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Essential Amino Acid Residues in the Single-stranded DNA-binding Protein of Bacteriophage T7

IDENTIFICATION OF THE DIMER INTERFACE*

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Gene 2.5 of bacteriophage T7 is an essential gene that encodes a single-stranded DNA-binding protein. T7 phage with gene 2.5 deleted can grow only on *Escherichia coli* cells that express gene 2.5 from a plasmid. This complementation assay was used to screen for lethal mutations in gene 2.5. By screening a library of randomly mutated plasmids encoding gene 2.5, we identified 20 different single amino acid alterations in gene 2.5 protein that are lethal *in vivo*. The location of these essential residues within the three-dimensional structure of gene 2.5 protein assists in the identification of motifs in the protein. In this study we show that a subset of these alterations defines the dimer interface of gene 2.5 protein predicted by the crystal structure. Recombinantly expressed and purified gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S do not form dimers at salt concentrations where the wild-type gene 2.5 protein exists as a dimer. The basis of the lethality of these mutations *in vivo* is not known because altered proteins retain the ability to bind single-stranded DNA, anneal complementary strands of DNA, and interact with T7 DNA polymerase.

Gene 2.5 of bacteriophage T7 is essential for phage growth (1). It encodes a single-stranded DNA (ssDNA)[1]-binding protein that is functionally similar to the *Escherichia coli* SSB protein and the gene 32 protein of bacteriophage T4 (2, 3). Like these ssDNA-binding proteins, the gene 2.5 product (wt gene 2.5 protein) is important for DNA replication, recombination, and repair (1–12). However, neither the *E. coli* SSB protein nor the T4 gene 32 protein can replace gene 2.5 protein in cells infected by T7 phage lacking gene 2.5 (13). This specificity for gene 2.5 protein is not surprising as there is little sequence homology between the proteins, and wt gene 2.5 protein differs from the other proteins significantly in a number of biochemical properties. For instance, the T7 protein binds to DNA with a lower affinity than either *E. coli* SSB protein or T4 gene 32 protein (2). The oligomeric state of these proteins also differ with wt gene 2.5 protein existing as a stable dimer in solution (2), whereas *E. coli* SSB protein forms a tetrameter (14), and T4 gene 32 protein is a monomer that forms multimers at high concentrations (15, 16). In addition to interacting with itself, wt gene 2.5 protein also interacts specifically with T7 DNA polymerase and the product of T7 gene 4, a helicase/primase (17). *E. coli* SSB protein and T4 gene 32 protein feature acidic carboxyl-terminal motifs that are involved in protein-protein interactions (18–22). Similarly, the acidic carboxyl terminus of wt gene 2.5 protein is required to mediate interactions with other replication proteins (13, 23), including those that coordinate leading and lagging strand synthesis by T7 replication proteins on a minicircle template *in vitro* (11).

Because of its critical role in interactions with other replication proteins, mutagenesis studies on gene 2.5 protein to date have focused on the carboxyl terminus (13, 23). In one study (23), a truncated gene 2.5 protein missing the final 21 amino acids was produced. Expressing this altered gene 2.5 protein in *E. coli* did not support the growth of a T7 phage deleted in gene 2.5 (23). The truncated gene 2.5 protein itself is a monomer in solution but retains the ability to bind DNA (23). It neither stimulates DNA synthesis by T7 DNA polymerase nor does it interact physically with that protein (23). A second study examined chimeric proteins in which the carboxyl-terminal motif of wt gene 2.5 protein was replaced with the complementary motif of T4 gene 32 protein and *E. coli* SSB protein (13). The chimeric proteins could support phage growth, form dimers, and interact with T7 DNA polymerase (13). When the carboxyl-terminal motif of T7 wt gene 2.5 protein was used to replace that of *E. coli* SSB protein and T4 gene 32 protein, the chimeric proteins could not substitute for wt gene 2.5 protein to support the growth of a gene 2.5-deleted phage (13). These results suggest that although the carboxyl terminus is required for protein-protein interactions, it does not account for the specificity of those interactions (13).

Recently a three-dimensional crystal structure of a carboxyl terminus deleted form of T7 gene 2.5 protein was published (24). The protein has a conserved oligosaccharide/oligonucleotide binding fold (25), similar to that of T4 gene 32 protein (26) and *E. coli* SSB protein (27, 28). The structure suggests models for DNA binding and dimerization (24); however, there are no mutagenesis studies to either support or refute these models. In fact, outside of the studies on the carboxyl terminus described above, there is a lack of experimental evidence to define the functional domains of wt gene 2.5 protein. To begin mapping these domains, we developed a screen for lethal mutations in gene 2.5. A similar screen was successfully used to identify lethal mutants of the T7 helicase/primase (29). Presumably, mutations that are lethal will occur in regions critical to wt gene 2.5 protein functions or proper folding. In the present study we characterize three of the altered proteins biochemi-

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[1] The abbreviations used are: ssDNA, single-stranded DNA; wt, wild-type; DTT, dithiothreitol; NTA, nitrilotriacetic acid; SPR, surface plasmon resonance.

cally, and we show that they define the interface for dimer formation, demonstrating that dimerization is an essential property of gene 2.5 protein.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Phage—E. coli XL1-Red (*end*A1 *gyr*A96 *thi*1 *hsd*R17 *sup*E44 *rel*A1 *lac mut*D5 *mut*S *mut*T *Tn*10 (Tet^r)) (Stratagene) was used to generate a library of randomly mutated plasmids. *E. coli* HMS262 (F$^-$ *hsdR pro leu*$^-$ *lac*$^-$ *thi*$^-$ *supE tonA*$^-$ *trxA*$^-$) and *E. coli* HMS 89 (*xth1 thi argE mtl xyl str-R ara his galK lacY proA leu thr tsx supE*) were used as hosts for phage experiments. *E. coli* BL21 (DE3) (F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)) (Novagen) was used to express wild-type gene 2.5 and mutant gene 2.5. Construction of the T7 deletion phage (T7Δ2.5) was described previously (1). T7Δ2.5 phage used in the *in vivo* DNA synthesis experiments was provided by Jaya Kumar (Harvard Medical School).

Plasmids, Oligonucleotides, and Proteins—The plasmids encoding gene 2.5, pETGP2.5 and pETGP2.5-PPS were provided by James Stattel (Harvard Medical School). The parent vector of pETGP2.5-PPS, pET19bPPS, which encodes a tag of 10 histidine residues and a rhinovirus C protease (PreScission protease, Amersham Biosciences) cleavage site located upstream of the start codon, was kindly provided by Tapan Biswas (Harvard Medical School). The following oligonucleotides were purchased from Oligos Etc.: T72.5NdeI, 5′-CGTAGGATCCATATGGCTAAGAAGATTTTCACCTC-3′; T72.5BamHI, 5′-CGTAGGATCCACTTAGAAGTCTCCGTC-3′; and Oligo 70, 5′-GACCATATCCTCCACCCTCCCCAATATTGACCATCAACCCTTCACCTCACTTCACTCCACTATACCACTC-3. The following oligonucleotides were purchased from Integrated DNA Technologies: T7 promoter, 5′-TAATACGACTCACTATAGGGG-3′; pET17up, 5′-CTTTAAGAAGGAGATATACATATG-3′; T7 terminator, 5′-GCTAGTTATTGCTCAGCGG-3′; and DS17b, 5′-GCTTCCTTTCGGGCTTTG-3′. The oligonucleotide BCMP206, 5′-TAACGCCAGGGTTTTCCCAGTCACG-3′, was synthesized by the Biopolymer Laboratory, Harvard Medical School. M13 (mGP1-2) DNA and T7 DNA polymerase lacking exonuclease activity (30) were kindly provided by Stan Tabor (Harvard Medical School). Wild-type and altered gene 2.5 proteins were purified as described below. Gene 2.5 protein-Δ26C was provided by Eric Toth (Harvard Medical School). His-gene 2.5 protein-Δ26C was provided by James Stattel (Harvard Medical School). T7 DNA polymerase was provided by Don Johnson and Joon-Soo Lee (Harvard Medical School).

Random Mutagenesis of DNA—A library of randomly mutated plasmids was created using the mutator *E. coli* strain XL1-Red (Stratagene). The plasmid pETGP2.5 was transformed into XL1-Red, and transformants were plated on LB plates supplemented with 60 μg/ml ampicillin and incubated overnight at 37 °C. The next day, 2 ml of LB were added to plates to facilitate the scraping of the colonies. Ampicillin was added to a concentration of 60 μg/ml, and the culture of pooled colonies were grown overnight at 37 °C. The next day plasmid DNA was prepared from the bacteria using an RPM kit (Qbiogene).

Selection of Lethal Mutations in Gene 2.5—Selection of lethal mutations in gene 2.5 was based on the complementation assay described previously (1). When gene 2.5 is expressed on a plasmid, the phage T7Δ2.5 can grow in *E. coli* HMS262. The screen was performed in a manner similar to that used to uncover lethal mutants of bacteriophage T7 gene 4 (29) with alterations noted below.

Randomly mutated plasmids generated from pETGP2.5 were introduced into *E. coli* HMS262 by electroporation using an *E. coli* Pulser Transformation Apparatus (Bio-Rad) in 19 separate experiments. Electrocompetent *E. coli* HMS262 cells were prepared according to the manufacturer's recommendation (Bio-Rad). In each experiment, 1 ng of DNA was mixed with 40 μl of electrocompetent cells and incubated on ice for 5 min. The mixtures were transferred to 0.1-cm cuvettes (Bio-Rad). Cuvettes were pulsed at 1.80 kV. One ml of SOC (2% bactotryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 20 mM glucose) was added immediately after pulsing, and the mixture was then transferred to a 15-ml polystyrene tube. Cells were allowed to recover by shaking for 1 h at 37 °C. One hundred fifty μl of cells were plated on LB plates containing 60 μg/ml ampicillin, which were overlaid with 2.5 ml of top agar (1% tryptone, 0.5% yeast, 0.5% NaCl, 0.7% agar (pH 7.0)) containing 60 μg/ml ampicillin either alone or with 10^7 plaque-forming units of T7Δ2.5 phage. Plates were incubated at 37 °C overnight. The next morning, colonies that formed on the LB plates with ampicillin were counted to determine the efficiency of electroporation.

