

C-terminal Phenylalanine of Bacteriophage T7 Single-stranded DNA-binding Protein Is Essential for Strand Displacement Synthesis by T7 DNA Polymerase at a Nick in DNA*

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Single-stranded DNA-binding protein (gp2.5), encoded by gene 2.5 of bacteriophage T7, plays an essential role in DNA replication. Not only does it remove impediments of secondary structure in the DNA, it also modulates the activities of the other replication proteins. The acidic C-terminal tail of gp2.5, bearing a C-terminal phenylalanine, physically and functionally interacts with the helicase and DNA polymerase. Deletion of the phenylalanine or substitution with a nonaromatic amino acid gives rise to a dominant lethal phenotype, and the altered gp2.5 has reduced affinity for T7 DNA polymerase. Suppressors of the dominant lethal phenotype have led to the identification of mutations in gene 5 that encodes the T7 DNA polymerase. The altered residues in the polymerase are solvent-exposed and lie in regions that are adjacent to the bound DNA. gp2.5 lacking the C-terminal phenylalanine has a lower affinity for gp5-thioredoxin relative to the wild-type gp2.5, and this affinity is partially restored by the suppressor mutations in DNA polymerase. gp2.5 enables T7 DNA polymerase to catalyze strand displacement DNA synthesis at a nick in DNA. The resulting 5'-single-stranded DNA tail provides a loading site for T7 DNA helicase. gp2.5 lacking the C-terminal phenylalanine does not support this event with wild-type DNA polymerase but does to a limited extent with T7 DNA polymerase harboring the suppressor mutations.

Single-stranded DNA (ssDNA)³-binding proteins have been assigned the role of removing secondary structure in DNA and protecting ssDNA from hydrolysis by nucleases (1). However, in addition to these mundane roles, ssDNA-binding proteins are now recognized as a key component of the replisome where they physically and functionally interact with other replication proteins and with the primer-template (2–4). ssDNA-binding proteins are also engaged in DNA recombination and repair (5).

In view of these multiple roles, it has been difficult to identify the specific defect in genetically altered ssDNA-binding proteins that leads to an observed phenotype.

The crystal structures of several prokaryotic ssDNA-binding proteins have been determined (6–8). These proteins have a conserved oligosaccharide-oligonucleotide binding fold (OB-fold) that is thought to bind the ssDNA by means of stacking and electrostatic interactions (6). Prokaryotic ssDNA-binding proteins also have an acidic C-terminal tail that is essential for bacterial and phage growth (9–13).

The ssDNA-binding protein of bacteriophage T7 is encoded by gene 2.5 (14). The gene 2.5 protein (gp2.5) is a homodimer in solution, a structure that is stabilized by its C-terminal tail (9, 15). The C-terminal tail of one monomer of gp2.5 binds in a *trans* mode to the ssDNA-binding cleft of the other subunit, thus stabilizing the dimer interface observed in the crystal structure (6). The current model proposes that the positively charged DNA-binding cleft is shielded by the electrostatic charges of the C-terminal tail in the absence of ssDNA, thus facilitating oligomerization of gp2.5. Upon binding ssDNA, the dimer dissociates to allow the C-terminal tail to interact with other replication proteins (16). The tail modulates the affinity for ssDNA and protein-protein interactions by functioning as a two-way switch (6, 17). This mode of function is applicable to other prokaryotic ssDNA-binding proteins, namely *Escherichia coli* SSB protein and T4 gp32 (10, 13, 15, 18–22).

gp2.5 is one of four proteins that include the T7 replisome. The other three proteins are the T7 gene 5 DNA polymerase (gp5), its processivity factor, *E. coli* thioredoxin (trx), and the multifunctional gene 4 helicase-primase (gp4). gp5 and trx bind with high affinity (K_D of 5 nM), and the two proteins are normally found in complex (gp5/trx) at a stoichiometry of one to one (23). The acidic C-terminal tail of gp2.5 is critical for the interactions of the protein with gp5/trx and gp4 (9, 24). The C-terminal tail binds to a positively charged segment located in the thumb subdomain of the gp5 (25). This fragment, designated the trx binding domain (TBD), is also the site of binding of the processivity factor, *E. coli* trx, and the C terminus of gp4. The multiple interactions of the C terminus of gp2.5 could thus function to coordinate the dynamic reactions occurring at the replication fork. gp2.5 is known to be critical for establishing coordination during leading and lagging strand DNA synthesis (26, 27).

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³ The abbreviations used are: ssDNA, single-stranded DNA; trx, thioredoxin; dsDNA, double-stranded DNA; TBD, thioredoxin binding domain; DTT, dithiothreitol; nt, nucleotide; SSB, single strand DNA-binding protein.

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This C-terminal tail of gp2.5 is an acidic 26-amino acid segment with an aromatic phenylalanine as the C-terminal residue. The C-terminal tail is not seen in the crystal structure because gp2.5 Δ 26, lacking the tail, was used for crystallization; the wild-type protein did not yield crystals that diffracted (6). gp2.5 Δ F designates a genetically modified gp2.5 lacking the C-terminal phenylalanine. gp2.5 Δ F does not support the growth of T7 Δ 2.5 phage lacking gene 2.5 (28). Interestingly, T7 gene 4 protein also has an acidic C-terminal tail with a C-terminal phenylalanine (29). Again, the phenylalanine is critical for the interaction of gp4 with gp5/trx (29). Further evidence for overlapping binding sites of the C termini of these two proteins comes from studies with chimeric proteins (28, 29). The C-terminal tails of gp2.5 and gp4 can be exchanged, and the chimeric proteins support the growth of T7 phage lacking the corresponding wild-type protein.

We recently designed a screen for suppressors of dominant lethal mutations of gp2.5 (30). The screen identified mutations in gene 5, the structural gene for T7 DNA polymerase (Fig. 1), which suppresses the lethal phenotype of gp2.5 mutant in which the C-terminal phenylalanine was moved to the penultimate position (gp2.5 Δ F232InsF231). One of the altered suppressor genes (gp5, gp5-sup1) encodes a gp5 in which where glycine at position 371 is replaced by lysine (G371K). Whereas the other (gp5-sup2) encodes a protein in which threonine 258 and alanine 411 are replaced by methionine and threonine, respectively (T258M and A411T). The suppressor mutations in gp5 are necessary and sufficient to suppress the lethal phenotype of gp2.5 Δ F232InsF231. The affected residues map in proximity to aromatic residues and to residues in close proximity to DNA as seen in the crystal structure of gp5/trx in complex with DNA (31). Throughout this study, gp2.5 Δ F232InsF231 mutant will be referred to as gp2.5-FD because it effectively switches the positions of the C-terminal phenylalanine and the adjacent aspartic acid. *E. coli* SSB protein also has a C-terminal phenylalanine, and recent studies have shown that this residue inserts into a hydrophobic region consisting of exonuclease I of *E. coli* (45, 46).

In this study, we have purified the two suppressor DNA polymerases and characterized them individually and in interaction with the other T7 replication proteins. Whereas wild-type gp5 binds with low affinity to gp2.5-FD, the DNA polymerases harboring the suppressor mutations bind with a higher affinity. An interesting finding is that whereas wild-type gp2.5 enables gp5/trx to catalyze strand displacement synthesis at a nick in DNA, gp2.5-FD does not support this reaction. Strand displacement synthesis is necessary for the initiation of leading strand DNA synthesis at a nick because it creates a 5'-single-stranded DNA tail for loading of the T7 helicase (32).

EXPERIMENTAL PROCEDURES

Purification of Proteins—Wild-type gp5, gp5-sup1, and gp5-sup2 plasmids have been described previously in the study identifying gp5 suppressors of the dominant lethal gp2.5-FD (30). Wild-type gp5, gp5-sup1, and gp5-sup2 were purified from *E. coli* HMS 174(DE3)/pLysS cells overexpressing their genes as described previously (23). The 1:1 complex of polymerase and thioredoxin was purified to apparent homogeneity using three

chromatographic steps as follows: phosphocellulose (Whatman), anion exchange Poros HQ (PerSeptive Biosystems), and ceramic hydroxylapatite (Bio-Rad) as described (23). Wild-type gp2.5, gp2.5-FD, and gp2.5 Δ 26 were purified from BL21(DE3)pLysS cells overexpressing their genes as described previously (33). gp4 was purified as described (24).

