 h and purified using Bio-Rad micro-biospin column P-30. Oligonucleotide (3.3 nM) was incubated for 15 min on ice with increasing concentrations (up to 10.6 μM) of purified wild-type gp2.5, gp2.5-Y158C, gp2.5-K109I, gp2.5-K152E, and gp2.5-Y111C/Y158C. All proteins were diluted in 20 mM Tris-Cl, pH 7.5, 10 mM β-mercaptoethanol, 500 μg/ml bovine serum albumin. Final concentrations of the components (in 15 μl) were 15 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 10% glycerol, and 0.01% bromphenol blue. Samples were loaded onto 10% TBE pre-cast gels (Bio-Rad) and run at 80 V for 2 h at 4 °C using 0.5× Tris glycine running buffer (12.5 mM Tris base, 95 mM glycine, 0.5 mM EDTA). Gels were dried, exposed to a FujiX phosphorimaging plate, and the fraction of DNA bound by gene 2.5 protein was measured using ImageQuant software. This measurement facilitated the calculation of the dissociation constants (K_d) for each protein using the Langmuir isotherm formula.

Annealing Assay—The ability of gene 2.5 protein to mediate homologous base pairing was assayed for each altered gene 2.5 protein using circular M13 ssDNA and a ^{32}P-labeled linear single-stranded fragment of M13. The labeled substrate was prepared by initially annealing 1 μM "BCMP 206" oligonucleotide to 0.13 μM mGPI-2 M13 in the presence of 50 mM NaCl and 25 mM Tris-Cl, pH 7.5, at 55 °C. The primer was then partially extended using an exonuclease-deficient T7 DNA polymerase in the presence of 8 μM dATP, dCTP, dGTP, and dTTP, 3 μCi/μl [α-^{32}P]dGTP, 5 mM DTT, 2 μM bovine serum albumin, and 10 mM $MgCl_2$, at room temperature for 10 min. To fully extend the primer 80 μM dATP, dGTP, dCTP, and dTTP were then added and the reaction was incubated for 15 min at room temperature. Reactions were then incubated for a further 10 min at 70 °C to denature the polymerase. Next 55 nM *E. coli* SSB protein was added and the DNA was digested with Acc65 I for 2 h at 37 °C. DNA was extracted with 50 μl of phenol: chloroform:isoamyl alcohol (25:24:1) and then separated from unincorporated nucleotides using a MicroSpin S-400HR column (Amersham Biosciences). To produce ssDNA fragments, the DNA was denatured with 100 mM NaOH at 25 °C for 5 min followed by neutralization on ice with 100 mM HCl and 100 mM Tris, pH 7.5. Separation of the fragments was achieved by gel electrophoresis on a 1.4% agarose gel run at 50 V for 90 min. The radioactive band corresponding to the Acc65 1 digested a 310-nt fragment that was extracted from the gel and purified using the gel extraction kit from Qiagen.

Annealing of 0.09 nM of the 310-nt fragment to 2 nM circular M13 single-stranded DNA (molar ratio = 1:22) was assayed under the following conditions: 20 mM Tris-Cl, pH 7.5, 1 mM DTT, 10 mM $MgCl_2$, and 50 mM NaCl, in the presence of increasing concentrations (up to 12 μM) of wild-type and altered gene 2.5 proteins. The reaction was incubated at 30 °C for 8 min prior to running on a 0.8% native agarose gel for 2 h at 80 V. Gels were dried under vacuum and the DNA was visualized by exposing the gel to a Fujix phosphorimaging plate and quantitated using ImageQuant software. For those proteins that mediated the annealing of DNA under these conditions, *i.e.* wild-type gene 2.5 protein and gp-K109I, a time course was obtained based on a 30-s interval from 0 to 7 min. To terminate the annealing activity at each time point 5 μl of stop solution (0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol, and 0.5% SDS) was added to the sample.

RESULTS

Essential Residues in the Proposed ssDNA Binding Site—In a separate report (35) we described a random mutagenesis and genetic selection to obtain lethal mutants in the cloned T7 gene 2.5. In the present study we biochemically characterize the altered gene 2.5 proteins that have amino acid changes located in the region of the protein predicted by the crystal structure to interact with ssDNA (16). The location of these residues on the crystal structure of gp2.5-Δ26C is shown in Fig. 1 and the phenotype of these mutants is summarized in Table I. The aromatic residues tyrosine 158 and tyrosine 111, structurally conserved among other ssDNA-binding proteins, lie in the core of the OB fold and comprise the trinucleotide-binding motif (16). The screen identified a single lethal mutation, Y158C. A mutation leading to an amino acid change at tyrosine 111, on the other hand, was not found alone, but rather in a clone containing two other mutations (35). Thus, site-directed mutagenesis was used to generate a gene 2.5 protein with a Y111C substitution. However, a plasmid expressing gp2.5-Y111C was shown to retain the ability to support the growth of T7 phage lacking wild-type gene 2.5 protein (Table I) and was not characterized further. A double mutant generated with the substitutions Y111C and Y158C was found to be unable to support the growth of T7Δ2.5 phage. In addition, the original screen detected independent lethal mutations at two lysine residues resulting in variants K109I and K152E. As well as being positively charged, both residues lie within the β barrel of the OB fold and flank the trinucleotide binding motif making them likely candidates to interact with ssDNA. None of these muta-