Colonies that formed on the plates overlaid with T7Δ2.5 phage were counted, then streaked on LB plates with 60 μg/ml ampicillin, and cross-streaked with T7Δ2.5 phage to confirm that the cells could not support the replication of the gene 2.5 deleted phage. Approximately 0.6% of the colonies screened could not support the growth of T7Δ2.5 phage. After streaking, a collection of 291 cultures of transformants that are unable to support the growth of T7Δ2.5 phage were frozen as glycerol stocks.

Sequencing of Plasmids from Transformants That Do Not Support the Growth of T7Δ2.5 Phage—Plasmid DNA was prepared from 5-ml cultures of 216 independent transformants. Each plasmid was analyzed by restriction digests with *Nde*I and *Bam*HI (New England Biolabs) to ensure that a 699-bp fragment was released. This analysis eliminated 14 plasmids from further consideration. The remaining 202 plasmids were sequenced by the Dana-Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility using the sequencing primers pET17up and DS17b. Readable sequence was obtained for 190 plasmids.

In Vivo DNA Synthesis Assay—DNA synthesis was measured by a method modified from Richardson and co-workers (31, 32). A culture of Davis minimal media supplemented with 60 μg/ml ampicillin was inoculated with *E. coli* HMS262 transformed with pETGP2.5, pETGP2.5-P22L, pETGP2.5-F31S, or pETGP2.5-G36S and grown at 30 °C in a gyratory shaker. Cells were grown to a density of 3×10^8 cells per ml and then infected with T7Δ2.5 phage at a multiplicity of infection of 7. At 5-min intervals post-infection, 200-μl samples were removed, and [^{3}H]thymidine (50 μCi/ml) was added. Reactions were incubated at 30 °C for 90 s and then terminated by adding 40 μl of an ice-cold solution of 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 2% SDS. Sixty μl of the terminated reactions were spotted onto DE81 filters. Filters were washed 3 times in 0.3 M ammonium formate, 2 times in ethanol, and then air-dried. [^{3}H]Thymidine incorporation into DNA was then determined by liquid scintillation counting.

Expression and Purification Gene 2.5 Proteins—Wild-type and altered gene 2.5 protein were purified by a procedure developed by Stattel and Richardson.[2] The plasmids pET2.5, pET2.5-P22L, pET2.5-F31S, and pET2.5-G36S were transformed into *E. coli* BL21(DE3) (Novagen). One- (pET2.5-P22L and pET2.5-F31S) or 8-liter cultures (pET2.5, pET2.5-G36S) were grown in LB with 60 μg/ml ampicillin to an OD of 1.0. Cells were induced for 4 h after adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Cells were then collected by centrifugation and resuspended in 20 ml/liter of culture lysis buffer (50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 10% sucrose), frozen in dry ice, and stored at −70 °C. Lysozyme (Sigma) was added to thawed cells (final concentrated 1 mg/ml) and stirred in the cold for 1 h. Lysed cells were warmed to 20 °C in a 37 °C bath, then chilled on ice, and centrifuged at 4 °C for 45 min at 100,000 × *g*. Polyethyleneimine (pH 7.5) was added to the supernatant (final concentration, 0.1%), and the mixture was stirred at 4 °C for 1 h. The mixture was centrifuged at 4 °C for 15 min at 21,000 × *g*. The resulting pellet was suspended in 75 ml of Buffer A (50 mM Tris-Cl (pH 7.5), 0.1 EDTA, 1 mM dithiothreitol (DTT), 10% glycerol) containing 1 M NaCl, stirred for 1 h at 4 °C, and then centrifuged at 21,000 × *g* for 15 min at 4 °C. The supernatant was collected and then diluted with Buffer A to a final volume of 150 ml. To precipitate the proteins, $(NH_4)_2SO_4$ was added to 80% saturation, and the solution was stirred for 1 h at 4 °C and then centrifuged at 21,000 × *g* for 15 min. The pellet was suspended in 60 ml of Buffer A and filtered through a 0.22-μm syringe filter. The sample was loaded onto a POROS HQ column (PE Biosystems) and gene 2.5 protein eluted in a 50 mM to 1 M NaCl gradient. Fractions containing gene 2.5 protein were pooled, and the protein was precipitated by adding $(NH_4)_2SO_4$ to 60% saturation. The solution was centrifuged at 21,000 × *g* for 15 min. The resulting pellet was resuspended in Buffer G (50 mM KPO_4 (pH 7.0), 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol) to a concentration of no more than 5 mg/ml. The sample was loaded onto a Superose 12 column (Amersham Biosciences). Fractions that contained gene 2.5 protein were pooled, dialyzed against Buffer S (50 mM Tris-Cl (pH 7.5), 0.1 mM EDTA, 1 mM DTT, 50% glycerol), and then stored at −20 °C. Purified wt gene 2.5 protein, gene 2.5 protein-P22L, and gene 2.5 protein-F31S were over 99% pure as determined by denaturing polyacrylamide gel electrophoresis followed by Coomassie Blue staining and were free of contaminating DNA-dependent nuclease activity (data not shown). Protein concentrations were calculated from UV spectrophotometer readings at 280 mM, using the calculated extinction coefficients at 280 nM (33) of 2.58×10^4 M^{-1} cm^{-1}. This procedure consistently yielded only small amounts of gene 2.5 protein-G36S, and the preparations were contaminated with a DNA nuclease. For this reason

[2] J. Stattel and C. C. Richardson, unpublished data.

gene 2.5 protein-G36S was expressed and purified as a 10-histidine fusion protein as described below.

Expression and Purification of Histidine-tagged Gene 2.5 Proteins—Separate 1-liter cultures of BL21(DE3) cells transformed with pET19b2.5PPS, pET19b2.5PPS-P22L, pET19b2.5PPS-F31S, and pET19b2.5PPS-G36S were grown, induced, and harvested as described above. Pellets were resuspended in 20 ml of Buffer B (50 mM Tris-Cl (pH 7.5), 500 mM NaCl) containing 70 mM imidazole, then frozen in dry ice, and stored at −70 °C. Lysozyme (Sigma) was added to thawed cells (final concentration 1 mg/ml), and the suspension was stirred at 4 °C for 2 h. One hundred twenty five units of Benzonase nuclease (Novagen) was added to lysates that were then rapidly warmed to ~20 °C in a 37 °C bath, chilled on ice, and centrifuged at 4 °C for 1 h at 8,000 × *g*. Supernatants were loaded onto a 5-ml column packed with nickel-NTA-agarose (Qiagen). The column was washed with 10 column volumes of Buffer B containing 70 mM imidazole and proteins eluted in 2 column volumes of Buffer B containing 500 mM imidazole. Histidine-tagged gene 2.5 protein (His-gene 2.5 protein), His-gene 2.5 protein-P22L, His-gene 2.5 protein-F31S, and His-gene 2.5 protein-G36S were dialyzed against Buffer S, and stored at −20 °C. An aliquot of His-gene 2.5 protein-G36S was then processed to remove the amino-terminal tag.

To cleave the histidine tag, 50 μg of PreScission protease was added to the eluted fraction, and the entire protein solution was dialyzed for18 h against Buffer C (50 mM Tris-Cl (pH 8.0), 225 mM NaCl, 0.1 mM EDTA, 2 mM DTT) using 10-kDa cut-off dialysis membrane (Pierce). The dialyzed protein solution was passed through a 1-ml GSTrap column (Amersham Biosciences) at a rate of 0.5 ml/min to remove the PreScission protease. Proteins were then re-applied to a 5-ml Ni-NTA column to ensure removal of any protein that still contained the histidine tag. Purified proteins were dialyzed into Buffer S and stored at −20 °C. Proteins prepared in this manner were determined to be over 95% pure and free of contaminating nuclease activity.

Size Determination by Gel Filtration—Gel filtration analysis was performed as described previously (2). Briefly, in three independent experiments 50 μg of wt gene 2.5 protein, gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S diluted in Buffer S (final concentration 4 μM) were applied to a Superdex 75 column (Amersham Biosciences) at a flow rate of 0.50 ml/min. The elution of each protein was monitored by absorbance at 280 nm. Chromatography was carried out at 4 °C in Buffer G (50 mM KPO_4 (pH 7.0), 150 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol). The running buffer for high salt experiments was 50 mM KPO_4 (pH 7.0), 250 mM NaCl, 0.1 mM EDTA, 0.1 mM DTT, and 10% glycerol. The peak elution volume (v_e) was taken to be the average of the volumes at which each protein eluted in three experiments. The void volume (v_0) and total volume (v_t) were determined by independently applying blue dextran and xylene cyanol, respectively. The fractional retention (K_{av}) was calculated using the formula $K_{av} = (v_e - v_0)/(v_t - v_0)$, where v_e is the peak elution volume. A standard curve of K_{av} *versus* log M_r was generated by applying both high and low molecular weight protein standards (Amersham Biosciences) to the column under the conditions described above. Standard curves were generated at both salt concentrations examined in this study.

Gel Shift Assay for ssDNA Binding—The oligodeoxynucleotide 70 was end-labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{33}P]ATP and then purified using micro BioSpin 6 chromatography columns (Bio-Rad). The 15-μl reactions included 3 nM ^{33}P-labeled 70-mer oligonucleotide, 15 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 10% glycerol, 0.01% bromphenol blue, and various concentrations (from 0 to 10 μM) of either wt gene 2.5 protein, gene 2.5 protein-P22L, gene 2.5 protein-F31S, or gene 2.5 protein-G36S diluted in a buffer of 20 mM Tris (pH 7.5), 10 mM β-mercaptoethanol, and 500 μg/ml bovine serum albumin. Reactions were immediately put on ice and then loaded onto a 10% TBE Ready Gel (Bio-Rad) running in 0.5× Tris/glycine buffer (12.5 mM Tris base, 95 mM glycine, and 0.5 mM EDTA). Gels were run at 80 V for 2 h at 4 °C and then dried and exposed to a Fujix PhosphorImager plate for quantitation using ImageQuant software. Dissociation constants were calculated from the average of three experiments using the Langmuir isotherm formula. In the experiments where the salt concentration was varied, KCl was replaced by NaCl at a variety of concentrations (0, 50, 100, 150, 200, 250, 300, or 400 mM). In these experiments gene 2.5 protein concentration was 1.3 μM.