Physical Interactions of Proteins—Surface plasmon resonance analysis was performed using a Biacore 3000 instrument. Wild-type and genetically altered gp2.5 were immobilized (150 response units) on a carboxymethyl-5 chip using *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide and *N*-hydroxysuccinimide chemistry. Immobilization was performed in 10 mM sodium acetate, pH 5.0, except for gp2.5 Δ 26 protein, which was immobilized at pH 4.5 at a flow rate of 10 μ l/min. Binding studies were performed in 20 mM HEPES (pH 7.5), 10 mM MgCl₂, 250 mM potassium glutamate, 5 mM DTT at a flow rate of 40 μ l/min (25, 28). The chip surface was regenerated using 1 M NaCl at a flow rate of 100 μ l/min. As a control, a flow cell was activated and blocked in the absence of protein to account for changes in the bulk refractive index. Apparent binding constants were calculated under steady-state conditions, and the data were fitted using BIAEVAL 3.0.2 software (Biacore).

Polymerase Assay—DNA polymerase activity was measured using M13 ssDNA as a template as described previously (23). The reaction contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 50 mM NaCl, 20 nM M13 mGP1–2 ssDNA annealed to a 24-nt oligonucleotide, with 500 μ M each of dATP, dCTP, dGTP, and [³H]dTTP (2 cpm/pmol), 50 μ g/ml bovine serum albumin, and 0.3 nM gp5/trx in a total volume of 10 μ l. Reaction mixtures were incubated at 37 °C for the indicated times and stopped by addition of 5 μ l of 0.25 M EDTA (pH 7.5). The incorporation of [³H]dTTP was measured on DE81 filter disks as described (23).

Exonuclease Assay—The 3'-5'-exonuclease activity of T7 DNA polymerase was measured using uniformly labeled M13 [³H]dsDNA as described previously (34). The M13 [³H]dsDNA was prepared by annealing the 24-nt oligonucleotide to M13 mGP1–2 DNA and then extending the primer by 200 nM T7 DNA polymerase in a 50- μ l reaction mixture containing 40 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 50 mM potassium glutamate, 0.5 mM each of dATP, dCTP, dGTP, and [³H]dTTP (3000 Ci/mmol). After incubation at 37 °C for 10 min, the DNA was extracted with phenol/chloroform and then purified by passing through Biospin 6 columns (Bio-Rad) to remove free nucleotides. M13 [³H]ssDNA was prepared by alkali denaturation of ³H-labeled M13 dsDNA (34). M13 [³H]dsDNA was treated by 50 mM NaOH at 37 °C for 15 min followed by neutralization with HCl. Reaction mixtures (10 μ l) contained 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 5 mM DTT, 50 mM NaCl, 0.5 nmol (in terms of total nucleotides) of ³H-labeled M13 mGP1–2 dsDNA, and 0–20 nM gp5/trx. After incubation for 20 min, the reaction was stopped by the addition of EDTA at a final concentration of 125 mM. The hydrolysis of DNA was measured by spotting the reaction mixture on DE81 filters and by determining the amount of radioactivity remaining in DNA by scintillation counting.

Leading Strand DNA Synthesis—Leading strand DNA synthesis catalyzed by gp5/trx and gene 4 helicase was measured

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Ala → Thr at positions 258 and 411, respectively, in gp5-sup2 does not significantly affect their polymerization activity.

Exonuclease Activity of gp5-sup1 and gp5-sup2—T7 DNA polymerase, like other members of the polymerase I family of DNA polymerases, has an N-terminal exonuclease domain (31). We measured the 3′–5′-exonuclease activities of the two

suppressor polymerases in comparison with wild-type gp5/trx on both dsDNA and ssDNA at 37 °C (Fig. 2B). The fraction of DNA hydrolyzed was measured as a function of enzyme concentration. gp5-sup1/trx and gp5-sup2/trx hydrolyzed the dsDNA as well as ssDNA at approximately the same rate as that observed with wild-type gp5/trx when examined over a range of concentration (0–20 nM) of enzyme. Thus the substitution of glycine at position 371 (gp5-sup1) as well as the substitution of threonine and alanine at positions 258 and 411 (gp5-sup2) do not affect the polymerase or exonuclease activities of gp5. These normal activities of the altered gp5 proteins are in agreement with their ability to support the growth of T7Δ5 phage lacking gp5 (30).

Physical Interaction of Suppressor DNA Polymerases with Gene 2.5 Proteins—The mutations identified in gene 5 detected through the suppressor screen of gp2.5-FD resulted in amino acid changes in T7 DNA polymerase. One obvious biochemical explanation for the suppressor effect is an alteration in their interaction of gp5/trx relative to wild-type gp2.5. The C-terminal phenylalanine of gp2.5 is known to interact with gp5/trx (31). We have used surface plasmon resonance to examine the physical interaction of wild-type gp5/trx and the two suppressor DNA polymerases with wild-type gp2.5, gp2.5-FD. We have also examined the interaction of gp2.5Δ26 lacking the entire acidic C-terminal tail. In these experiments either gp2.5, gp2.5-FD, or gp2.5Δ26 was immobilized on a carboxymethyl-5 chip, and gp5/trx, gp5-sup1/trx, or gp5-sup2/trx was flowed over the chip. Upon binding of the two proteins, the resulting change in mass is reflected as a change in the resonance angle that is detected in real time. The affinities of the binding partners were examined over a range of concentrations to obtain the dissociation constants (K_D) presented in Table 1. A representative binding titration curve is shown in Fig. 3A, where gp2.5 is immobilized on the carboxymethyl-5 chip, and gp5-sup1/trx flows at a range of concentrations. The relevant binding constants were calculated using steady-state kinetics. Fig. 3B shows the binding curve used to calculate the K_D of 1.1 μM for the interaction of gp2.5 with gp5-sup1/trx. The K_D values presented in Table 1 for the other interactions were calculated in an identical manner.

As reported previously (25, 28), wild-type gp5/trx physically interacts with gp2.5 with a K_D of 2.5 μM and to a significantly lesser extent to gp2.5-FD ($K_D = 23 \mu\text{M}$), lacking the C-terminal phenylalanine (Table 1). Elimination of the entire C-terminal tail, gp2.5Δ26, which eliminates both the negative charge and the phenylalanine reduces the affinity of the two proteins even more ($K_D = 69 \mu\text{M}$) (28). These results are in agreement with the finding that neither of these two altered gp2.5 mutants can complement T7Δ2.5 phage for growth (28). The suppressor

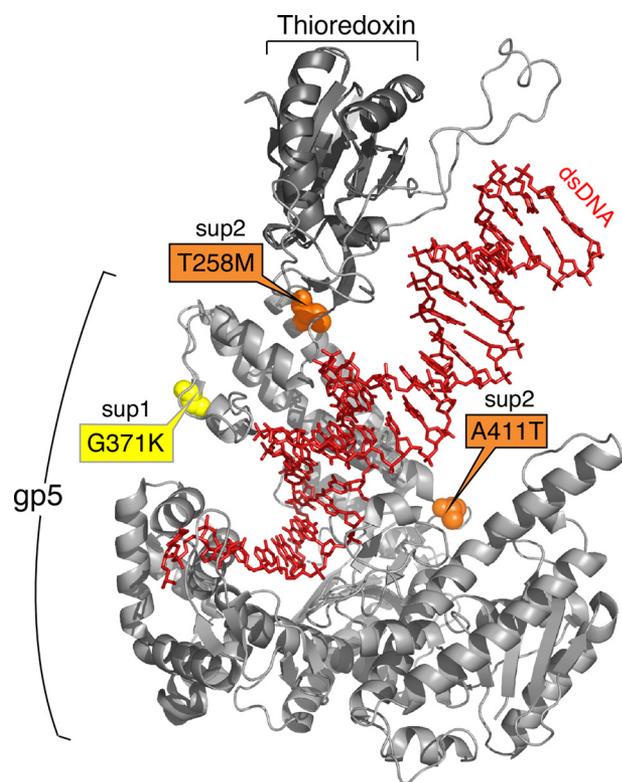


FIGURE 1. Amino acid changes in gp5 suppressor mutant polymerase(s). The amino acid changes in gp5 arising from the suppressor mutations in gene 5 are identified in the crystal structure of gp5/trx in complex with a primer-template and a nucleoside triphosphate (31). gp5 (light gray), trx (dark gray), and primer/template (red) are depicted. The suppressor mutation G371K (gp5-sup1) is shown in yellow and T258M and A411T (gp5-sup2) in orange.