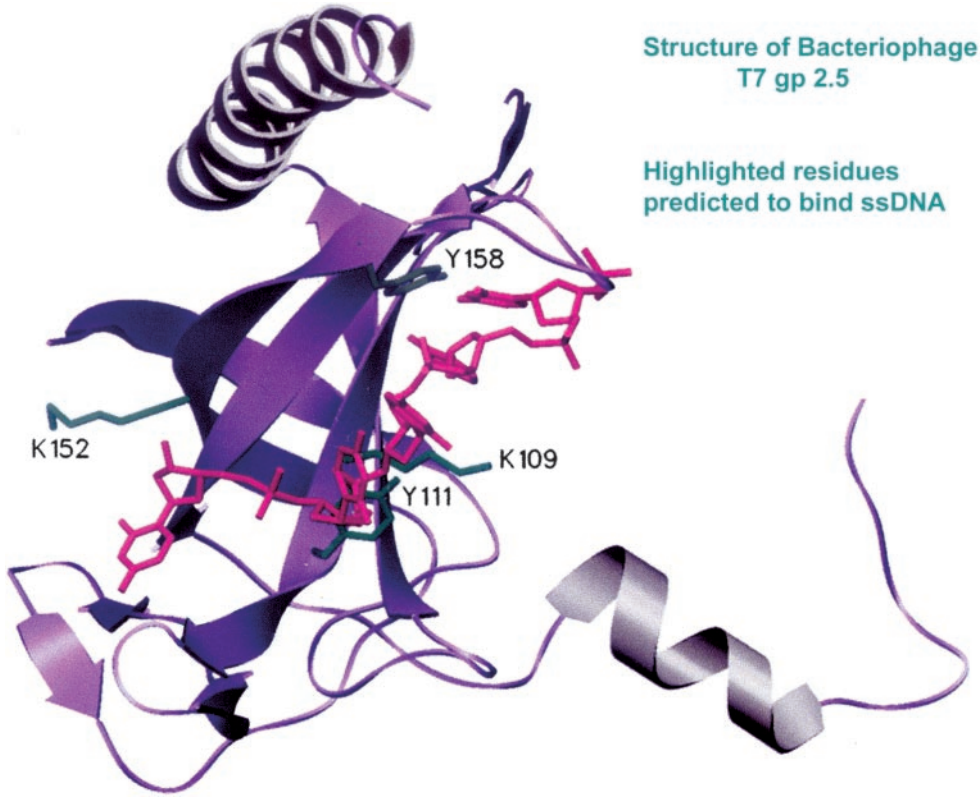

FIG. 1. **Proposed model for ssDNA binding in the crystal structure of T7 gene 2.5 protein and location of the altered amino acids.** The crystal structure of gp2.5-Δ26, determined at 1.9-Å resolution (16). The five-stranded anti-parallel β-barrel, depicted in *purple*, capped by a α-helix (*gray*), comprises the conserved oligosaccharide/oligonucleotide binding fold. Single-stranded DNA (*pink*) is modeled in the structure at the predicted binding site. The side chains of the residues characterized in this study are highlighted in *green* and their position in the polypeptide note.

TABLE I
Ability of gene 2.5 proteins to complement the growth of T7Δ2.5 phage or suppress the growth of wild-type T7 phage

Plasmid	Efficiency of plating[a]	
	Δ2.5::trxA phage[b]	T7 wild-type phage
pETGP2.5	1	1
pETGP2.5-Y111C	0.705	1.22
pETGP2.5-Y111C/Y158C	0	0.83
pETGP2.5-K152E	1.1×10^{-4}	1.33
pETGP2.5-Y158C	0	1.05
pETGP2.5-K109I	0	1.11
pETGP2.5-Δ26	0	0.72

[a] Plating efficiencies were determined by averaging the number of plaques present on individual plates from three separate experiments, and dividing this number by the number of plaques formed by cells expressing the wild-type gene 2.5 protein.
[b] As described Kim *et al.* (2).

tions could complement the growth of T7Δ2.5, nor were they dominant lethal, as they could not suppress the growth of wild-type T7 bacteriophage (Table I).

Amino Acid Alterations Do Not Disrupt Dimer Formation—In solution wild-type gene 2.5 protein forms a homodimer with molecular weight of 51,124. One model for dimerization proposes that the carboxyl-terminal tail of one protomer interacts with the predicted ssDNA binding groove of another (16). Furthermore, stable dimer formation has been shown to involve protein-protein interactions between specific residues along the dimer interface (35). To ascertain whether these altered proteins selected for this study were misfolded, we assessed their ability to promote these interactions and form dimers. Using gel filtration analysis to approximate their molecular weights we initially calibrated a Superdex 75 column using four standard proteins: ovalbumin, chymotrypsinogen, ribonuclease, and bovine serum albumin. From their individual elution volumes a standard curve was generated (Fig. 2) for the estimation of the molecular weights of the gene 2.5 variant proteins. Wild-type gene 2.5 protein displayed a fractional retention (K_{av}), equal to 0.078. This value corresponds to an estimated molecular weight of 47,000. Gp2.5-Y158C, gp2.5-K109I, and gp2.5-Y111C/Y158C eluted in volumes consistent with wild-type gene 2.5 protein with a comparable molecular weight of 47,000 for each protein. Gp2.5-K125E eluted slightly later than the native protein with its molecular weight calculated to be 46,000. From this analysis we conclude that each protein retains the ability to form homodimers in solution, strongly suggesting that the overall structure of these proteins is not affected by the amino acid substitutions.