DNA Annealing Assay—The ability of wt gene 2.5 protein to facilitate the annealing of homologous strands of DNA was assessed using an *in vitro* annealing assay developed by Tabor and Richardson.[3] The assay measures the annealing of a radiolabeled ssDNA fragment of M13 DNA to unlabeled circular M13 ssDNA. The labeled fragment was generated in a 60.5-μl reaction by annealing 60 pmol of the oligonucleotide BCMP206 to 8 pmol of M13 (mp1-2) in a buffer containing 25 mM Tris-Cl (pH 7.5), and 50 mM NaCl. The annealed primer was partially extended by T7 DNA polymerase-Δ28 in a 77.75-μl reaction containing 10 mM $MgCl_2$, 3.9 mM DTT, 0.13 mg/ml bovine serum albumin, 2.5 μCi [α−^{32}P]dGTP, and a limiting (8 μM each) quantity of dATP, dCTP, dGTP, and dTTP. After 10 min at room temperature, the reaction was supplemented with 80 μM each of dATP, dCTP, dGTP, and dTTP, and DNA synthesis was completed in 15 min at room temperature. Reactions were then incubated for 10 min at 70 °C to inactivate the polymerase. Next, *E. coli* SSB protein was added, and the DNA was digested with Acc65-1 (New England Biolabs) for 2 h at 37 °C. Reactions were extracted with phenol/chloroform/isoamyl alcohol (50:49:1), and DNA was purified using microspin S-400 columns (Amersham Biosciences). ssDNA fragments were generated by adding NaOH to a final concentration of 100 mM and incubating at room temperature for 5 min. HCl and Tris-Cl (pH 7.5) were each added to a final concentration of 100 mM, and DNA fragments were separated on a 1.4% agarose gel. After electrophoresis the 310-bp band was cut from the gel, and DNA was isolated using a QIAquick gel extraction kit (Qiagen).

DNA annealing was assayed in 20-μl reactions containing 4 nM ^{32}P-labeled ssDNA fragment, 20 μM M13 mGP1–2 ssDNA, 20 mM Tris-Cl (pH 7.5), 1 mM DTT, 10 mM $MgCl_2$, 50 mM NaCl, and 0–30 μM wt gene 2.5 protein or altered gene 2.5 proteins. Reactions were incubated at 30 °C for 8 min and then analyzed on a 0.8% agarose gel at 75 V for 1 h at room temperature, then dried, and exposed to a Fujix PhosphorImager plate. Time course experiments were carried out under the same conditions except all reactions contained a constant amount of gene 2.5 protein (gene 2.5 protein, 10 μM; gene 2.5 protein-P22L, 10 μM; gene 2.5 protein-F31S, 10 μM; gene 2.5 protein-G36S, 30 μM). Reactions were stopped by adding SDS to a final concentration of 0.5% and then immediately put on ice.

T7 DNA Polymerase-Gene 2.5 Protein Interaction Using Surface Plasmon Resonance—The interaction between gene 2.5 protein and T7 DNA polymerase was assayed by surface plasmon resonance (SPR) using the BIAcore 3000 system. Histidine-tagged gene 2.5 protein, gene 2.5 protein-P22L, gene 2.5 protein-F31S, gene 2.5 protein-G36S, and gene 2.5 protein-Δ26C were immobilized on a nickel-charged Sensor-chip NTA (BIAcore). Experiments were performed in a running buffer consisting of 100 mM HEPES (pH 7.5), 50 μM EDTA, 0.1 mM DTT, and 100 mM NaCl at a flow rate of 10 μl per min at 25 °C. All proteins were diluted in the running buffer supplemented with 500 μg/ml of bovine serum albumin. The BIAcore 300 allows four channels to be monitored simultaneously. In each experiment, up to four different histidine-tagged proteins were immobilized onto separate channels on the chip in each experiment. The chip was charged by injecting 10 μl of running buffer plus 0.5 mM $NiCl_2$. After charging, 10 μl of 500 nM His-gene 2.5 protein, His-gene 2.5 protein-P22L, His-gene 2.5 protein-F31S, His-gene 2.5 protein-G36S, or His-gene 2.5 protein-Δ26C were each immobilized to a separate lane of the chip. This amount of protein correlated to ~7,000 resonance units. Once all four proteins were immobilized, a stable base line was established by passing 20 μl of running buffer over the chip. Then 10 μl of 0–500 nM T7 DNA polymerase or bovine serum albumin was passed over the chip. Dissociation of T7 DNA polymerase was monitored for 10 min while passing 100 μl of running buffer over the chip. At the end of this time the chip was regenerated by passing 20 μl of running buffer supplemented with 0.35 M EDTA. Each analysis was performed in triplicate and repeated on three separate days. Representative data are shown in the figures. To assess further the stability of this interaction, these experiments were repeated with running buffer containing varying concentrations (0–200 mM) of NaCl. To look at the kinetics of the gene 2.5 protein-T7 DNA polymerase interaction, 50 nM of either wild-type or mutant histidine-tagged gene 2.5 protein was passed over to the nickel-charged chip and then 10 μl of 0–50 nM T7 DNA was passed over the chip. BIAevaluation software was used to determine dissociation constants (K_D), which were solved using the simultaneous k_a/k_d data fit.

RESULTS

Selection of Gene 2.5 Mutants That Do Not Support T7 Growth—The product of gene 2.5 (wt gene 2.5 protein) is required for the growth of T7 phage (2). Gene 2.5 expressed from a plasmid can complement the growth of a phage deleted for gene 2.5 (T7Δ2.5 phage) (2). In the present study, we have exploited this system to screen for mutations in gene 2.5 that

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

TABLE I
Location of lethal mutations in gene 2.5 and the predicted amino acid alterations

Mutation	No. clones	Predicted protein alteration
Mutations leading to a single amino acid change		
9 G → C	1	K3N
22 T → C	1	S8P
65 C → T	3	P22L
92 T → C	3	F31S
106 G → A	4	G36S
244 C → T	5	R82C[a]
250 A → G	2	K84E
275 G → T	1	G92V
326 A → T	1	K109I
329 G → A	1	C110Y
337 T → C	1	S113P
454 A → G	1	K152E
460 T → C	2	S154P
473 A → G	7	Y158C
477 A → G	1	W160R
494 G → A	1	G165D
497 C → T	2	A166V
500 G → T	2	S167I
502 G → T	1	V168F
694 T → C	1	F232L
Mutations leading to multiple amino acid changes		
34 A → C; 73 G → A	1	T12P, G25S
68 A → G; 488 C → T	1	D23G, A163V
74 G → A; 563 A → T; 674 T → C	1	G65D, D188V, E225G, M94T, T103S
201[b] T → C; 281 T → C; 294 T → C; 307 A → T	1	
331 T → C; 464 T → G; 477 A → G	1	Y111H, L155R, K159R
405 A → C; 497 C → T	1	K135T, A166V
442 A → G; 448 A → G; 454 A → G	1	K148E, K150E, K152E
507 G → T; 562 G → T, 666 G → T	1	K169N, D188Y, E222D
637 T → G; 667 T → C, 668 C → T, 681 C → G; 693 C → G	1	D212A, E222G, D227H, D231H[b]
Single base mutations leading to insertions or deletions		
661 G → T	1	Δ12
648 G → A	1	Δ17
640 G → T	1	Δ18
511 C → T	2	Δ62
448 A → T	2	Δ80
329 G → A	1	Δ122
701 T → A	1	+30

[a] One of the isolates also had the nucleotide changes 297 C → G and 309 G → C which are both silent in the predicted protein sequence.
[b] Denotes isolates where one or more nucleotide change is silent in the predicted protein sequence.

cannot support the growth of T7Δ2.5 phage. The screen was based on one successfully employed by Rosenberg *et al.* (29) to identify lethal mutations in bacteriophage T7 gene 4. We used a commercially available mutator strain of *E. coli*, XL1-Red, to create a library of randomly mutated plasmids that encode gene 2.5. This library was introduced into *E. coli* and plated on LB plates supplemented with ampicillin, and cells were infected by T7Δ2.5 phage. Cells that could support the growth of T7Δ2.5 phage were lysed by the phage. Those that either could not be infected by T7Δ2.5 phage or did not make a functional gene 2.5 protein survived and grew into a colony. This selection identified 291 clones that could not support the growth of T7Δ2.5 phage. Further analysis (described under "Experimental Procedures") reduced the collection to 202 plasmids. Readable DNA sequence was obtained for 190 of these plasmids.

Identification of Mutations in Gene 2.5—DNA sequence analysis of the plasmids identified above uncovered mutations leading to single amino acid alterations, multiple amino acid alterations, and truncated proteins of various sizes (Table I and Fig. 1*A*). Thirty five of the plasmids contained single-base insertions or deletions. Ninety five of the plasmids contained no mutations in gene 2.5. It is likely that these clones arose either from mutations in the promoter regions of the plasmid that prevented the expression of gene 2.5 or that they were isolated from *E. coli* with host-range mutations that rendered them resistant to infection by T7 phage. Twenty seven distinct single mutations were identified that are lethal to T7. Six of these single nucleotide changes gave rise to nonsense mutations that lead to the production of truncated proteins. One of the single mutations changed the stop codon (TAA) to one coding for the amino acid lysine (AAA), presumably resulting in a protein extended by 46 amino acids. The remaining single nucleotide changes lead to single amino acid alterations in gene 2.5 protein. In addition to these single mutations, nine plasmids contained multiple mutations that did not support phage growth. Of these, two were found to have mutations (454 A → G or 497 C → T) that also occurred alone in plasmids harboring only a single mutation.

Location of Single Amino Acid Alterations in Gene 2.5 Protein—The predicted amino acid alterations encoded by 19 of the 20 single residues affect residues that are present in the crystal structure of gene 2.5 protein (24), whereas one (F232L) lies in the carboxyl-terminal motif that has not yet been crystallized. Their locations are depicted in Fig. 1*B*. Two of the alterations (R82C and K84E) lie in disordered regions of the structure. The majority, however, is located in the β-barrel including four of the alterations (K3N, K109I, K152E, and Y158C) that lie in the predicted DNA binding domain, and three (P22L, F31S, and G36S) that reside at the interface of the crystallographic dimer (24). Three other alterations (R82C, K84E, and G92V) lie in the loop connecting the α-helix to the end of the barrel. The remaining alterations (S8P, C110Y, S113P, S154P, W160R, G165D, A166V, S167I, and V168F) map to the β-barrel. As these amino acids are buried in the structure (Fig. 1*B*), it is possible that their alteration results in a misfolded protein.