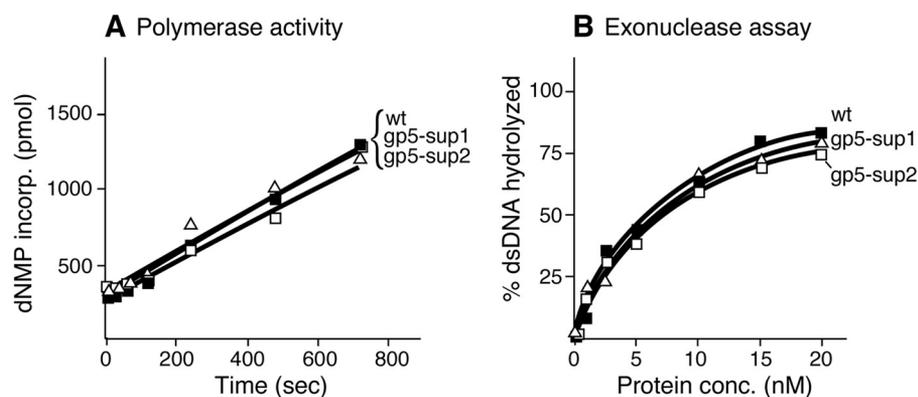


FIGURE 2. DNA synthesis and exonuclease activity catalyzed by wild-type gp5/trx, gp5-sup1/trx, and gp5-sup2/trx. A, rates of DNA synthesis. A 24-nt oligo annealed to circular M13 ssDNA (20 nM) was used as the primer-template in a standard polymerase assay with gp5/trx (■), gp5-sup1/trx (△), or gp5-sup2/trx (□) (0.3 nM). The rate of incorporation (*incorp.*) of [^3H]dTMP was measured at 37 °C on DE81 filter disks as described under “Experimental Procedures.” The data have been presented as the rates of incorporation of total deoxyribonucleoside monophosphate. The length of the template is 9950 bases. B, 3′–5′-exonuclease activity on M13 dsDNA. Uniformly ^3H -labeled M13 dsDNA was prepared by annealing a 24-nt oligonucleotide to M13 DNA and then extending the primer using gp5/trx in the presence of [^3H]dTTP. Acid-soluble radioactivity was measured as described under “Experimental Procedures.” The percentage of DNA hydrolyzed has been presented as a function of polymerase concentration (*conc.*). 100% dsDNA corresponds to 0.5 nmol of M13 DNA (in terms of total nucleotides). wt, wild type.

TABLE 1
Physical interaction between gp2.5 and gp5/trx, apparent dissociation constants

The apparent binding constant, K_D , was calculated using the steady-state fit model provided by BIAEVAL 3.0.2 software (Biacore). The experimental values for each interaction were performed in triplicate. Wild-type gp2.5, gp2.5-FD, and gp2.5 Δ 26 were coupled to three different flow cells on a carboxymethyl-5 chip, and then the indicated gp5/trx was flowed over a range of concentrations to obtain steady-state binding. Binding studies were carried out as described under "Experimental Procedures." A representative binding profile of the interaction of gp2.5-wt with gp5-sup1/trx is shown in Fig. 3, A and B, which shows the binding curve used to calculate the K_D of 1.1 μ M for the interaction of gp2.5 with gp5-sup1/trx. Other interactions were examined in the identical manner. Approximately 150 response units of gp2.5 were coupled to the chip. A control flow cell lacking gp2.5 is used to subtract the response units resulting from nonspecific interaction and bulk refractive index; coupling step was omitted and base line adjusted to zero.

	gp2.5	gp2.5-FD	gp2.5(Δ 26)
gp5	2.5 (\pm 0.8)	23.6 (\pm 3.0)	69.5 (\pm 8.0) μ M
gp5-sup1	1.0 (\pm 0.2)	3.8 (\pm 0.3)	4.7 (\pm 1.0)
gp5-sup2	1.7 (\pm 0.2)	3.7 (\pm 0.4)	11.2 (\pm 1.0)

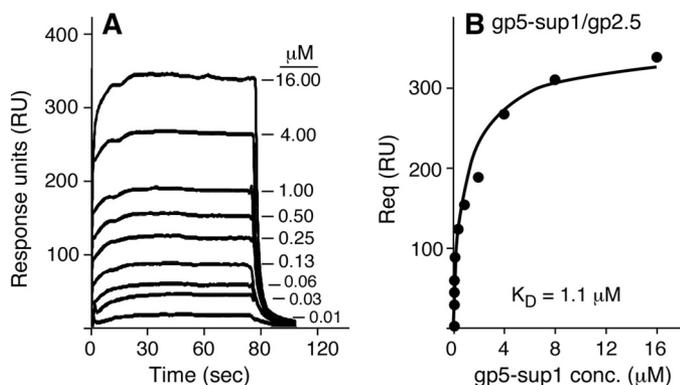


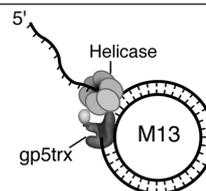
FIGURE 3. Binding of gp5-sup1/trx to gp2.5. The interaction of gp5 with gp2.5 as measured by surface plasmon resonance. gp2.5 (150 response units (RU)) was immobilized on a Biacore carboxymethyl-5 chip, and increasing concentrations (*conc.*) of gp5-sup1/trx were flowed over the surface of the chip. A, sensorgrams of the binding of gp5-sup1/trx (0.002–16 μ M) to gp2.5. Only 9 of 14 concentrations tested are shown for clarity. B, K_D determination of the binding of gp5-sup1/trx to gp2.5. Data points represent the equilibrium average response for the last 10 s of the injection in each of the experiments shown in A, where steady-state conditions have been obtained. The K_D of 1.1 μ M was calculated using the steady-state fit model provided by BIAEVAL 3.0.2 software (Biacore). It should be noted that the calculated K_D value is apparent and not absolute due to the random immobilization of gp2.5 on the surface of the chip with some molecules having a conformation that may not support interaction with the polymerase.

mutants, gp5-sup1/trx and gp5-sup2/trx, however, bind much stronger to gp2.5-FD compared with wild-type gp5/trx, with K_D values of 3.8 and 3.7 μ M, respectively. We were surprised to find that gp5-sup1/trx and gp5-sup2/trx also bind to gp2.5 Δ 26 with higher affinity compared with wild-type gp5, with K_D values of 4.7 and 11.2 μ M, gp5-sup1/trx and gp5-sup2/trx, respectively. Both suppressor polymerases bind somewhat tighter ($K_D = 1$ –1.7 μ M) to wild-type gp2.5 relative to the wild-type gp5/trx. Both suppressor DNA polymerases support the growth of T7 Δ 5 phage where they must interact with wild-type gp2.5, but the efficiency of plating is somewhat reduced (0.6 for sup1 and 0.9 for sup2 compared with 1 for wild-type gp5). The tighter binding between the gp5 mutants and gp2.5 may prevent the dynamic interactions of other proteins essential for replication.