Interaction of Gene 2.5 Proteins with Other T7 Replication Proteins—Gene 2.5 protein physically and functionally interacts with T7 DNA polymerase, an interaction that is dependent on its carboxyl-terminal amino acids (9, 17, 21). To further establish whether the mutations affected the integrity of the protein, the interaction of each altered gene 2.5 protein with the T7 DNA polymerase was examined using surface plasmon resonance. In these experiments histidine-tagged gene 2.5 proteins were immobilized on the chip surface and then T7 DNA polymerase was flowed over the chip. The dissociation of the polymerase from the bound gene 2.5 protein was monitored over a 10-min period. A typical wild-type gene 2.5 binding curve is presented in Fig. 3*A*. Binding curves consistent with that of the native protein were obtained for mutants gene 2.5 protein-Y158C, gp2.5-Y111C/Y158C, gp2.5-K109I, and gp2.5-K152E (Fig. 3*B, bottom*). This result demonstrates that the amino acid substitutions do not disrupt the ability of the altered proteins to physically interact with T7 DNA polymerase. Because this specific interaction is mediated by the carboxyl terminus of gene 2.5 (21), we also examined the ability of the truncated

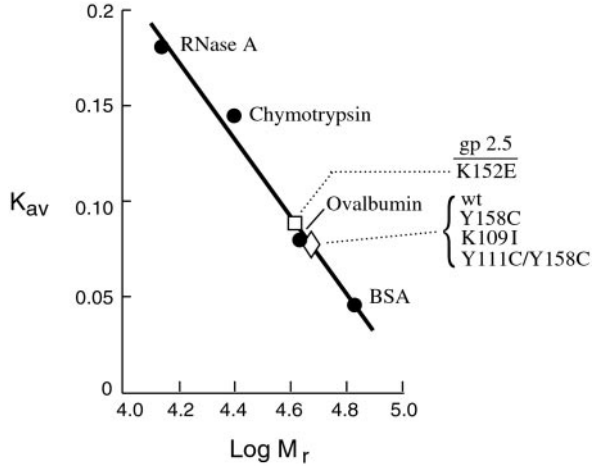

FIG. 2. **Estimation of the molecular weight of wild-type and variant gene 2.5 proteins by gel filtration analysis.** Gel filtration was performed using a Superdex 75 column as described under "Experimental Procedures." The protein standards, ovalbumin (43 kDa), chymotrypsinogen (25 kDa), bovine serum albumin (67 kDa), and ribonuclease A (13.7 kDa), were used to calibrate the column. The elution volumes of blue dextran and xylene cyanol determined the void volume and total volume of the column, respectively. A plot of K_{av} *versus* the log M_r of the standard proteins was generated and the best-fit line was determined. Wild-type gene 2.5 protein, gp2.5-K109I, gp2.5-K152E, gp2.5-Y158C, and gp2.5-Y111C/Y158C were applied to the column in three independent experiments. The K_{av} for each variant was calculated based on their elution volumes. Their positions on the standard curve are noted.

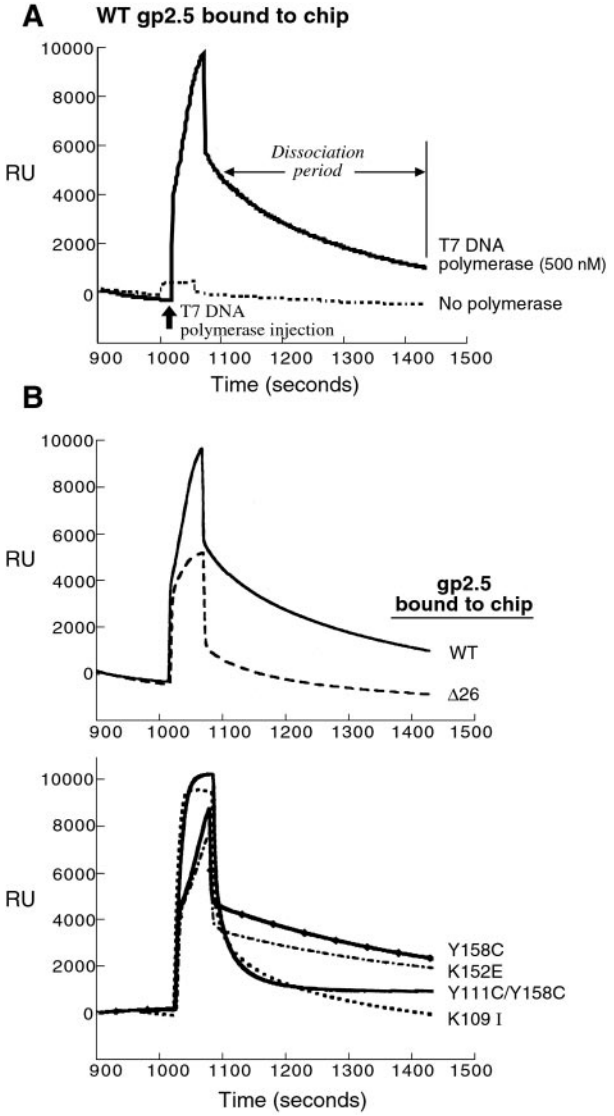

FIG. 3. **Interaction of gene 2.5 proteins with T7 DNA polymerase.** Surface plasmon resonance was used to detect the interaction between the gene 2.5 proteins and T7 DNA polymerase. The surface of the chip was activated by saturating the nitrilotriacetic sites with running buffer (100 mM Hepes-NaOH, pH 7.5, 50 μM EDTA, 0.1 mM DTT, 50 mM NaCl) containing 0.5 mM $NiCl_2$. Five nmol of the histidine-tagged gene 2.5 proteins were then immobilized on the chip surface. Following a period of stabilization, 5 nmol of DNA polymerase was passed over the chip. Its association and dissociation with the gene 2.5 proteins was monitored for 10 min through changes in the refractive index, measured in arbitrary resonance units (*RU*). *A*, an overlay plot where either 0 or 500 nM DNA polymerase was injected onto a nitrilotriacetic chip in which wild-type gene 2.5 had been immobilized. The injection point of T7 DNA polymerase and its dissociation period is noted. *B*: *top*, an overlay plot depicting the interaction of 500 nM T7 DNA polymerase with either wild-type gp2.5 (*solid line*) or gp.2.5Δ26C (*dashed line*) immobilized to the chip. *Bottom*, an overlay plot depicted the interaction of 500 nM T7 DNA polymerase with gp2.5-Y158C (*closed circles*), gp2.5-K152E (*dashed line*), gp2.5-Y111C/Y158C (*solid line*), or gp2.5-K109I (*dotted line*).

protein, gp2.5-Δ26C, that lacks the 26 carboxyl-terminal amino acids to interact with the T7 DNA polymerase (Fig. 3*B*, *top*). As expected, this protein did not appreciably bind the polymerase. These findings, combined with the gel filtration results presented above, increase our confidence that the variant proteins are not grossly misfolded.