Amino Acid Alterations at the Dimer Interface—Wt gene 2.5 protein has been shown by gel filtration and sedimentation velocity analysis to exist as a dimer in solution (2). In the present study, we have chosen to study a subset of the above lethal mutants with modifications within the predicted dimer interface (24). Although the truncated gene 2.5 protein-Δ26C is a monomer at the low protein concentrations used for *in vitro* assays, it crystallizes as a dimer. The crystal packing arrangement of gene 2.5 protein-Δ26C suggested a model for dimerization (Fig. 2), in which the acidic carboxyl-terminal motif from one monomer binds in the DNA binding groove of the second monomer. This model predicts that the carboxyl-terminal motif acts as a protein mimic of ssDNA, in a manner analogous to the binding of uracil-DNA glycosylase inhibitor protein to *E. coli* uracil-DNA glycosylase (34). Three of the lethal mutations uncovered in our screen, P22L, F31S, and G36S, affect the putative dimer interface, suggesting that amino acid residues in this region are critical for gene 2.5 protein *in vivo*.

To begin our characterization, we were interested in the ability of these altered proteins to inhibit the function of the native protein. For this reason, we looked at the ability of altered gene 2.5 proteins expressed from plasmids to inhibit the growth of wild-type phage. Interestingly, whereas these mutations could not complement the growth of T7Δ2.5, none of the three inhibited the growth of wild-type bacteriophage T7 (Table II).

Effect of Alteration at the Dimer Interface on T7 DNA Synthesis—To test whether the alteration in gene 2.5 protein led to a defect in DNA synthesis, we followed phage DNA synthesis *in vivo* by radioactively labeling DNA synthesized in T7Δ2.5-infected *E. coli* expressing wild-type and altered gene 2.5 proteins. When wt gene 2.5 protein is overexpressed from a plas-

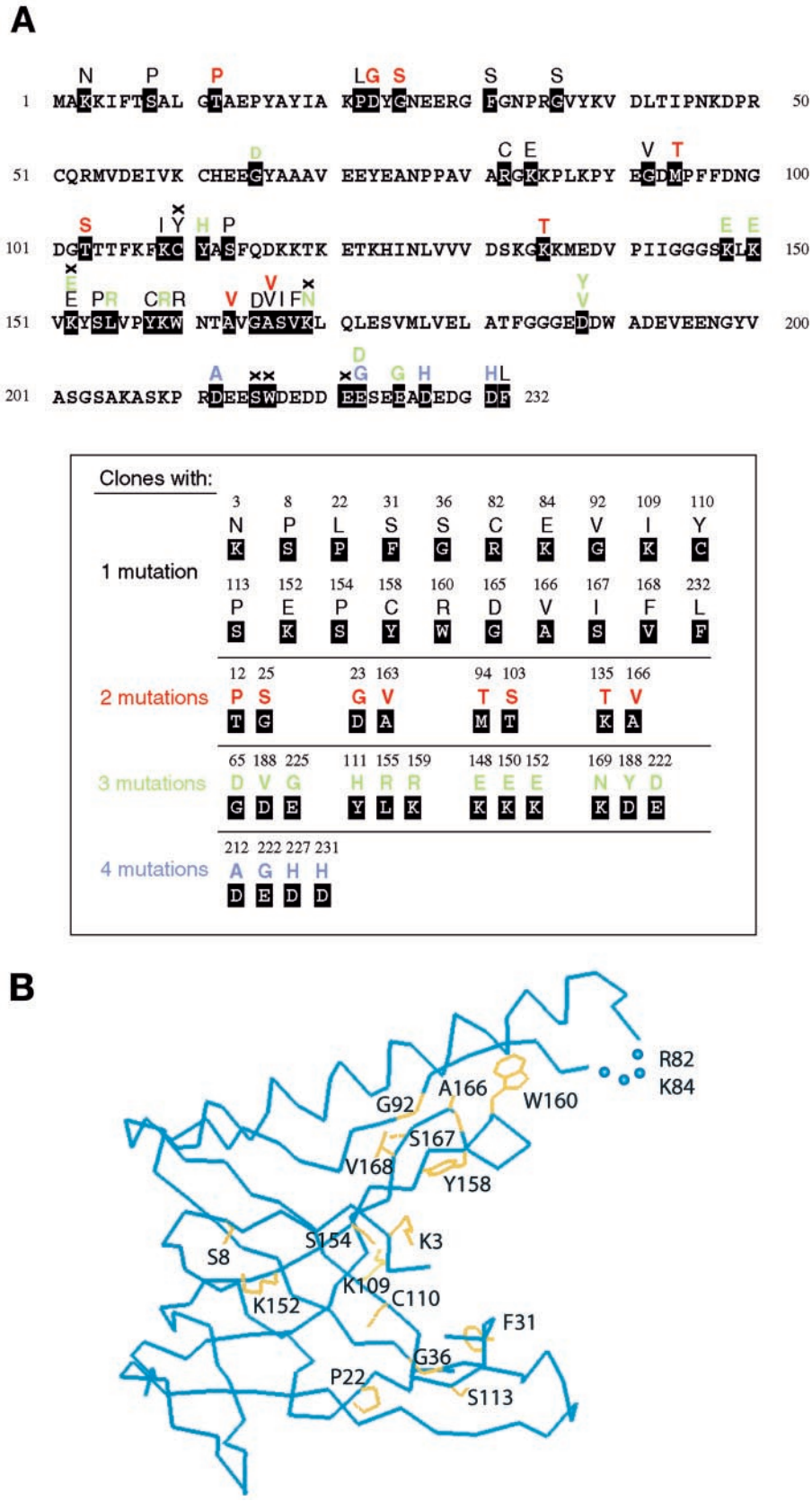

FIG. 1. **Location of amino acid alterations in wt gene 2.5 protein.** *A*, the primary structure of wt gene 2.5 protein is depicted with the residues with alterations uncovered in this study *highlighted*. The single letter abbreviations for amino acids found altered alone in a lethal mutant are denoted in *black*. The *letter X* above an amino acid denotes that it is the first amino acid deleted as a result of a nonsense mutation. The amino acid residue changes found in clones with two mutations in gene 2.5 are written in *red*, those found in clones with three mutation in *green*, and those found in the clone containing four mutations are in *blue*. *B*, diagram depicting the location on the gp2.6-Δ26C crystal structure (24) of the amino acid residues found to be altered in the screen for lethal mutations in gene 2.5. The backbone of gene 2.5 protein-Δ26C is depicted in *blue*, with the side chains of 13 of these amino acids shown in *gold*. Three of the residues altered are glycines in the wild-type protein and are located by *arrows* on the structure. Disordered regions of the structure are represented by the *green dotted lines*. Two of the alterations, R82C and K84E, lie in the disordered regions between the α-helix and the β-barrel. The final residue, F232L, is in the carboxyl-terminal residue, which is deleted and is not a part of this structure.

mid, DNA synthesis peaks at 30 min after phage infection (Fig. 3). Little DNA synthesis occurred in cells infected with each of the three altered gene 2.5 proteins that contained alterations at the putative dimer interface (gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S). In these cells, DNA synthesis falls after phage infection and then continues to decrease throughout the time course. These data show that all these lethal mutations give rise to defective gene 2.5 proteins that cannot support T7 DNA synthesis.

Homodimer Evaluation—The predicted molecular weight of

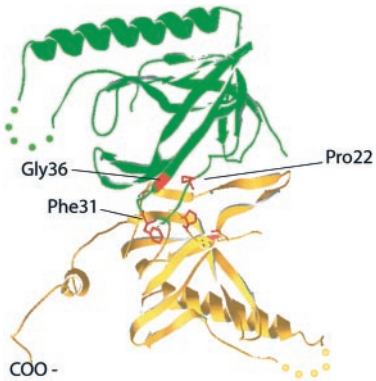

FIG. 2. **Model for dimer formation and location of the amino acid alterations at the interface.** Crystal structure of gene 2.5-Δ26C dimer. The two monomers in the crystal structure are depicted in *green* and *gold*, with the amino acid residues altered in this study highlighted in *red*. Note that residue Phe-31 is ordered in one protamer and disordered in the other.

TABLE II
Plating efficiency of T7 and T7Δ2.5 on E. coli strains containing plasmids expressing wild-type or mutant T7 gene 2.5 proteins

E. coli cells harboring plasmids expressing either wild-type or mutant gene 2.5 were infected with either bacteriophage T7 or T7 phage missing gene 2.5 (T7Δ2.5). Plating efficiencies were determined by dividing the number of plaques observed when cells expressed wild-type gene 2.5 by the number of plaques that are observed when cells expressed the mutant gene 2.5.