Effect of gp2.5 on Leading Strand DNA Synthesis Mediated by gp5/trx and T7 DNA Helicase—gp5/trx catalyzes highly processive DNA synthesis on ssDNA templates but is unable to

TABLE 2
Stimulation of leading strand synthesis of gp5 by gp2

Strand displacement synthesis catalyzed by gp5/trx and T7 gene 4 helicase was measured using M13 circular dsDNA with a preformed replication fork (see structure) in the presence of gp2.5. Reaction mixtures contain 10 nM DNA, 10 nM gp4 (hexamer), 10 nM of the indicated gp5/trx, and 4 μ M of the indicated gp2.5. The incorporation of [α - 32 P]dAMP is measured at 37 $^{\circ}$ C for 10 min as described under "Experimental Procedures." The activity observed with wild-type gp5/trx (400 pmol of DNA synthesis/min) was normalized to 100%, and all other values are expressed relative to this value. The results are derived from two independent experiments. The rate of strand displacement synthesis of gp5-sup1/trx and gp5-sup2/trx was similar to gp5/trx (data not shown).



	no gp2.5	gp2.5 wt	gp2.5-FD	gp2.5 Δ 26
gp5	100 (\pm 10)	260 (\pm 15)	150 (\pm 7)	19 (\pm 2)
gp5-sup1	98 (\pm 15)	310 (\pm 12)	175 (\pm 10)	80 (\pm 3)
gp5-sup2	96 (\pm 12)	275 (\pm 10)	185 (\pm 9)	70 (\pm 2)

polymerize nucleotides on duplex DNA (23). In order for gp5/trx to polymerize nucleotides on duplex DNA, the gene 4 helicase must unwind the duplex DNA to expose the ssDNA template. In this reaction gp5/trx and the helicase form a stable complex that increases the processivity of nucleotide polymerization dramatically (38). Although the effect of gp2.5 on leading strand synthesis with DNA helicase has not been previously examined, it would not be surprising if it did influence the reaction. The hexameric helicase must first load onto the nontemplate strand for its subsequent 5'–3'–translocation along the DNA (see structure in Table 2), a process that could potentially be affected by gp2.5 bound to this segment of ssDNA. Aside from its interaction with DNA, gp2.5 also physically interacts with both gp5/trx and with the gene 4 helicase (25). In fact, the interaction with gp5/trx involves the C-terminal tail of gp2.5 that binds to two basic loops in the TBD of gp5. These same basic loops also interact with the acidic C-terminal tail of the helicase. Finally, gp2.5 is essential to establish coordination in leading and lagging strand DNA synthesis that progress at the same rate (26).

We first examined the ability of the two suppressor DNA polymerases and T7 DNA helicase to mediate leading strand synthesis (Table 2). In this assay we have used a primer-template consisting of a circular M13 dsDNA molecule in which one of the two strands bears a 36-nt ssDNA tail resembles a replication fork (see structure in Table 2). The 5'-ssDNA provides a site for the assembly of the hexameric gene 4 protein. Upon the addition of T7 DNA polymerase and deoxyribonucleotides, the two proteins act together to mediate leading strand DNA synthesis (39). In Table 2, we have compared the rate of DNA synthesis catalyzed by gp5/trx, gp5-sup1/trx, and gp5-sup2/trx in the presence of gene 4 helicase. The values are expressed as a percentage of that obtained with wild-type gp5/trx and helicase expressed as 100. Leading strand synthesis by gp5-sup1/trx and gp5-sup2/trx is essentially identical to that of gp5/trx.

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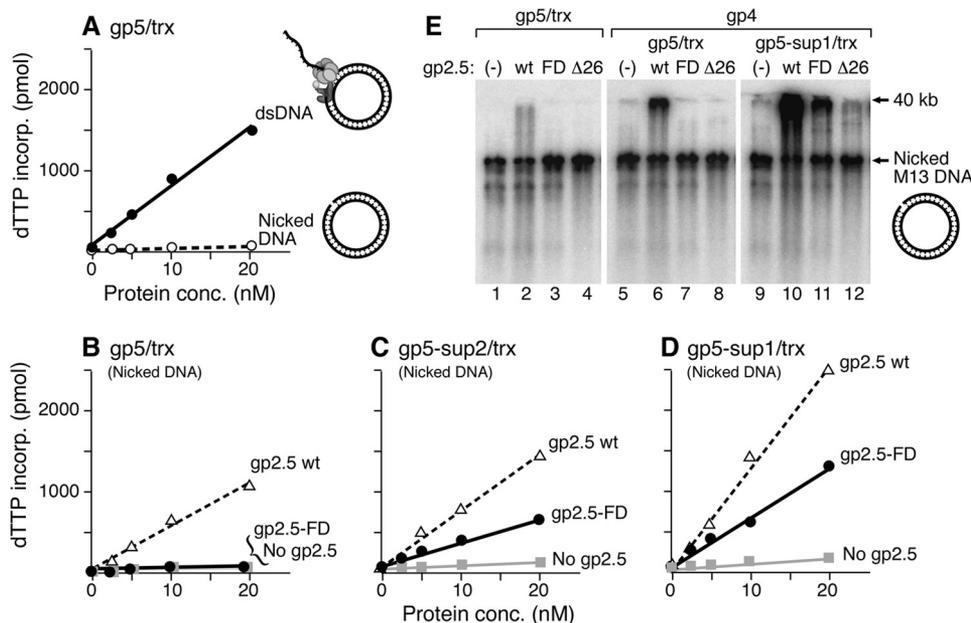


FIGURE 4. Effect of gp2.5 on strand displacement DNA synthesis catalyzed by gp5/trx at a nick in DNA. Strand displacement synthesis catalyzed by gp5/trx on nicked DNA was monitored by coupling the reaction to leading strand synthesis mediated by gp5/trx and DNA helicase. *A*, strand displacement synthesis on M13 nicked circular DNA and M13 circular dsDNA bearing a 5'-ssDNA tail of 36 nucleotides (see inset). Each reaction contained 10 nM DNA, 10 nM helicase, and the indicated amounts of gp5/trx (0–20 nM) in a total reaction volume of 10 μ l. After incubation for 10 min at 37 $^{\circ}$ C, the amount of [32 P]dTTP incorporated (*incorp.*) into DNA was measured. *B*, effect of gp2.5 on strand displacement synthesis at a nick. The reaction with nicked DNA described in *A* was carried out with the addition of 4 μ M gp2.5 or gp2.5-FD as indicated. *C*, effect of gp2.5 and gp2.5-FD on strand displacement synthesis catalyzed by gp5-sup2/trx. gp5-sup2/trx was substituted for wild-type gp5/trx in the reaction described in *B*, and the effect of wild-type gp2.5 or gp2.5-FD was examined. *D*, effect of gp2.5 and gp2.5-FD on strand displacement synthesis catalyzed by gp5-sup1/trx. gp5-sup1/trx was substituted for wild-type gp5/trx in the reaction described in *B*, and the effect of wild-type gp2.5 or gp2.5-FD was examined. *E*, radioactive products of strand displacement synthesis. The DNA products of the reaction shown in *B* and *C* for a single concentration (*conc.*) of gp5/trx (10 nM) were denatured and analyzed by electrophoresis on 0.6% alkaline-agarose gel.

We next examined the effect of wild-type gp2.5 on leading strand synthesis. Leading strand synthesis with all three DNA polymerases was stimulated \sim 3-fold (Table 2). Whether this stimulation is due to a facilitated loading of gene 4 protein or to a coating of the ssDNA produced is not known. gp2.5-FD provides only a 50% stimulation with wild-type gp5 and a slight but significant increase over this value with the two suppressor polymerases. gp2.5 Δ 26 lacking the entire C-terminal tail inhibits leading strand synthesis with all three DNA polymerases. Perhaps more striking is the dramatic increase in stimulation by gp2.5 Δ 26 when used with the two suppressor DNA polymerases.

Effect of gp2.5 on Initiation of Strand Displacement Synthesis Catalyzed by gp5/trx at a Nick in DNA—Wild-type gp5/trx is unable to initiate DNA synthesis at a nick in duplex DNA (32). However, reduction or elimination of the 3'-5'-exonuclease activity of gp5, either by chemical modification or genetic alterations (41, 42), enables gp5/trx to catalyze limited strand displacement synthesis of several hundred nucleotides (32, 43). The resulting 5'-ssDNA tail provides a site to which the helicase can assemble, an event that is followed by extensive strand displacement synthesis. The inability of wild-type gp5/trx and helicase to initiate leading strand synthesis at a nick is shown in Fig. 4*A* where there is no detectable synthesis of M13 dsDNA containing a nick in one strand (Fig. 4*A*, inset). As a control, the

circular dsDNA containing a 5'-ssDNA tail of 36 nucleotides allowed for extensive leading strand synthesis.