Next we assessed the physical interaction between the wt and altered gene 2.5 proteins and the 63-kDa form of the gene 4 helicase/primase protein. Previous studies have shown that these proteins interact physically and functionally (7, 9). The interaction is weaker than that observed with T7 DNA polymerase and cannot be detected using surface plasmon resonance.[3] Therefore, we examined the interaction of gp2.5 with gene 4 protein using affinity chromatography. The 63-kDa gene 4 protein binds to a wt gp 2.5 affinity column and elutes over a broad range of salt concentrations (100 to 250 mM NaCl) (data not shown). Similarly, the 63-kDa gene 4 protein stably binds to gp2.5-K109I, gp2.5-K152E, gp2.5-Y158C, or gp2.5-Y111C/Y158C immobilized on a column. Gene 4 protein also elutes from these columns over a broad range of salt concentrations (50–250 mM NaCl) (data not shown). We conclude that the altered proteins interact with the 63-kDa gene 4 protein in a manner similar to wt gp2.5.

Binding of Gene 2.5 Protein to ssDNA—In an earlier study we assessed the binding of wild-type gene 2.5 protein to ssDNA using either a nitrocellulose filter binding assay or by measuring fluorescence quenching (8). In the present study we have employed an electrophoretic mobility shift assay, using radioactively labeled oligonucleotides of 38 and 70 nucleotides in length. In the experiment shown in Fig. 4*A* a fixed amount (3.3 nM) of the ^{33}P-labeled 38-nucleotide oligonucleotide (38-mer) was incubated with increasing amounts of wild-type gene 2.5 protein in the presence of 15 mM $MgCl_2$. The 38-mer and 38-mer·protein complexes were then resolved by electrophoresis through a nondenaturing polyacrylamide gel. The first detectable shift for the wild-type gene 2.5 protein binding to the 38-mer occurs at a protein concentration of 1330 nM. Using the Langmuir isotherm the dissociation constant (K_d) was calculated to be 7.9×10^{-6} M. The K_d values are listed in Table II for the wild-type protein as well as for the altered gene 2.5 proteins discussed below.

Several of the genetically altered gene 2.5 proteins clearly have a reduced ability to bind to the 38-mer oligonucleotide. Gp2.5-Y158C binds to the 38-mer only at the highest concen-

[3] L. F. Rezende and C. C. Richardson, unpublished data.