Plasmid	T7	T7Δ2.5
pETGP2.5	1	1
pETGP2.5-P22L	1.11	1.6×10^{-6}
pETGP2.5-F31S	1.02	3.06×10^{-7}
pETGP2.5-G36S	1.09	1.73×10^{-7}

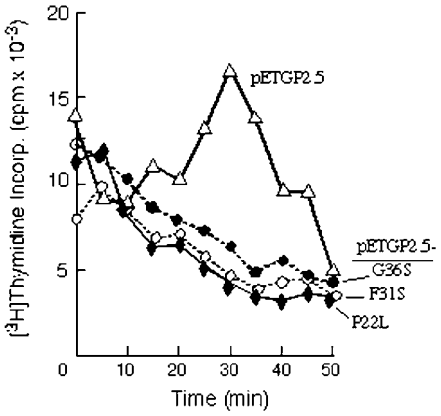

FIG. 3. ***In vivo* DNA synthesis.** *In vivo* DNA synthesis was followed by measuring the incorporation of ^{3}H-labeled thymidine into DNA (*y* axis) at 5-min intervals after T7Δ2.5 infects *E. coli* expressing either wild-type or mutant gene 2.5 (*x* axis) as described under "Experimental Procedures." The graph shows a comparison of *in vivo* DNA synthesis when T7Δ2.5 infects *E. coli* expressing wt gene 2.5 protein (*squares*), gene 2.5 protein-P22L (*diamonds*), gene 2.5 protein-F31S (*circles*), and gene 2.5 protein-G36S (*triangles*).

the wt gene 2.5 protein monomer is 25,562 (35). Gel filtration analysis has shown previously (2) that native gene 2.5 protein forms a stable dimer in solution. To ascertain the ability of gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S to form stable dimers, we estimated their molecular weight by gel filtration at 150 mM NaCl (Fig. 4). wt gene 2.5 protein, gene 2.5 protein-P22L, and gene 2.5 protein-F31S eluted from a Superdex 75 column at the same volume. By using a standard curve derived from the elution volumes of a number of commercially available protein standards, the molecular weight of these proteins was estimated to be 58,200, which is in good agreement with the predicted molecular weight of the gene 2.5 protein dimer, 51,124 (Fig. 4*A*). Gene 2.5 protein-G36S eluted in a broader peak (data not shown) with a calculated molecular weight of 55,300, a value that is also consistent with a dimer. Finally, gene 2.5 protein-Δ26C eluted at a larger volume that is consistent with previous studies (23) showing the protein being a monomer in solution.

This finding was intriguing since we had hypothesized that the residues altered in this study were part of the dimer interface. We were curious whether electrostatic interactions between the acidic residues in the carboxyl-terminal motif and basic residues in the DNA binding cleft were holding the dimer together and masking the contribution of other amino acids in dimer formation. To reduce these effects, we investigated the stability of the dimer by increasing the concentration of salt in our running buffer. When gel filtration was carried out at 250 mM NaCl, the altered proteins eluted differently than did the wild-type protein (Fig. 4*B*). At this elevated salt concentration, wt gene 2.5 protein remains a dimer with an apparent molecular weight of 58,100. The altered proteins, in contrast, eluted with the apparent molecular weight of 31,300, suggesting they are monomers at this salt concentration. Again, gene 2.5 protein-Δ26C behaves as a monomer with a calculated molecular weight of 23,000. Raising the salt concentration to 500 mM NaCl disrupted dimerization of all four proteins (data not shown).

ssDNA Binding Properties of Gene 2.5 Proteins—Gene 2.5 binds to ssDNA (2). We have used a gel shift assay (36) to examine the ssDNA binding ability of gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S at 50 mM KCl (Fig. 5). The dissociation constant for wild-type gene 2.5 protein was calculated to be 2.6×10^{-6} M (Table III). All three altered proteins bind the 70-mer with similar affinity to the wild-type protein. One of the altered proteins, gene 2.5 protein-G36S, did not bind all of the labeled DNA in the reaction even at the highest concentration (Fig. 5). These data suggest that the overall structure of these altered proteins is similar to wild-type gene 2.5 protein, as it is unlikely that a misfolded protein would retain ssDNA binding activity.

Because the homodimer of all three altered proteins is less stable at higher concentrations of NaCl, we examined the effect of salt concentration on DNA binding (Fig. 5*B*). Like other ssDNA-binding proteins (37, 38), gene 2.5 protein DNA binding is affected by salt concentration. DNA binding activity of wt gene 2.5 protein increases with NaCl concentration up to 100 mM; beyond 100 mM NaCl, however, higher concentrations of salt are inhibitory (Fig. 5, *B* and *C*). In contrast, the altered gene 2.5 proteins continue to bind DNA at higher salt concentrations. The binding of one of these proteins, gene 2.5 protein-P22L, is inhibited at 150 mM NaCl, a concentration at which it elutes from a gel filtration column as a dimer but stimulated at higher concentrations (Fig. 5*C*). These data show that at salt concentrations where the altered gene 2.5 protein is a monomer, it binds ssDNA with greater affinity. We have observed that the monomeric gene 2.5 protein-Δ26C binds ssDNA with greater affinity than wt gene 2.5 protein.[4] Taken together, these results suggest that when gene 2.5 protein is in the monomer form, its affinity for ssDNA is increased.

Homologous Base Pairing Mediated by Gene 2.5 Protein—We have observed previously (10)[3] that wt gene 2.5 protein can facilitate annealing of homologous strands of DNA. This prop-

[4] E. Hyland, L. F. Rezende, and C. C. Richardson, unpublished data.

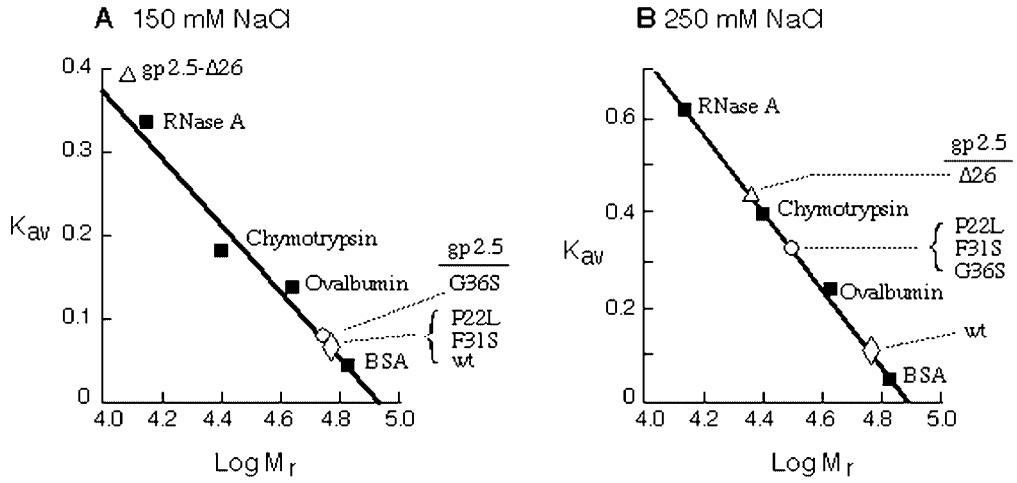

FIG. 4. **Determination of the molecular weight of altered gene 2.5 proteins by gel filtration.** Gel filtration was carried out as described under "Experimental Procedures." Wild-type gene 2.5 protein, gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S were loaded on a Sephadex 75 column in three independent experiments. Gel filtration was carried out in buffer containing either 150 mM NaCl (*A*) or 250 mM NaCl (*B*). Standard curves were generated by plotting K_{av} *versus* log M_r for known molecular weight standards. The position of wt gene 2.5 protein, gene 2.5 protein-P22L, gene 2.5 protein-F31S, gene 2.5 protein-G36S, and gene 2.5 protein-Δ26C are noted with a *dashed line*. The following standards were used in this experiment: albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

erty of gene 2.5 protein has been used previously (9, 10) in preparing DNA substrate for strand transfer mediated by T7 DNA helicase. In the experiment shown in Fig. 6*A,* we have used a concentration of a radiolabeled 310 nucleotide fragment of M13 DNA such that it cannot anneal to its complementary region in M13 ssDNA in an 8-min incubation at 30 °C in the presence of 10 mM $MgCl_2$ and 50 mM NaCl. However, the addition of gene 2.5 protein results in annealing within this period. Under these salt concentrations, both the wild-type and altered gene 2.5 proteins should exist as a dimer. Because the altered gene 2.5 proteins can bind DNA, it was of interest to see if they can also facilitate DNA annealing. As shown in Fig. 6*A*, all three proteins can facilitate this annealing. Two of these proteins, gene 2.5 protein-P22L and gene 2.5 protein-F31S, were required at similar levels as gene 2.5 protein, whereas 3-fold more gene 2.5 protein-G36S was required. Next we investigated whether there were any differences in the rate of DNA annealing (Fig. 6*B*). Both wt gene 2.5 protein and gene 2.5 protein-F31S facilitated the complete annealing of a 310 nucleotide fragment in 1 min 20 s, whereas the reaction with gene 2.5 protein-P22L was slightly slower, 2 min 40s. The reaction with the third protein, gene 2.5 protein-G36S was even slower, requiring up to 4 min for the complete annealing of DNA. These data demonstrate that although gene 2.5 proteins with alterations at the dimer interface are able to mediate the annealing of homologous strands of DNA, two do so somewhat more slowly than the native protein.

Interaction of Gene 2.5 Protein with T7 DNA Polymerase—Studies using both affinity chromatography and fluorescence emission anisotropy have shown that gene 2.5 protein interacts with T7 DNA polymerase (17). We investigated this interaction using SPR by immobilizing histidine-tagged wild-type and altered gene 2.5 proteins on a chelating NTA chip using methods developed to analyze the interaction between GroEL and GroES (39). First, we tested whether SPR could also be used to analyze the well established interaction between gene 2.5 protein and T7 DNA polymerase. We expressed and purified fusion proteins of gene 2.5 protein and gene 2.5 protein-Δ26C with 10 histidines on the amino terminus (His-gene 2.5 protein and His-gene 2.5 protein-Δ26C). The proteins were then immobilized onto a nickel-charged NTA chip. Subsequently, various concentrations (0–500 nM) of T7 DNA polymerase are passed over the chip at room temperature; buffer is then passed over the chip for 10 min to measure the dissociation of T7 DNA polymerase. A typical experiment demonstrating the binding of T7 DNA polymerase to wt gene 2.5 protein is depicted in Fig. 7*A*. The dissociation constant was calculated as 2.97×10^{-6} M, which is in agreement with the value previously calculated using fluorescence emission anisotropy (1.1×10^{-16}) (17). This binding of wild-type gene 2.5 protein is stable in buffers with NaCl concentrations up to 200 mM (Fig. 7*B*), the same concentration of salt where T7 DNA polymerase elutes from a wt gene 2.5 protein affinity column (17). Previous studies (23) have shown that the carboxyl-terminal motif of gene 2.5 protein is required for gene 2.5 protein-T7 polymerase interaction. When gene 2.5 protein-Δ26C is immobilized to the chip, T7 DNA polymerase is not stably bound (Fig. 7*C*), even at low concentrations of NaCl.