The detection of limited strand displacement synthesis is difficult as the resulting DNA product with an ssDNA tail of only \sim 100 nucleotides has physical properties similar to that of the original nicked DNA. A sensitive assay for this limited strand displacement synthesis is an amplification of the event by coupling it to leading strand DNA synthesis mediated by gp5/trx and helicase. Provided the strand displacement synthesis proceeds for 40 or more nucleotides, gene 4 helicase can assemble on the resulting 5'-ssDNA tail, interact with gp5/trx, and initiate extensive leading strand DNA synthesis. We initially used this assay to detect the strand displacement synthesis mediated by gp5/trx having reduced exonuclease activity (32). Using this assay we have previously shown that wild-type gp2.5 enables wild-type gp5/trx to carry out limited strand displacement synthesis from a nick (44). The ability of gp2.5 to enable gp5/trx to catalyze strand displacement synthesis using this assay is shown in

Fig. 4*B*, and the high molecular weight products ($>$ 40 kb) of leading strand DNA synthesis are seen in the gel presented in Fig. 4*E*, lane 6.

We were surprised to find that gp2.5-FD does not support this reaction (Fig. 4, *B* and *E*) (28). The inability of gp2.5-FD to enable leading strand synthesis clearly demonstrates an interaction of gp2.5 with the polymerase rather than just a role in forming and stabilizing the ssDNA tail because it binds to ssDNA as well as does wild-type gp2.5 (28). Not surprisingly, gp2.5 Δ 26, lacking the entire C-terminal tail, does not support leading strand synthesis (Fig. 4*E*, lane 8). gp2.5 Δ 26 has a 10-fold higher affinity for ssDNA compared with wild-type gp2.5 (28).

Like wild-type gp5/trx, both suppressor DNA polymerases together with helicase are unable to catalyze strand displacement synthesis at a nick sufficient to allow loading of the helicase (Fig. 4, *C* and *D*). However, wild-type gp2.5 does promote strand displacement synthesis with both of these suppressor polymerases. In fact, the observed leading strand synthesis is significantly enhanced over that observed with wild-type gp5/trx, 1.5-fold for gp5/trx-sup1 and 2.5-fold for gp5/trx-sup2, suggesting that initial strand displacement synthesis was more efficient. Most significant is the finding that gp2.5-FD enables gp5-sup1/trx and gp5-sup2/trx to mediate strand displacement synthesis (Fig. 4, *C–E*, lanes 7 and 11). Recall that gp2.5-FD does not allow for synthesis to initiate with wild-type DNA polymer-

ase. The observed leading strand synthesis is less than that observed with wild-type gp2.5, but the activity is significant. In the case of gp5-sup1/trx, synthesis is ~50% that observed with wild-type gp2.5. The high molecular weight of the products of these reactions documents the extensive leading strand synthesis (Fig. 4E). In this analysis we also examined the ability of the two suppressor DNA polymerases to allow gp2.5 Δ 26 to stimulate strand displacement synthesis. Limited but detectable strand displacement synthesis is observed with both suppressor DNA polymerases in the presence of gp2.5 Δ 26 (Fig. 4E). Recall that gp2.5 Δ 26 also has a stronger affinity for the two suppressor DNA polymerases than it does for wild-type gp5/trx (Table 2). The nicked DNA in the above experiments was prepared by completing the synthesis of the complementary strand of primed M13 DNA using T7 DNA polymerase. T7 DNA polymerase cannot catalyze any strand displacement synthesis, leaving a nick after completion of synthesis on the circular DNA (45). However, we have also carried out the experiments presented in Fig. 4E using nicked DNA prepared by converting supercoiled pBR322 DNA to the relaxed form with the nicking enzyme Nb.BsmI. Essentially identical results as presented in Fig. 4E were obtained (data not shown).

Effect of gp2.5 on the Coordination of Leading and Lagging Strand Synthesis—Leading strand synthesis is continuous, whereas that of the lagging strand is discontinuous leading to formation of Okazaki fragments. Although lagging strand synthesis requires multiple initiation events, the rates of synthesis of both strands are equal strongly indicating that leading and lagging strand synthesis are coordinated (26, 46). As gp2.5 is known to be essential for the coordination of leading and lagging strand synthesis, it was of interest to see if gp2.5-FD is able to support coordinated DNA synthesis with wild-type DNA polymerase as well as with the two suppressor DNA polymerases. We have used a mini-circular DNA to examine coordinated DNA synthesis as described previously (26). In this reaction, [α - 32 P]dTTP and [α - 32 P]dATP are used to measure leading and lagging strand synthesis, respectively, because the incorporation of these nucleotides into the two strands is dictated by the nucleotide composition of each chemically synthesized strand. The radioactive products of synthesis with [α - 32 P]dTTP (leading strand) or [α - 32 P]dATP (lagging strand) are displayed on an alkaline agarose gel (Fig. 5).

Wild-type gp5/trx, gp4, and gp2.5 mediate coordinated DNA synthesis as seen by the production of high molecular weight leading strand product and of Okazaki fragments on the lagging strand (Fig. 5, lanes 1 and 2). The majority of the products of leading strand synthesis exceeds the resolving power of the agarose gel, having lengths greater than 30,000 nucleotides (46), whereas the Okazaki fragments range in length from 800 to 3000 nt (27). Substitution of gp2.5-FD for wild-type gp2.5 does not affect coordinated DNA synthesis but does lead to Okazaki fragments of a slightly greater length (Fig. 5, lanes 3 and 4). The increase in length of the Okazaki fragments are well within the range previously observed for coordinated DNA synthesis (46).

Both suppressor DNA polymerases mediate coordinated DNA synthesis with wild-type gp2.5, although again the length of the Okazaki fragments is slightly increased (Fig. 5, lanes 5, 6, 9, and 10). When wild-type gp2.5 is replaced with gp2.5-FD,

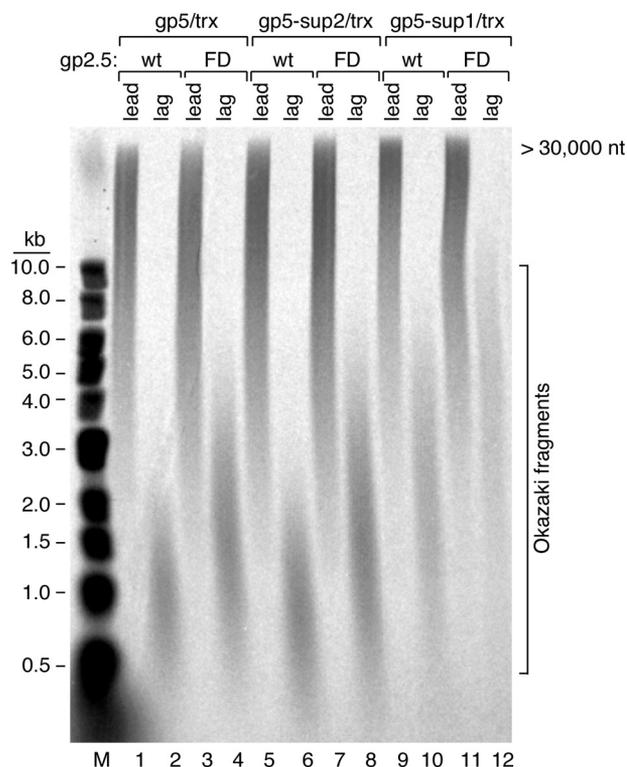


FIGURE 5. Effect of gp2.5 on coordinated DNA synthesis using mini-circle DNA. Coordinated synthesis of gp5/trx variants is studied in the presence of gp2.5 and helicase on a mini-circle DNA. The effect of gp2.5-FD on coordinated DNA synthesis was also examined on gp5/trx, gp5-sup1/trx, and gp5-sup2/trx. The leading and lagging strand synthesis of each polymerase is studied by formation of DNA products. The product of leading strand synthesis is more than 30,000 nucleotides that lie beyond the resolution of the gel. The product of lagging strand synthesis, Okazaki fragments, forms within a size range of 800–3000 nucleotides, beyond which the system loses the coordination with the leading strand polymerase. Marker, M, on left measures length of the Okazaki fragments. wt, wild type.

coordination of synthesis is decreased as seen by the greater length of lagging strand products, particularly with gp5-sup1/trx (Fig. 5, lanes 7, 8, 11, and 12). The Okazaki fragment of gp5-sup1/trx in the presence of wild-type gp2.5 is about 3000 nt, a length beyond which coordination of synthesis is lost (27, 46). However, gp5-sup1/trx in the presence of gp2.5-FD is not coordinated as evidenced by the elongated Okazaki fragments of around 6000 nt in length (Fig. 5, lane 12).