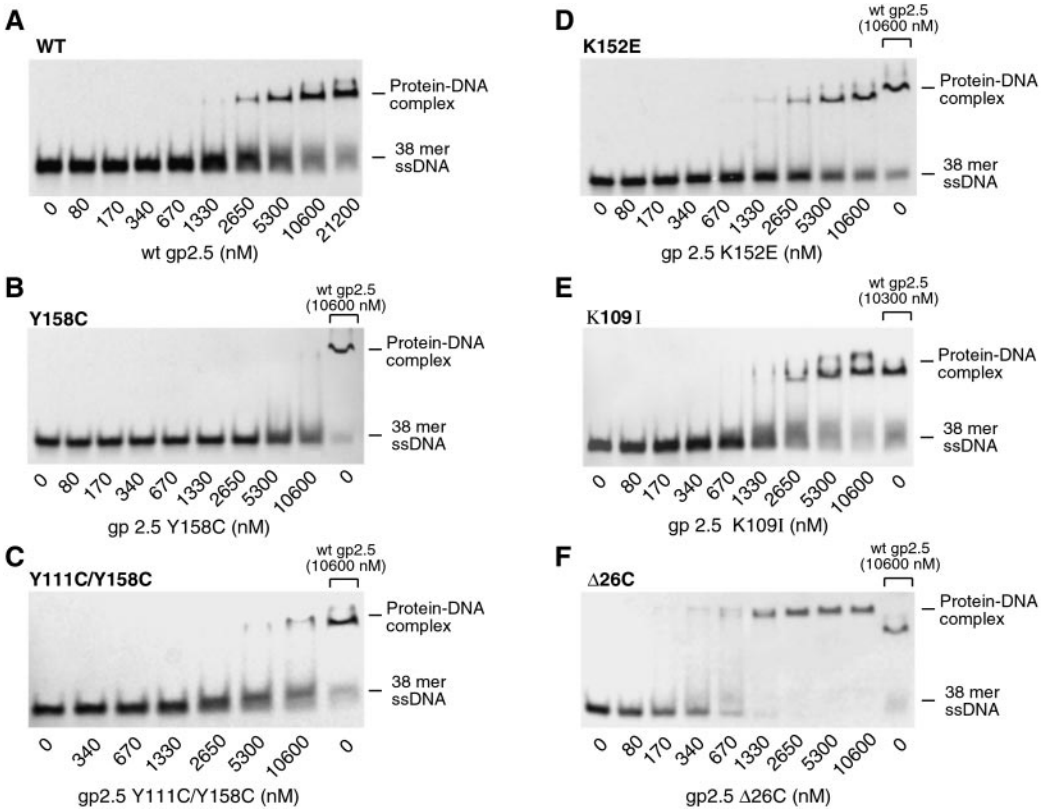

FIG. 4. **Binding of gene 2.5 proteins to a 38-nucleotide oligonucleotide.** An electrophoretic mobility shift assay was employed to assess the binding of the gene 2.5 proteins to ssDNA. 3.3 nM 5′-^{33}P-Labeled oligonucleotide was incubated for 15 min on ice with increasing amounts of gene 2.5 proteins, as described under "Experimental Procedures." Reaction products were resolved on a 10% nondenaturing polyacrylamide gel. The proteins examined were wild-type gene 2.5 protein (wt) (*A*), gp2.5-Y158C (Y158C) (*B*), gp2.5-Y111C/Y158C (Y111C/Y158C) (*C*), gp2.5-K152E (K152E) (*D*), gp2.5-K109I (K109I) (*E*), and gp2.5-Δ26C (*F*).

TABLE II
Dissociation constants of gene 2.5 proteins to ssDNA

Protein	Dissociation constants, K_d (M)[a]		
	38-mer	70-mer	
		Fast mobility	Slow mobility
Wt gp 2.5	7.9×10^{-6}	3.3×10^{-6}	5.4×10^{-7}
Gp2.5-Y111C/Y158C	2.5×10^{-5}	3.3×10^{-5}	—[b]
Gp2.5-K152E	6.6×10^{-5}	3.6×10^{-5}	2.3×10^{-6}
Gp2.5-Y158C	2.9×10^{-5}	1.0×10^{-5}	2.6×10^{-6}
Gp2.5-K109I	8.1×10^{-6}	—[b]	5.7×10^{-7}
Gp2.5-Δ26C	5.6×10^{-7}	—[b]	3.6×10^{-8}

[a] Dissociation constants (K_d) were calculated based on results obtained from three individual electrophoretic mobility shift assays, using the Langmuir isotherm ($r = [A]/K_d + [A]$, r = ssDNA bound, A = total ssDNA).
[b] No shift detected.

tration of protein tested, thus requiring almost 10-fold more protein than the native protein (Fig. 4*B*). As expected, a similar pattern of diminished binding is also observed with the gene 2.5 protein containing the two amino acid substitutions Y111C/Y158C (Fig. 4*C*). Likewise gp2.5-K152E (Fig. 4*D*) has a lower affinity for the 38-mer, and more protein is required to obtain a band shift relative to the wild-type protein. Although gp2.5-K109I is not able to support T7 growth and the amino acid change lies within the OB fold the K109I alteration has no detectable affect on binding to the ssDNA (Fig. 4*E*) with a K_d comparable with that of wild-type gene 2.5 protein. Interestingly, the truncated form of the protein, gp2.5-Δ26C, binds much tighter to the 38-mer compared with the native protein (Fig. 4*F*) with a corresponding K_d that is 10-fold lower.

The binding of the altered gene 2.5 proteins to a 70-mer oligonucleotide was then assessed to determine whether the length of ssDNA influenced the ssDNA binding patterns of the variant protein. Results obtained mimicked that found with the 38-mer except for the appearance of two individual bands in the gel shift for a number of these gene 2.5 proteins. Wild-type gene 2.5 protein initially binds to the 70-mer at a protein concentration of 670 nM, where two species of 70-mer·protein complexes are observed. As the amount of gene 2.5 protein is increased the amount of unbound 70-mer decreases such that by 2650 nM all of the 70-mer is found in the slower migrating species (Fig. 5*A*). The K_d was calculated to be 3.3×10^{-6} for the more rapidly migrating complex and 5.4×10^{-7} for the slower migrating complex. No bandshift was observed in the case of gp2.5-Y158C until a protein concentration of 2650 nM was reached, which was 4-fold the concentration required for the native protein (Fig. 5*B*). Furthermore, the slower migrating complex is observed only at the highest concentration of protein. The K_d values for both complexes are ~10-fold higher than that observed with the wild-type protein (Table II). The variant gp2.5-Y111C/Y158C has a similar affinity to that of gp2.5-Y158C (Fig. 5*C*) but with this altered protein the slower