This technique was used to assess the interaction between T7 DNA polymerase and the histidine fusion proteins: His-gene 2.5 protein-P22L, His-gene 2.5 protein-F31S, and His-gene 2.5 protein-G36S. T7 DNA polymerase binds His-gene 2.5 protein-P22L, His-gene 2.5 protein-F31S, and His-gene 2.5 protein-G36S as well as it does to wild-type gene 2.5 protein (Fig. 7*D*), whereas it does not bind to surface coated with His-gene 2.5 protein-Δ26C (Fig. 7*C*). These experiments were carried out in a buffer containing 100 mM NaCl, where the wild-type and altered proteins exist as a dimer. The dissociation constant for these interactions was calculated to be 3.15×10^{-6} M (gene 2.5 protein-P22L), 5.43×10^{-6} M (gene 2.5 protein-F31S), and 1.54×10^{-6} M (gene 2.5 protein-G36S). The interaction between the altered gene 2.5 proteins and T7 DNA polymerase is disrupted by increasing the concentration of salt to 200 mM (data not shown), just as it is in the wild-type protein. Therefore, we cannot test the interactions with T7 DNA polymerase under the high salt concentrations required to disrupt dimer formation in the altered proteins. This experiments demonstrates that the alterations at the dimer interface do no affect the ability of the protein to interact physically with the T7 DNA polymerase, suggesting that these residues are located away from the site of interaction with the T7 DNA polymerase. Because these altered proteins retain this vital function of gene 2.5 protein, it is likely that the amino acid changes do not affect the overall fold of the protein.

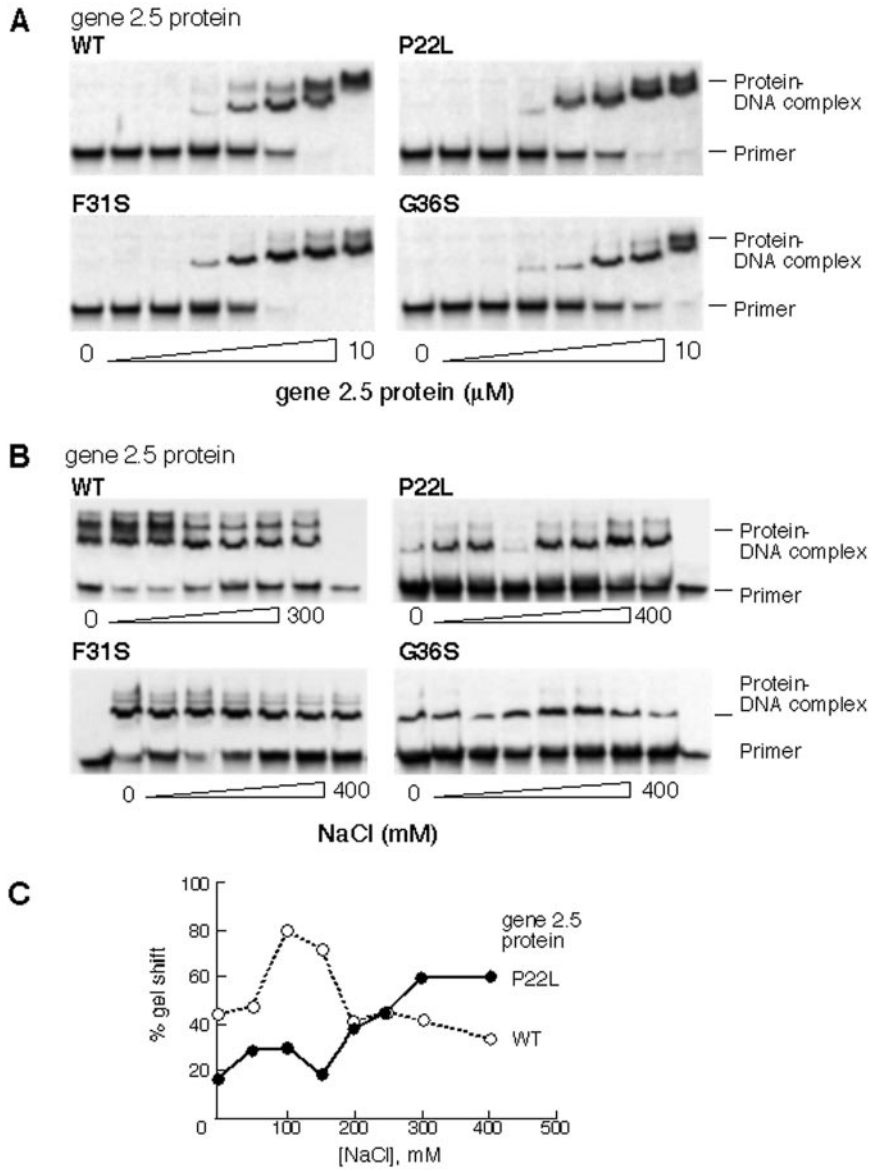

FIG. 5. **Binding of gene 2.5 protein to ssDNA.** An electrophoretic mobility shift assay was used to examine the ability of gene 2.5 protein to bind to ssDNA. *A*, varying concentrations (0, 0.5, 1, 2, 4, 8, 16, or 32 μM) of wt gene 2.5 protein (*top left*), gene 2.5 protein-P22L (*top right*), gene 2.5 protein-F31S (*bottom left*), and gene 2.5 protein-G36S (*bottom right*) were bound to a 5′-^{33}P-labeled 70-mer oligodeoxyribonucleotide. The reactions were analyzed on a 10% polyacrylamide gel. *B*, effect of varying the concentration of NaCl (0, 50, 100, 150, 200, 250, 300, or 400 mM unless otherwise noted) on the DNA binding activity of gene 2.5 protein (*top left*, highest concentration of NaCl is 300 mM), gene 2.5 protein-P22L (*top right*), gene 2.5 protein-F31S (*bottom left*), and gene 2.5 protein-G36S (*bottom right*). Gene 2.5 protein concentration is held constant at 1.3 μM in all lanes. *C*, bands representing the electrophoretic mobility shift of wt gene 2.5 protein and gene 2.5 protein-P22L were quantified, and the average for three experiments was plotted. The concentration of NaCl during the binding reaction is denoted on the *x* axis; the percentage of radiolabeled oligodeoxyribonucleotide shifted is plotted on the *y* axis.

TABLE III
Dissociation constants of wild-type and altered gene 2.5 proteins ssDNA

Dissociation constants (K_d) were calculated based on the average of three individual electrophoretic mobility shift assays, using the Langmuir isotherm ($r = [A]/K_d + [A]$, r = ssDNA bound, A = total ssDNA).

Protein	Dissociation constant
	M
wt gene 2.5 protein	2.6×10^{-6}
Gene 2.5 protein-P22L	1.8×10^{-6}
Gene 2.5 protein-F31S	0.9×10^{-6}
Gene 2.5 protein-G36S	2.1×10^{-6}

DISCUSSION

Gene 2.5 of bacteriophage T7 is an essential gene that encodes an ssDNA-binding protein. In the present study we have employed a genetic screen to identify residues essential for the function of gene 2.5 protein. The identities of these residues in conjunction with the recently determined crystal structure of the protein (24) are helpful in mapping important domains in the protein. Our screen uncovered 20 independent single amino acid alterations in gene 2.5 protein that could not support the growth of a gene 2.5-deleted phage. In the current study we have characterized three altered gene 2.5 proteins: gene 2.5 protein-P22L, gene 2.5 protein-F31S, and gene 2.5 protein-G36S. These alterations map to the interface of the crystallographic dimer seen in the structure of gp2.6-Δ26C (24).

Gene 2.5 protein forms a dimer in solution (2), whereas the carboxyl-terminal deleted versions of the protein gene 2.5 protein-Δ21C and gene 2.5 protein-Δ26C appear to be a monomer (23) (Fig. 4). The crystal structure of gene 2.5 protein-Δ26C suggested a mechanism of dimerization whereby the acidic carboxyl-terminal tail mimics DNA and binds in the DNA binding groove (24). This model provides an explanation as to why gene 2.5 proteins with deletions in the carboxyl-terminal tail are monomers in solution. However, a carboxyl-terminal deletion of the protein crystallized as a dimer, suggesting that additional interactions are involved in dimer formation. Because three of the amino acid alterations uncovered in our screen mapped to the interface of the dimer, it was of interest to see if proteins with these alterations could form dimers in solution. Under our standard conditions, including 150 mM NaCl, all three of the genetically altered proteins elute from a gel filtration column as a dimer. Conceivably, electrostatic interactions between the acidic tail and the DNA binding domain could stabilize the dimer, overshadowing other protein inter-