DISCUSSION

Acidic C termini and OB-folds are common structural features of prokaryotic ssDNA-binding proteins regardless of their amino acid sequences. The essential C-terminal phenylalanine found in T7 gp2.5 is conserved among gp2.5 and *E. coli* SSB protein homologs (28). On the other hand, the ssDNA-binding protein encoded by bacteriophage T4, gp32, as well as all gp32 homologs have either a leucine or isoleucine as the C-terminal residue. Interestingly, the gene 4 helicase-primase of phage T7 has an acidic C-terminal tail with a C-terminal phenylalanine. As is the case for gp2.5, both the acidic charge and the C-terminal phenylalanine of the C-terminal tail of gp4 are critical for function (29).

C-terminal Phenylalanine Is Essential for gp2.5 Function in Vivo and in Vitro—The C-terminal phenylalanine of gp2.5 is essential for T7 DNA synthesis *in vivo* (28). Either deletion of

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the phenylalanine or switching it with the adjacent residue results in a gp2.5 that cannot function *in vivo*. The critical location of the phenylalanine became most apparent during the isolation of the suppressor mutations used in this study; most suppressor mutations restored the phenylalanine to the C-terminal position (30). These studies also revealed that gp2.5-FD is dominant lethal.

The major biochemical alteration initially found in gp2.5-FD is a 10-fold lower affinity for gp5/trx (28). The extragenic suppressor mutations found in gene 5 were therefore not unexpected (30). gp2.5-FD binds to ssDNA with the same affinity as the wild-type protein (28). However, one altered gp5, gp5-F232L, in which the phenylalanine has been replaced with leucine, has a 3-fold higher affinity for ssDNA (40). Interestingly, gp2.5-F232L allows gp5/trx to catalyze strand displacement synthesis on nicked DNA even in the absence of DNA helicase (40).

gp2.5 Enables gp5/trx to Catalyze Strand Displacement Synthesis at a Nick in DNA—T7 gp5/trx is highly processive on ssDNA templates with a processivity of ~800 nucleotides polymerized per binding event (47). However, gp5/trx is unable to polymerize nucleotides through duplex regions of DNA unless associated with T7 gene 4 helicase. The helicase and gp5/trx form a stable complex, an interaction that increases the processivity to >16,000 nucleotides (38). However, in order for this association to occur both gp5/trx and the gp4 helicase must load onto the primer template. gp5/trx can bind to the 3'-hydroxyl group at a nick in duplex DNA and initiate either 3' to 5' hydrolysis of the DNA or extend the 3'-hydroxyl terminus at the nick by invading the duplex region and displacing the opposite strand (40). This latter polymerization reaction requires a significant reduction in the 3'-5'-exonuclease activity of gp5 (40, 42). Normally, gp5/trx "idles" at a nick, removing and replacing nucleotides.

Loading of the helicase onto DNA poses a greater problem. gp4 helicase cannot load onto duplex DNA, instead requiring a single-stranded stretch of DNA (39). To load onto DNA at a nick, a 5'-ssDNA tail of at least 36 nucleotides is required. Once the hexameric helicase is assembled on the 5'-tail it can translocate 5'-3' to contact gp5/trx bound on the opposite strand. The two proteins then mediate leading strand DNA synthesis. The strict requirement for an ssDNA for loading obviously precludes the ability of the helicase to interact with gp5/trx at a nick.

At the T7 origin of replication, it is likely that a replication bubble is an early intermediate in the initiation of DNA replication (4). If so then the helicase can load directly onto one or both of the single strands of DNA. The requirement for a region of ssDNA for initiation at a nick necessitates that either T7 DNA polymerase or one of the *E. coli* DNA polymerases catalyze strand displacement synthesis or that some other component separate the duplex DNA. *E. coli* DNA polymerase I can catalyze strand displacement synthesis and T7 gp5/trx as well, provided that its exonuclease activity is reduced (32, 41). On the other hand, gp2.5 or *E. coli* SSB protein allows T7 gp5/trx to catalyze strand displacement synthesis in a reaction dependent on stoichiometric amounts of these proteins to bind the exposed ssDNA (44). In this reaction *E. coli* SSB protein is far

more efficient in promoting strand displacement synthesis by gp5/trx, perhaps a result of its 10-fold higher affinity for ssDNA. Here we show that the resulting ssDNA tail provides an effective site for loading of the helicase to provide for extensive DNA synthesis by gp5/trx and helicase.

C-terminal Phenylalanine of gp2.5 Is Essential for Strand Displacement Synthesis by gp5/trx—Does the ability of gp2.5 and SSB protein to enable gp4 and gp5/trx to initiate DNA synthesis at a nick derive solely from their ability to partially denature the DNA at a nick? This scenario is unlikely because gp2.5 Δ 26 binds considerably more tightly to ssDNA but yet is unable to mediate strand displacement synthesis with gp5/trx alone (43). gp2.5-FD does not enable gp5/trx to catalyze strand displacement synthesis as monitored by the inability of gp5/trx to catalyze sufficient strand displacement synthesis to generate a 5'-ssDNA tail sufficient for the assembly of DNA helicase. The inability to establish this reaction most likely resides in a defective interaction of gp2.5 with gp5/trx because no strand displacement synthesis is seen even in the absence of the helicase. The results obtained with the interaction of suppressor DNA polymerases and gp2.5 support this interpretation (see below).

Interactions of C-terminal Tail of gp2.5 with gp5/trx Required for Strand Displacement Synthesis at Nicks—Inasmuch as the C-terminal tail of gp2.5 is known to interact with gp5, it is plausible that the defect leading to the lethal phenotype of gp2.5-FD arises from a defective interaction with gp5 *in vivo*. Indeed, gp2.5-FD has a 10-fold lower affinity for gp5/trx (28). Consequently, we recently carried out a screen for suppressor mutations in bacteriophage T7 that would allow for the growth of T7 phage requiring the use of gp2.5-FD (30). We had anticipated that extragenic suppressor mutations would be found in gene 5, the DNA polymerase gene, and indeed two such suppressor mutants were identified. Here we designate the products of the mutated genes as gp5-sup1 and gp5-sup2 bearing a single amino acid and a double amino acid substitution, respectively (Fig. 1).

In our characterization of these two suppressor DNA polymerases, the only defect observed is an inability of either to support leading strand synthesis by gp5/trx and DNA helicase at a nick. Both of these suppressor polymerases mediate leading strand synthesis with wild-type gp2.5 to a greater extent than observed with wild-type gp5/trx. More important, both suppressor DNA polymerases can function, albeit to a lesser extent, with gp2.5-FD and DNA helicase to initiate leading strand synthesis at a nick. Clearly, this initiation event involves a physical interaction of the C terminus of gp2.5 with gp5/trx.