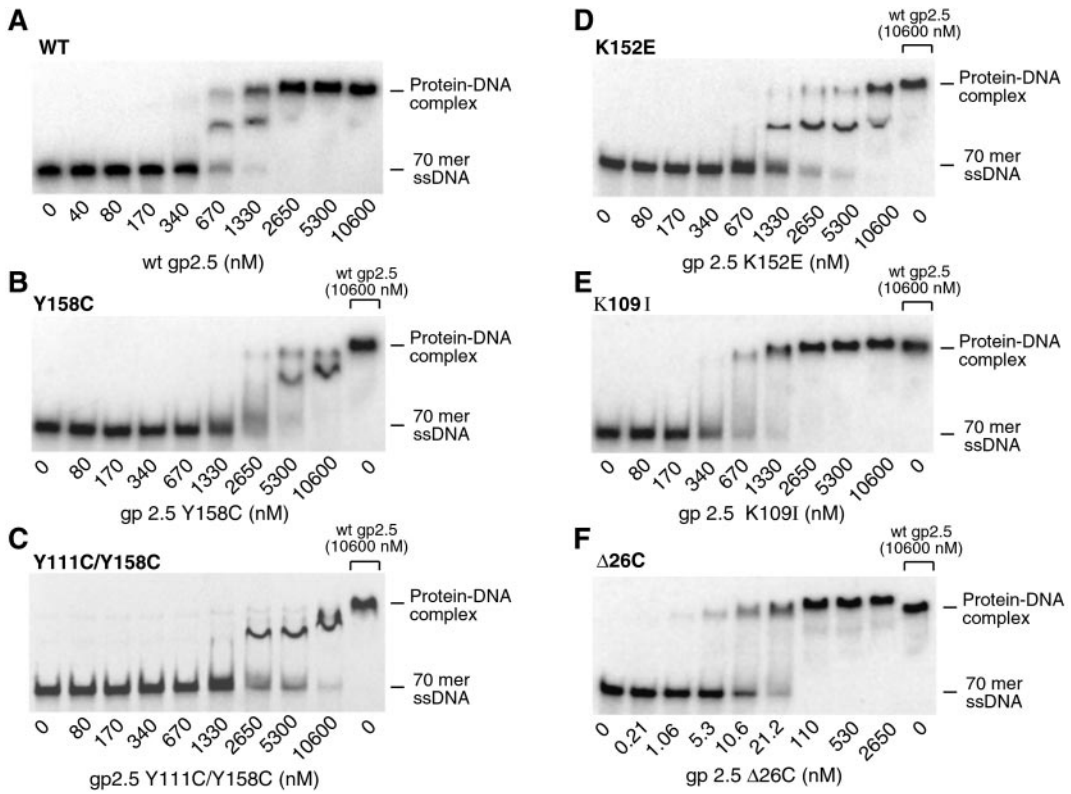

FIG. 5. **Binding of gene 2.5 proteins to a 70-nucleotide oligonucleotide.** The experiment in Fig. 4 was repeated with a 70-mer oligonucleotide and the same abbreviations are used.

migrating complex is never observed. In addition gp2.5-K152E has a lower affinity for the 70-mer and considerably higher concentrations of this altered protein are required to bind all of the oligonucleotide and to achieve the slower migrating complex (Fig. 5*D* and Table II). As with the 38-mer, gp2.5-K109I appears to bind to the 70-mer with the same affinity as the native protein. However, no rapidly migrating complex is observed with gp2.5-K109I (Fig. 5*E*). We have also examined the binding of the altered protein gp2.5-Δ26C, where the protein is found to have a higher affinity for the 70-mer and, like gp2.5-K109I, fails to produce the rapidly migrating complex (Fig. 5*F*).

Homologous Base Pairing Mediated by Gene 2.5 Proteins—Gene 2.5 protein facilitates the annealing of complimentary strands of ssDNA,[2] a property that has been used in preparing substrate for studies on the strand transfer mediated by the T7 helicase (10, 11). We have measured the ability of the altered gene 2.5 proteins to facilitate this reaction to determine the effect of the decreased affinity for ssDNA. Wild-type gene 2.5 protein is capable of successfully annealing the homologous ssDNA at a concentration of 4 μM (Fig. 6*A*). A time course experiment revealed that the wild-type gene 2.5 protein can accomplish this base pairing after approximately 1 min incubation at 30 °C (Fig. 6*B*). The genetically altered gene 2.5 proteins defective in ssDNA binding do not anneal the two species of ssDNA at the concentrations tested (Fig. 6, *C–E*). Presumably this lack of activity is because of the decreased binding affinity of these proteins, because we have observed a small percentage (<25%) of annealed products at high protein concentrations (data not shown). Therefore, it is likely that the defect in annealing observed for these proteins is a reflection of the decreased affinity for ssDNA. Interestingly, whereas gp2.5-K109I shows an equal affinity for ssDNA in the electrophoretic mobility shift assay, it is not as efficient in annealing homologous ssDNA requiring twice as much protein (Fig. 6*F*), and taking 1 min longer to form the annealed product (compare Fig. 6, *G–B*).

DISCUSSION

There is a significant lack of knowledge on the structure-function relationship of the product of bacteriophage T7 gene 2.5, a ssDNA-binding protein. In the current study we sought to define the DNA binding domain of gp2.5. This study provides the first experimental evidence for the identity of the DNA binding domain of gp2.5. In a separate report (35) we describe a genetic screen for lethal mutations in bacteriophage T7 gene 2.5. By examining the crystal structure of the protein (16) we noted that a subset of these generated mutations lay in the postulated ssDNA binding domain. In this study we have biochemically characterized these altered gene 2.5 proteins and show that two of them, gp2.5-Y158C and gp2.5-K152E, are indeed defective in their interaction with ssDNA. A third, gp2.5-K109I, appears to interact differently with ssDNA when assessed by an electrophoretic mobility shift assay. We feel confident that the defective phenotypes do not arise from a misfolding of the protein as they physically interact with both T7 DNA polymerase and the gene 4 protein, a helicase/primase. In addition, all four proteins form dimers in a manner similar to the wild-type protein.

At the onset of these studies the amino acids involved in the interaction of gene 2.5 protein with ssDNA were unknown. Previous studies on other ssDNA-binding proteins have shown that aromatic residues have the potential to intercalate between the nucleic acid bases thus stabilizing the protein-ssDNA interaction (27, 41–43). In the *E. coli* SSB protein for example mutational studies have implicated phenylalanine 60 (Phe^{60}) and tyrptophan 54 (Trp^{54}) in binding ssDNA (44–46). Similarly, in T4 gene 32, protein site-directed mutagenesis has identified numerous tyrosine residues necessary for the proteins interaction with ssDNA (47, 48). T7 gene 2.5 protein