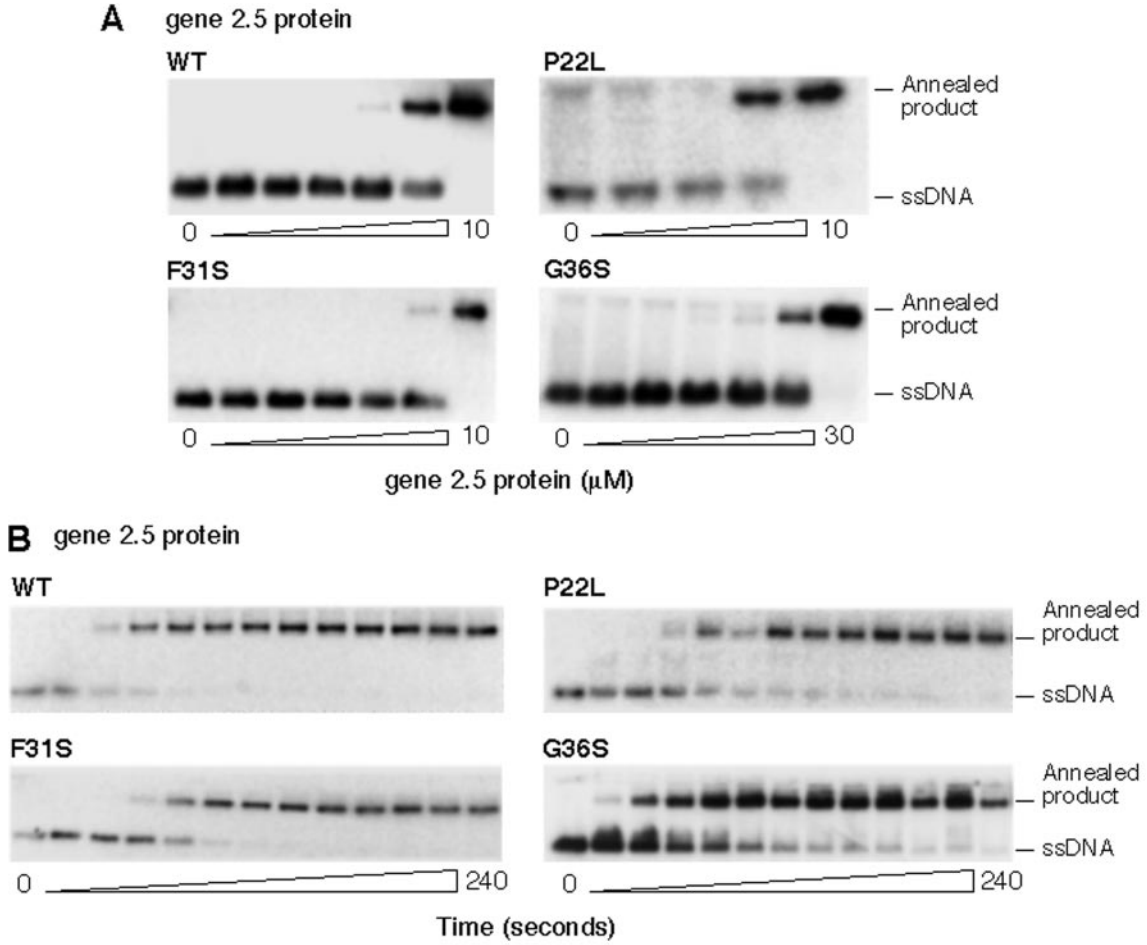

FIG. 6. **Homologous base pairing mediated by gene 2.5 protein.** In this assay a radiolabeled 310 nucleotide fragment of M13 is incubated with M13 ssDNA in the presence of gene 2.5. *A*, agarose gels demonstrating the effect of increasing protein concentration (0, 0.63, 1.3, 2.5, 5, or 10 μM, unless noted otherwise) on the DNA annealing activity of wt gene 2.5 protein (*top left*), gene 2.5 protein-P22L (*top right,* protein concentrations 0,1.3, 2.5, 5, or 10 μM), gene 2.5 protein-F31S (*bottom left*), and gene 2.5 protein-G36S (0, 1.8, 3.8, 7.5, 15, or 30 μM) (*bottom right*). Reactions were incubated for 8 min at 30 °C. The migration position of the 310 nucleotide ^{32}P-labeled DNA fragments and the annealed products are denoted on the *right*. *B*, agarose gel analysis of time course experiments examining the annealing activity of wt gene 2.5 protein (*top left*), gene 2.5 protein-P22L (*top right*), gene 2.5 protein-F31S (*bottom left*), and gene 2.5 protein-G36S (*bottom right*). All reactions were carried out at 30 °C with either 10 μM of wt gene 2.5 protein, gene 2.5 protein-P22L, and gene 2.5 protein-F31S, or 30 μM of gene 2.5 protein-G36S. Time points were taken from 0 to 4 min at 20-s intervals after adding gene 2.5 protein.

actions at the dimer interface. At 250 mM NaCl, wt gene 2.5 protein remains a stable dimer, but proteins with alterations at the dimer interface eluted as monomers. Interestingly, these altered proteins also bind ssDNA differently than the wild-type gene 2.5 protein when the concentration of salt increases. Whereas the wt gene 2.5 protein DNA has a decreased affinity for ssDNA at 250 mM NaCl, gene 2.5 proteins with alterations at the dimer interface have increased binding affinity at this salt concentration. It is possible that the instability of the dimer at 250 mM NaCl leaves the DNA-binding surface of gene 2.5 protein more accessible and thus increases its DNA binding affinity.

The molecular explanation for the lethality of the mutations described in this study remains elusive. A defect in DNA replication is most likely responsible for the lethality observed since T7 DNA synthesis in cells harboring defective gene 2.5 proteins is drastically curtailed. Because the most obvious consequence of the single amino acid substitutions *in vitro* is the inability to dimerize at higher ionic strength, it is reasonable to propose it is this defect that gives rise to the problems observed *in vivo*. To date, however, we have not been able to identify a specific defect *in vitro* that arises from the inability of the protein to dimerize. The altered proteins bind ssDNA, and they physically interact with T7 DNA polymerase. Furthermore, they can mediate coordinated synthesis in a minicircle replication system involving several of the T7 replication proteins.[5] We do observe a slightly different affinity of the altered proteins for ssDNA depending on the ionic strength. Although the magnitude of these differences is not impressive, it is conceivable that under physiological conditions the altered proteins bind ssDNA differently than does the wild-type gene 2.5 protein. We also note that two of the proteins mediate homologous base pairing at a slightly lower rate. However, we have identified another single amino acid change that has far more drastic effects on the ability of gene 2.5 protein to mediate homologous base pairing and that protein forms dimers normally.[6]

In addition to the alterations at the dimer interface, our screen uncovered a number of other potentially interesting lethal mutations in gene 2.5. Four of these lead to the alterations K3N, K109I, K152E, and Y158C, which map to the proposed DNA binding domain (24). Lysine residues (40) and aromatic amino acids (41–44) have been implicated in the DNA binding activity of *E. coli* SSB protein. One of these residues, Tyr-158, is positioned at the end of β-strand β4, a position

[5] J. Lee and C. C. Richardson, unpublished data.
[6] L. F. Rezende and C. C. Richardson, unpublished data.

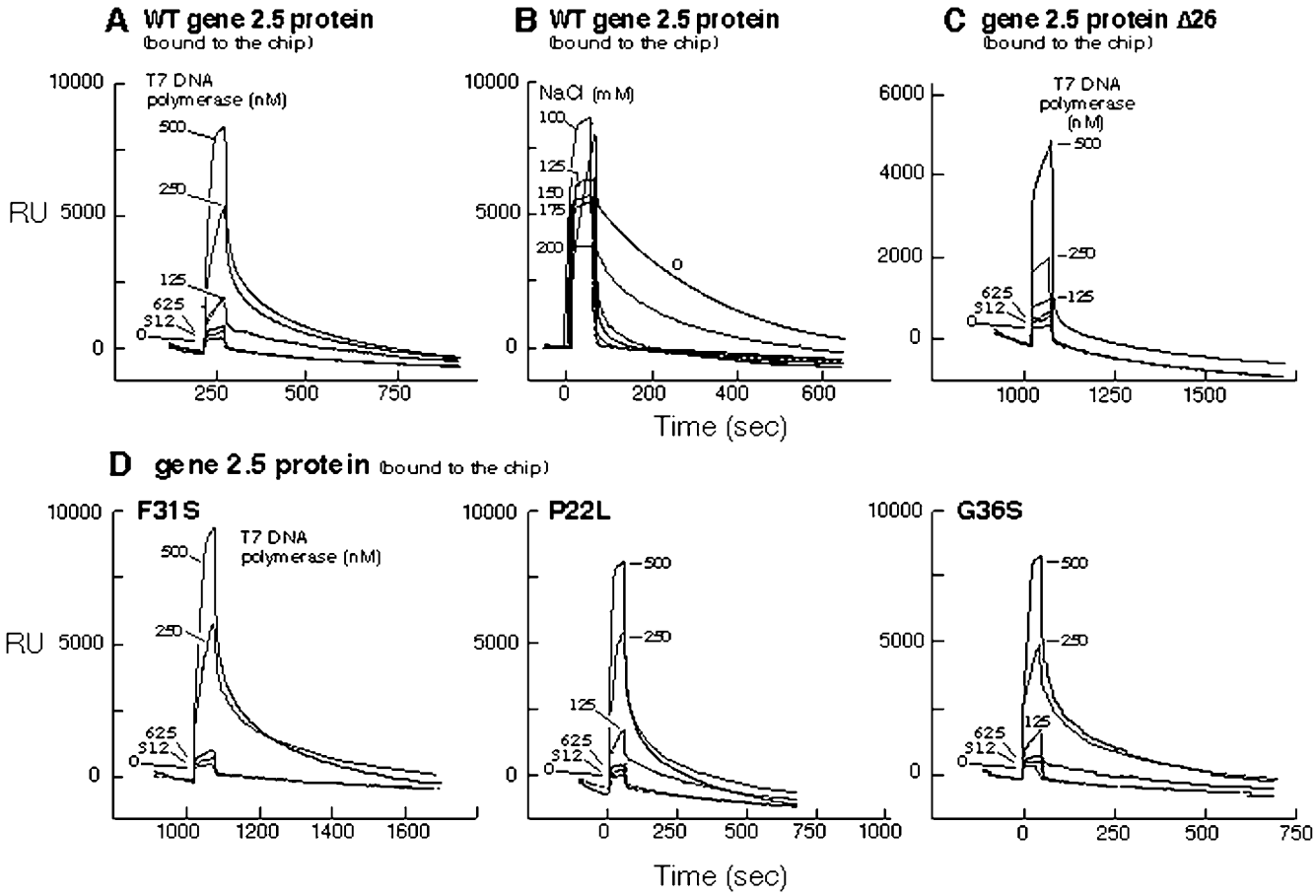

FIG. 7. **Interaction between gene 2.5 protein and T7 DNA polymerase.** The interaction between gene 2.5 protein and T7 DNA polymerase was monitored using surface plasmon resonance on a BIAcore 3000. In all panels the baseline has been normalized to zero. Unless otherwise noted, the running buffer contained 100 mM NaCl. In all graphs time(s) is plotted on the *x* axis; response units (*RU*) are plotted on the *y* axis. *A,* overlay plot of various concentrations (0–500 nM) of T7 DNA polymerase binding to wt gene 2.5 protein immobilized on an NTA chip charged with $NiCl_2$. T7 DNA polymerase was passed over the chip, then allowed to dissociate for 10 min. *B,* effect of increasing concentration of NaCl in the running buffer on the binding of T7 DNA polymerase to wt gene 2.5 protein. *C,* overlay plot of various concentrations (0–500 nM) of T7 DNA polymerase passing over gene 2.5 protein-Δ26C immobilized on an NTA chip charged with $NiCl_2$. *D,* overlay plot of various concentrations (0–500 nM) of T7 DNA polymerase binding to gene 2.5 protein-P22L (*left*), gene 2.5 protein-F31S (*center*), and gene 2.5 protein-G36S (*right*) immobilized on an NTA chip charged with $NiCl_2$. T7 DNA polymerase was passed over the chip, then allowed to dissociate for 10 min.

where other ssDNA-binding proteins encode aromatic amino acid residues, and is part of a conserved trinucleotide-binding motif (24). Therefore, it was surprising we did not uncover mutations at the second critical residue in that motif, Tyr-111. Interestingly, a mutation at that position, resulting in an amino acid change from a tyrosine to a histidine, was found in a plasmid containing multiple mutations. In a separate study, we have shown that a plasmid containing the mutation leading to that alteration alone can support T7 phage growth,[4] explaining why Tyr-111 was not identified in our screen.