Physical Interaction of gp2.5 with gp5/trx—Previous studies established that gp2.5 physically interacts with gp5/trx (25, 28). In the absence of DNA, the C termini of both gp2.5 and gene 4 helicase contact T7 DNA polymerase through two basic loops on the TBD, the 76-amino insert in the thumb subdomain to which the processivity factor thioredoxin binds (25). Both the acidic nature of the C-terminal tail and the terminal phenylalanine are, however, crucial for this electrostatic interaction of gp2.5 with gp5/trx (28). Elimination of the entire C-terminal tail, elimination of 4 of the 13 acidic residues, or replacement of the phenylalanine all reduce the binding by ~10-fold. Here we

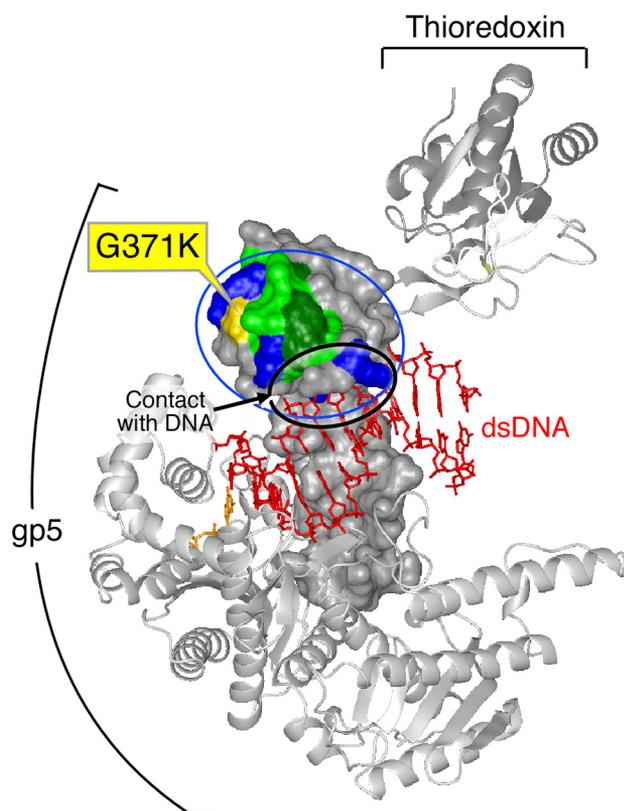
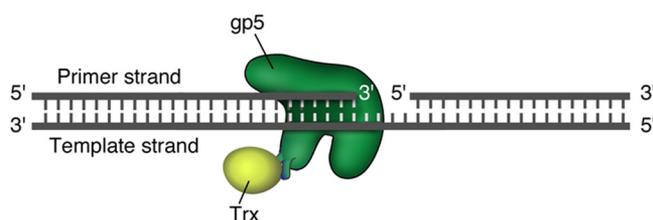
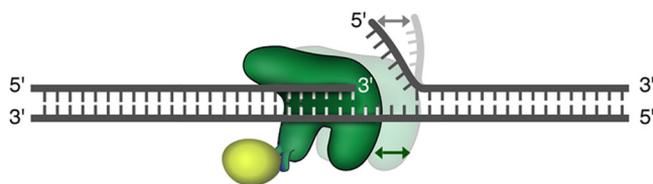


FIGURE 6. **Amino acid changes in gp5-sup1.** The location of the amino acid change in gp5-sup1 arising from the suppressor mutations in gene 5 is identified in the crystal structure of the gp5/trx in complex with a primer-template and a nucleoside triphosphate (31). gp5 with trx is depicted in gray. The primer-template is shown in red. Hydrophobic residues are displayed in green, and aromatic residues are displayed in dark green (Trp-351 and Tyr-356). Positively charged residues are displayed in blue (Glu-535 and Glu-546). Residues in direct contact with DNA (Val-364 and Asp-366) are circled in a black oval.

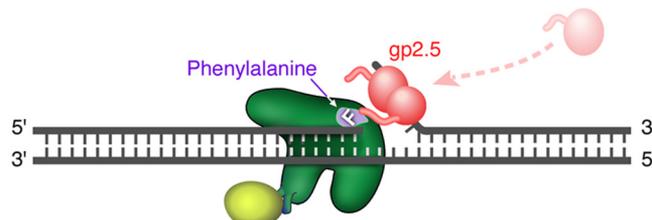
A gp5 idles at nick, and forms a gap



B Breathing of DNA with DNA synthesis



C gp2.5 binds to the displaced DNA strand. c-terminal tail of gp2.5 interacts with gp5



D Helicase replaces gp2.5

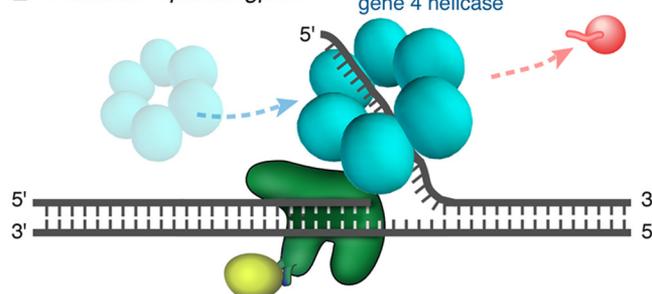


FIGURE 7. **Model for the role of gp2.5 in the initiation of leading strand synthesis at nicks in DNA.** A, gp5/trx binds to 3'-hydroxyl at a nick in duplex DNA. Alternate hydrolysis of the DNA by the 3'-5'-exonuclease activity of gp5 and nucleotide polymerization allows gp5/trx to idle at the nick. B, transient breathing of the 5' terminus at the nick allows ssDNA to give rise to 5'-ssDNA tail. C, gp2.5 binds to ssDNA tail, and the C-terminal tail of gp2.5 binds to gp5/trx to stabilize the complex and allow for limited strand displacement synthesis. D, when the 5'-tail is sufficiently long gp4 assembles as a hexamer and displaces gp2.5. gp4 and gp5/trx form a stable complex and initiate leading strand DNA synthesis.

have shown that, as anticipated, gp2.5-FD binds to each of the suppressor DNA polymerases with an affinity ~ 6 -fold greater (3.8 and 3.7 μM) than with wild-type gp5/trx ($K_D = 23.6 \mu\text{M}$). It is not unreasonable to propose that the restoration of this interaction leads to the ability of gp5-FD to promote leading strand synthesis with gp5-sup1/trx or gp5-sup2/trx.

The residues that affect the interaction of gp2.5-FD with gp5, glycine 371 in gp5-sup1 and threonine 258 and alanine 411 in gp5-sup2, are not in direct interaction with DNA (Fig. 6). However, from the crystal structure of gp5/trx in complex with the primer-template, residues adjacent to glycine 371 in gp5-sup1, *i.e.* valine 364 and aspartate 366 (Fig. 6, circled in a black oval), are in contact with DNA. These two residues are in close proximity to the amino acid change G371K (Fig. 6, shown in yellow). Likewise the residues Lys-404 and Asp-403 lie adjacent to the DNA interface, and they lie in the vicinity of the two amino acid changes (T258M and A411T) in gp5-sup2 (data not shown). The aromatic side chain residues in proximity to the suppressor mutations may be interacting with some of the aromatic residues (Fig. 6, shown in green). Unfortunately, the crystal structure and the identity of the suppressor mutations provide little insight into the mechanism by which gp2.5 enables gp5/trx and the helicase to initiate leading strand synthesis. Furthermore it is not known if the suppressor mutations lie within the binding pocket for the C-terminal phenylalanine of gp2.5 or if they have arisen elsewhere on gp5 to accommodate the new C terminus of gp2.5. The arrangement described above is somewhat similar to that observed in the crystal structure of *E. coli* exonuclease I in complex with the C-terminal peptide corresponding to the last 8 amino acids of the *E. coli* SSB protein (48, 49). SSB protein also has a C-terminal phenylalanine. This C-ter-

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minal phenylalanine inserts into a hydrophobic region composed of conserved residues of exonuclease I. The hydrophobic region and the α -carboxyl group of phenylalanine contribute to this stable interaction. The electron density map of the C-terminal peptide of *E. coli* SSB protein as seen in the crystal structure with exonuclease I is strongly suggestive of the interaction within the C-terminal tail, more so with the last 3 amino acids from a 10-mer peptide (48). These observations parallel our results on the importance of the C-terminal phenylalanine and the flexibility of the tail as a means to accommodate multiple interaction partners (50).

It is also plausible that the suppressor mutations result in conformational changes within the gp5/trx that alter the binding of gp2.5. Indeed, such a conformational change could in part bypass the role of the acidic C-terminal tail and the C-terminal phenylalanine rather than provide an altered binding site. In this regard it is interesting to note that gp2.5 Δ 26, lacking the C-terminal tail, binds considerably more tightly to the two suppressor polymerases than it does to wild-type DNA polymerase. Likewise, wild-type gp2.5 enables the two suppressor DNA polymerases to catalyze strand displacement synthesis considerably better than it does with wild-type DNA polymerase. These observations, along with the nonproximity of the suppressor mutations, clearly favor a conformational change in the polymerase that alters the interaction with gp2.5.