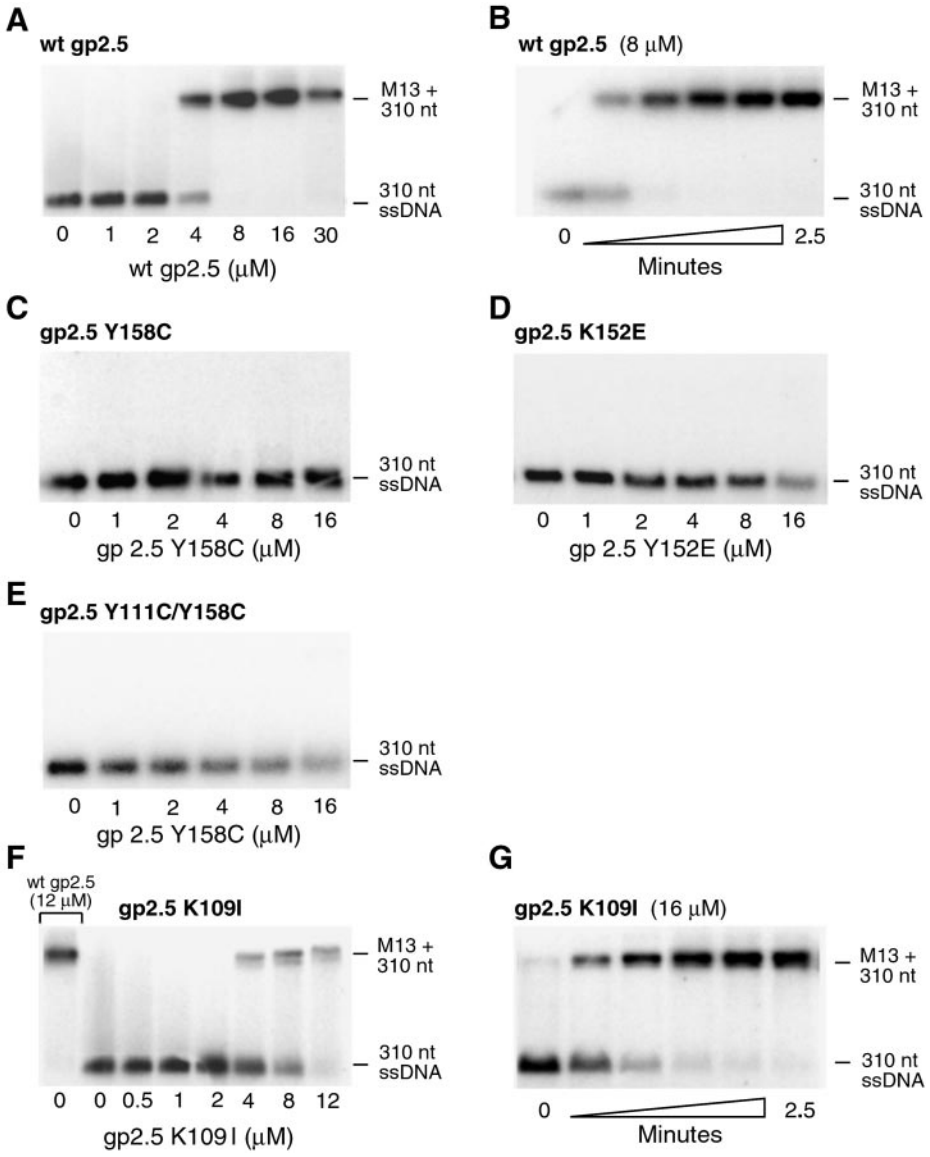

FIG. 6. **Homologous base pair annealing facilitated by gene 2.5 proteins.** Circular M13 ssDNA was incubated with a 310-nt ^{32}P-labeled complimentary ssDNA fragment, in the presence of increasing concentrations of gene 2.5 proteins as described under "Experimental Procedures." The annealing products were fractionated on a nondenaturing agarose gel and visualized by autoradiography. *A,* a concentration series of wild-type gene 2.5 protein illustrating the ability of this protein to anneal a 310-nt fragment to M13 ssDNA. *B,* a time course experiment using 8 μM wild-type gene 2.5 protein. Time points were taken at 30-s intervals and the reaction was terminated by adding 5 μl of stop solution (0.25% bromphenol blue, 0.25% xylene cyanol FF, 30% glycerol, and 0.5% SDS). *C–E*, annealing reactions were performed using increasing amounts of gp2.5-Y158C (*C*), gp2.5-K152E (*D*), and gp2.5-Y111C/Y158C (*E*). *F,* the annealing activity of gp2.5-K109I was assessed over a range of protein concentrations. *G,* the rate of homologous base pair annealing of a 310-nt fragment to M13 ssDNA mediated by gp2.5-K109I (16 μM). Reactions were performed as described above.

possesses one such structurally conserved aromatic residue found in the OB fold. Based on our observation that the lethal substitution Y158C gives rise to a gene 2.5 protein that has a higher dissociation constant for ssDNA than that of wild-type gene 2.5 protein, we can infer that this residue plays an essential role in binding to ssDNA. Similarly, the altered gp2.5-Y111C/Y158C is also defective in binding ssDNA although the binding constant is similar to that of gp2.5-Y158C alone, implying that tyrosine 111 is not essential for ssDNA binding. This result was unexpected as an aromatic residue is conserved at this site among other ssDNA-binding proteins, specifically phenylalanine 60 in *E. coli* SSB protein and phenylalanine 90 in human replication protein A protein (16).