A number of other lethal mutations affect residues that are conserved between T7 and closely related bacteriophage T3 and bacteriophage φYeO3-12 (35, 45, 46) but are not conserved in alignments with other prokaryotic ssDNA-binding proteins such as *E. coli* SSB protein or the T4 gene 32 protein (47, 48). Conserved residues include the three residues altered in the proteins described here. Another set of alterations, R82C and K84E, lie in a disordered loop between the α-helix and β-barrel domains of the protein. The remainder (S8P, C110Y, S113P, W160R, G165D, A166V, S167I, and V168F) lie in the β-barrel domain and may lead to disruption of the overall structure. Elucidation of the exact role of these residues awaits further analysis.

The small number of mutants with alterations in the carboxyl-terminal motif (Fig. 1) was surprising, as this was the one region of the protein known to be critical for gene 2.5 protein function (23). Whereas acidic residues in the carboxyl-terminal motif have been shown to be important in mediating protein-protein interactions (11, 13, 23), no single alteration of an acidic residue was uncovered in our screen. We did, however, isolate a plasmid with mutations leading to four amino acid alterations in the carboxyl-terminal tail (D212A, E222G, D227H, and D231H). This finding suggests that a reduction in the overall charge of the motif is critical rather than specific amino acid interactions. The only single amino acid alteration found in this motif was F232L, the terminal amino acid of the protein. Interestingly, the terminal amino acid of *E. coli* SSB protein is also a phenylalanine (47), and this residue is also conserved between T7, bacteriophage T3, and bacteriophage φYeO3-12. Further studies will explore the role of this residue in gene 2.5 protein function. The majority of the mutations in the carboxyl terminus leads to truncated proteins. We have previously studied gene 2.5 proteins with 21 (23) and 26 amino acid (24) deletions in the carboxyl terminus. In our screen we found that deletions as small at 12 amino acids from the carboxyl terminus result in proteins that cannot support the growth of a gene 2.5-deleted phage.

Previous studies (13) showed that the carboxyl-terminal motif of *E. coli* SSB protein could replace that of gene 2.5 protein both in *in vitro* assays and in *in vivo* complementation assays.

However, a chimeric protein in which the carboxyl terminus of gene 2.5 protein replaces that of *E. coli* SSB protein did not support phage growth in a complementation assay, suggesting that this motif alone cannot account for the specificity of its role in the T7 life cycle (13). It is likely that amino acid residues outside of the carboxyl terminus contribute to the specificity. The alterations uncovered in the screen described here may help us identify other regions of gene 2.5 protein that are critical for protein function *in vivo*. The current study begins this process by identifying three critical residues, Pro-22, Phe-31, and Gly-36, which are required for maintaining a stable dimer *in vitro*.

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REFERENCES

1. Kim, Y. T., and Richardson, C. C. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10173–10177
2. Kim, Y. T., Tabor, S., Bortner, C., Griffith, J. D., and Richardson, C. C. (1992) *J. Biol. Chem.* **267,** 15022–15031
3. Chase, J. W., and Williams, K. R. (1986) *Annu. Rev. Biochem.* **55,** 103–136
4. Reuben, R. C., and Gefter, M. L. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 1846–1850
5. Scherzinger, E., Litfin, F., and Jost, E. (1973) *Mol. Gen. Genet.* **123,** 247–262
6. Araki, H., and Ogawa, H. (1981) *Virology* **111,** 509–515
7. Araki, H., and Ogawa, H. (1981) *Mol. Gen. Genet.* **183,** 66–73
8. Nakai, H., and Richardson, C. C. (1988) *J. Biol. Chem.* **263,** 9831–9839
9. Kong, D., and Richardson, C. C. (1996) *EMBO J.* **15,** 2010–2019
10. Kong, D., Nossal, N. G., and Richardson, C. C. (1997) *J. Biol. Chem.* **272,** 8380–8387
11. Lee, J., Chastain, P. D., II, Kusakabe, T., Griffith, J. D., and Richardson, C. C. (1998) *Mol. Cell* **1,** 1001–1010
12. Yu, M., and Masker, W. (2001) *J. Bacteriol.* **183,** 1862–1869
13. Kong, D., and Richardson, C. C. (1998) *J. Biol. Chem.* **273,** 6556–6564
14. Weiner, J. H., Bertsch, L. L., and Kornberg, A. (1975) *J. Biol. Chem.* **250,** 1972–1980
15. von Hippel, P. H., Kowalczykowski, S. C., Lonberg, N., Newport, J. W., Paul, L. S., Stormo, G. D., and Gold, L. (1982) *J. Mol. Biol.* **162,** 795–818
16. Carroll, R. B., Neet, K., and Goldthwait, D. A. (1975) *J. Mol. Biol.* **91,** 275–291
17. Kim, Y. T., Tabor, S., Churchich, J. E., and Richardson, C. C. (1992) *J. Biol. Chem.* **267,** 15032–15040
18. Curth, U., Genschel, J., Urbanke, C., and Greipel, J. (1996) *Nucleic Acids Res.* **24,** 2706–2711
19. Genschel, J., Curth, U., and Urbanke, C. (2000) *Biol. Chem. Hoppe-Seyler* **381,** 183–192
20. Burke, R. L., Alberts, B. M., and Hosoda, J. (1980) *J. Biol. Chem.* **255,** 11484–11493
21. Reddy, M. S., Guhan, N., and Muniyappa, K. (2001) *J. Biol. Chem.* **276,** 45959–45968
22. Salinas, F., and Benkovic, S. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 7196–7201
23. Kim, Y. T., and Richardson, C. C. (1994) *J. Biol. Chem.* **269,** 5270–5278
24. Hollis, T., Stattel, J. M., Walther, D. S., Richardson, C. C., and Ellenberger, T. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9557–9562
25. Murzin, A. G. (1993) *EMBO J.* **12,** 861–867
26. Shamoo, Y., Friedman, A. M., Parsons, M. R., Konigsberg, W. H., and Steitz, T. A. (1995) *Nature* **376,** 362–366
27. Raghunathan, S., Ricard, C. S., Lohman, T. M., and Waksman, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 6652–6657
28. Raghunathan, S., Kozlov, A. G., Lohman, T. M., and Waksman, G. (2000) *Nat. Struct. Biol.* **7,** 648–652
29. Rosenberg, A. H., Griffin, K., Washington, M. T., Patel, S. S., and Studier, F. W. (1996) *J. Biol. Chem.* **271,** 26819–26824
30. Tabor, S., and Richardson, C. C. (1989) *J. Biol. Chem.* **264,** 6447–6458
31. Saito, H., and Richardson, C. C. (1981) *J. Virol.* **37,** 343–351
32. Kumar, J. K., Kremsdorf, R., Tabor, S., and Richardson, C. C. (2001) *J. Biol. Chem.* **276,** 46151–46159
33. Gill, S. C., and von Hippel, P. H. (1989) *Anal. Biochem.* **182,** 319–326
34. Mol, C. D., Arvai, A. S., Sanderson, R. J., Slupphaug, G., Kavli, B., Krokan, H. E., Mosbaugh, D. W., and Tainer, J. A. (1995) *Cell* **82,** 701–708
35. Dunn, J. J., and Studier, F. W. (1983) *J. Mol. Biol.* **166,** 477–535
36. Taylor, J., Ackroyd, A., and Halford, S. (1994) *Methods Mol. Biol.* **30,** 263–279
37. Lohman, T. M., and Ferrari, M. E. (1994) *Annu. Rev. Biochem.* **63,** 527–570
38. Lohman, T. M., Overman, L. B., Ferrari, M. E., and Kozlov, A. G. (1996) *Biochemistry* **35,** 5272–5279
39. Nieba, L., Nieba-Axmann, S. E., Persson, A., Hamalainen, M., Edebratt, F., Hansson, A., Lidholm, J., Magnusson, K., Karlsson, A. F., and Pluckthun, A. (1997) *Anal. Biochem.* **252,** 217–228
40. Chen, J., Smith, D. L., and Griep, M. A. (1998) *Protein Sci.* **7,** 1781–1788
41. Khamis, M. I., Casas-Finet, J. R., Maki, A. H., Murphy, J. B., and Chase, J. W. (1987) *J. Biol. Chem.* **262,** 10938–10945
42. Ferrari, M. E., Fang, J., and Lohman, T. M. (1997) *Biophys. Chem.* **64,** 235–251
43. Casas-Finet, J. R., Khamis, M. I., Maki, A. H., and Chase, J. W. (1987) *FEBS Lett.* **220,** 347–352
44. Bayer, I., Fliess, A., Greipel, J., Urbanke, C., and Maass, G. (1989) *Eur. J. Biochem.* **179,** 399–404
45. Schmitt, M. P., Beck, P. J., Kearney, C. A., Spence, J. L., DiGiovanni, D., Condreay, J. P., and Molineux, I. J. (1987) *J. Mol. Biol.* **193,** 479–495
46. Pajunen, M. I., Kiljunen, S. J., Soderholm, M. E., and Skurnik, M. (2001) *J. Bacteriol.* **183,** 1928–1937
47. Sancar, A., Williams, K. R., Chase, J. W., and Rupp, W. D. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4274–4278
48. Williams, K. R., LoPresti, M. B., Setoguchi, M., and Konigsberg, W. H. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 4614–4617