Basic residues, which can make electrostatic contacts with the negatively charged phosphates in ssDNA, are also likely candidates for DNA binding. Lysine residues have been shown to be involved in the ability of *E. coli* SSB protein to bind ssDNA (27, 49). Indeed we have shown that substituting lysine 152 with glutamic acid weakens its binding to ssDNA, as this variant protein exhibits a 10-fold decrease in its affinity for ssDNA. Interestingly, the protein·DNA complex resulting from this interaction has difficulty adopting the slower mobility complex at concentrations comparable with wild-type gene 2.5 protein, as discussed in more detail below. Perhaps Lys^{152} is involved in an interaction of gene 2.5 with ssDNA at higher concentrations, and this interaction leads to the higher order structure in a manner similar to the *E. coli* SSB protein, which demonstrates distinct binding modes at different protein concentrations (18). In addition, the side chain of Lys^{152} is oriented away from the prominent groove in the crystal structure, decreasing its direct accessibility to ssDNA bound at this site. Therefore to implicate Lys^{152} in binding ssDNA, the ssDNA would somehow have to wrap around the protein, suggesting that the interaction encompasses more residues than those lying directly within this groove. A structural based sequence alignment of other ssDNA-binding proteins does not reveal conservation at this particular residue (16) and therefore, perhaps this interaction is a unique feature of the gene 2.5 protein.

A second lethal mutation resulting in the alteration of a basic residue was identified at Lys^{109}. In contrast to gp2.5-K152E, this K109I variant gene 2.5 protein failed to inhibit the ability of gene 2.5 protein to bind ssDNA despite the loss of a positively charged residue in the OB fold. Interestingly gp2.5-K109I displayed an aberrant binding pattern in the gel shift assay, forming only the slower mobility complex. This binding pattern was also exhibited by gp2.5-Δ26C, which likewise failed to form the rapidly migrating complex. Originally this binding pattern was thought to indicate a level of cooperative binding to ssDNA

not characteristic of the native protein. However, upon closer examination, the appropriate kinetic calculations, *i.e.* Hill coefficients, could not support this theory. Further dissection of the binding mode(s) of the native protein may lead to an explanation of this observation.

Based on the crystal structure a model was proposed for DNA binding that assumes that gene 2.5 binds ssDNA as a monomeric species (16). The hypothesis is that the negatively charged, acidic carboxyl terminus competes with the proposed DNA binding site of an adjacent protomer leading to the formation of dimers in the absence of ssDNA. Therefore dissolution of the dimer would be necessary to expose the DNA binding domain and allow for ssDNA binding. In support of this model we have shown how a carboxyl-terminal truncated form of the protein gp 2.5-Δ26C that exists as a monomer in solution (35) has a 10-fold greater affinity for ssDNA as compared with wild-type gene 2.5 protein. Similar results have been seen with gp2.5-Δ21C, also a monomer in solution, where ssDNA binding was analyzed by surface plasmon resonance.[4]

The electrophoretic mobility shift assay employed in this study provided an insight into the mode by which wild-type gene 2.5 protein binds ssDNA. Over a protein concentration series from 80 to 10,600 nM, upon binding a 70-mer oligonucleotide, two distinct protein·DNA complexes were resolved. Conceivably these two complexes could represent the binding of one monomer of gene 2.5 protein and a subsequent second monomer at a higher concentration. This interpretation is supported by the absence of the slower mobility complex when wild-type gene 2.5 protein binds to a shorter oligonucleotide of 38 bases in length as presumably only one monomer can be accommodated on this length. However, this hypothesis does not agree with the published site size for the protein, which is seven nucleotides per monomer (8). This site size was calculated by assessing the binding of gene 2.5 protein to circular M13 ssDNA. In a subsequent study using surface plasmon resonance, stable binding of gene 2.5 protein required an oligonucleotide of at least 30 nucleotides in length.[4] Nonetheless this unexplained phenomenon presented by the distinct protein·DNA complexes warrants further study especially because other ssDNA-binding proteins have demonstrated different modes of binding ssDNA.

As gene 2.5 protein has the capacity to mediate homologous DNA annealing[2] we examined how efficiently the altered proteins could accomplish this activity. The altered proteins deficient in binding to ssDNA were also defective in annealing complimentary strands of ssDNA. However, we do observe some annealing at higher protein concentrations. For this reason, we feel the lack of annealing we observed is related to the affinity of the altered proteins for ssDNA rather than reflecting a defect in the basic mechanism of homologous base-pairing. Interestingly, gp2.5-Δ26C facilitates strand annealing more efficiently than the wild-type protein,[3] further supporting a relationship between ssDNA binding affinity and homologous base pair annealing. Furthermore, despite its unaltered affinity for ssDNA the variant protein K109I is defective in base pairing, requiring 2-fold more protein to completely anneal all the substrate. We are currently pursuing the mechanism of this reaction by studying another altered protein that binds ssDNA but cannot facilitate the annealing of homologous strands of ssDNA.[3] From the data presented here, we conclude that gp2.5 must be able to bind ssDNA to facilitate DNA annealing.

All of the proteins described in this study were expressed from lethal mutations in gene 2.5. It is likely that gp2.5-K152E, gp2.5-Y158C, and gp2.5-Y111C/Y158C are lethal because they have a lower affinity for ssDNA. Given that ssDNA binding is an essential function for gene 2.5 it is not surprising that amino acid changes that reduce binding affinity *in vitro* are lethal *in vivo*. In addition, we have shown that these proteins can still form dimers, and physically interact with the T7 DNA polymerase and the 63-kDa gene 4 protein. The mechanism underlying gp2.5-K109I lethality, on the other hand, remains unclear. In cells harboring a plasmid encoding for this genetic alteration, we detected a reduced level DNA synthesis *in vivo* upon infection by T7Δ2.5 bacteriophage,[5] suggesting that the function of this residue is important in the overall DNA replication of the bacteriophage. It is conceivable that both the impaired ability to facilitate annealing and the altered manner in which it binds ssDNA accounts for the *in vivo* phenotype. However, further investigation is necessary to probe the precise molecular basis of its involvement in the life cycle of bacteriophage T7.

Acknowledgments—We thank Tomas Hollis for preparation of Fig. 1, Stanley Tabor for assistance with the DNA annealing assay, and Don Crampton for critically reading the manuscript.

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