Model for gp2.5, gp5/trx, and Gene 4 Helicase Interactions at a Nick—In the model presented in Fig. 7 gp5/trx binds to the 3'-hydroxyl at the nick in duplex DNA (Fig. 7A). During idling at the nick transient, gaps occur facilitating breathing of the 5'-end at the nick to expose short stretches of ssDNA (Fig. 7B). In the absence of gp2.5, the 3'-5'-exonuclease activity of gp5 prevents strand displacement synthesis. However, when gp2.5 is present the transient ssDNA tail binds within the OB-fold of gp2.5, displacing the acidic C-terminal tail of gp2.5 (16) (Fig. 7C). Coating of the 5'-tail with gp2.5 is in itself not sufficient for strand displacement synthesis as evidenced from the results obtained with gp2.5-FD and gp2.5 Δ 26. Instead, we propose that the free C-terminal tail of gp2.5 establishes an electrostatic interaction with the basic patches in the TBD of gp5 and the C-terminal phenylalanine of gp2.5 with a hydrophobic region in gp5 (Fig. 7C). Whether these two modes of binding occur simultaneously or independently is not known. In any case the interaction of gp2.5 and gp5/trx allows for limited strand displacement synthesis until the length of the tail is sufficient for the assembly of the functional gene 4 hexamer, displacing gp2.5 (Fig. 7D). The displacement of gp2.5 may involve a competition of the C-terminal tails of each protein for the same binding sites on gp5. In fact, it is of interest to determine whether the suppressor mutations in gene 5 also suppress the phenotype of gene 4 proteins lacking the C-terminal phenylalanine. If so, then the C-terminal phenylalanines of gp2.5 and gene 4 proteins also have similar interactions with gp5/trx. Once polymerization of nucleotides is established within the complex, the electrostatic interaction of gp4 with gp5/trx shifts to a far more stable interaction that does not involve the C-terminal tail of gp4 (38). It is not unreasonable to postulate that strand displacement at nicks may occur during recombination, repair, and initiation of DNA replication.

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REFERENCES

1. Chase, J. W., and Williams, K. R. (1986) *Annu. Rev. Biochem.* **55**, 103–136
2. Benkovic, S. J., Valentine, A. M., and Salinas, F. (2001) *Annu. Rev. Biochem.* **70**, 181–208
3. O'Donnell, M. (2006) *J. Biol. Chem.* **281**, 10653–10656
4. Hamdan, S. M., and Richardson, C. C. (2009) *Annu. Rev. Biochem.* **78**, 205–243
5. Lohman, T. M., and Ferrari, M. E. (1994) *Annu. Rev. Biochem.* **63**, 527–570
6. 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
7. Savvides, S. N., Raghunathan, S., Fütterer, K., Kozlov, A. G., Lohman, T. M., and Waksman, G. (2004) *Protein Sci.* **13**, 1942–1947
8. Shamoo, Y., Friedman, A. M., Parsons, M. R., Konigsberg, W. H., and Steitz, T. A. (1995) *Nature* **376**, 362–366
9. Kim, Y. T., and Richardson, C. C. (1994) *J. Biol. Chem.* **269**, 5270–5278
10. Burke, R. L., Alberts, B. M., and Hosoda, J. (1980) *J. Biol. Chem.* **255**, 11484–11493
11. Hosoda, J., and Moise, H. (1978) *J. Biol. Chem.* **253**, 7547–7558
12. Williams, K. R., and Konigsberg, W. (1978) *J. Biol. Chem.* **253**, 2463–2470
13. Williams, K. R., Spicer, E. K., LoPresti, M. B., Guggenheimer, R. A., and Chase, J. W. (1983) *J. Biol. Chem.* **258**, 3346–3355
14. Kim, Y. T., and Richardson, C. C. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 10173–10177
15. Kim, Y. T., Tabor, S., Churchich, J. E., and Richardson, C. C. (1992) *J. Biol. Chem.* **267**, 15032–15040
16. Shokri, L., Marintcheva, B., Eldib, M., Hanke, A., Rouzina, I., and Williams, M. C. (2008) *Nucleic Acids Res.* **36**, 5668–5677
17. Marintcheva, B., Marintchev, A., Wagner, G., and Richardson, C. C. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 1855–1860
18. Kowalczykowski, S. C., Lonberg, N., Newport, J. W., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145**, 75–104
19. Newport, J. W., Lonberg, N., Kowalczykowski, S. C., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145**, 105–121
20. Lonberg, N., Kowalczykowski, S. C., Paul, L. S., and von Hippel, P. H. (1981) *J. Mol. Biol.* **145**, 123–138
21. Krassa, K. B., Green, L. S., and Gold, L. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 4010–4014
22. Nelson, S. W., Kumar, R., and Benkovic, S. J. (2008) *J. Biol. Chem.* **283**, 22838–22846
23. Tabor, S., Huber, H. E., and Richardson, C. C. (1987) *J. Biol. Chem.* **262**, 16212–16223
24. Kong, D., and Richardson, C. C. (1998) *J. Biol. Chem.* **273**, 6556–6564
25. Hamdan, S. M., Marintcheva, B., Cook, T., Lee, S. J., Tabor, S., and Richardson, C. C. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 5096–5101
26. Lee, J., Chastain, P. D., 2nd., Kusakabe, T., Griffith, J. D., and Richardson, C. C. (1998) *Mol. Cell* **1**, 1001–1010
27. Hamdan, S. M., Loparo, J. J., Takahashi, M., Richardson, C. C., and van Oijen, A. M. (2009) *Nature* **457**, 336–339
28. Marintcheva, B., Hamdan, S. M., Lee, S. J., and Richardson, C. C. (2006) *J. Biol. Chem.* **281**, 25831–25840
29. Lee, S. J., Marintcheva, B., Hamdan, S. M., and Richardson, C. C. (2006) *J. Biol. Chem.* **281**, 25841–25849
30. Marintcheva, B., Qimron, U., Yu, Y., Tabor, S., Richardson, C. C., and Richardson, C. (2009) *Mol. Microbiol.* **72**, 869–880
31. Doublé, S., Tabor, S., Long, A. M., Richardson, C. C., and Ellenberger, T. (1998) *Nature* **391**, 251–258
32. Lechner, R. L., Engler, M. J., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11174–11184
33. Rezende, L. F., Hollis, T., Ellenberger, T., and Richardson, C. C. (2002) *J. Biol. Chem.* **277**, 50643–50653
34. Kumar, J. K., Tabor, S., and Richardson, C. C. (2001) *J. Biol. Chem.* **276**, 34905–34912

35. Delagoutte, E., and von Hippel, P. H. (2001) *Biochemistry* **40**, 4459–4477
36. Johnson, D. E., Takahashi, M., Hamdan, S. M., Lee, S. J., and Richardson, C. C. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104**, 5312–5317
37. Mark, D. F., and Richardson, C. C. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 780–784
38. Hamdan, S. M., Johnson, D. E., Tanner, N. A., Lee, J. B., Qimron, U., Tabor, S., van Oijen, A. M., and Richardson, C. C. (2007) *Mol. Cell* **27**, 539–549
39. Notarnicola, S. M., Mulcahy, H. L., Lee, J., and Richardson, C. C. (1997) *J. Biol. Chem.* **272**, 18425–18433
40. He, Z. G., Rezende, L. F., Willcox, S., Griffith, J. D., and Richardson, C. C. (2003) *J. Biol. Chem.* **278**, 29538–29545
41. Tabor, S., and Richardson, C. C. (1989) *J. Biol. Chem.* **264**, 6447–6458
42. Tabor, S., and Richardson, C. C. (1990) *J. Biol. Chem.* **265**, 8322–8328
43. Lechner, R. L., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11185–11196
44. Nakai, H., and Richardson, C. C. (1988) *J. Biol. Chem.* **263**, 9831–9839
45. Engler, M. J., Lechner, R. L., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11165–11173
46. Lee, J., Chastain, P. D., 2nd, Griffith, J. D., and Richardson, C. C. (2002) *J. Mol. Biol.* **316**, 19–34
47. Wuite, G. J., Smith, S. B., Young, M., Keller, D., and Bustamante, C. (2000) *Nature* **404**, 103–106
48. Lu, D., and Keck, J. L. (2008) *Proc. Natl. Acad. Sci. U.S.A.* **105**, 9169–9174
49. Shereda, R. D., Reiter, N. J., Butcher, S. E., and Keck, J. L. (2009) *J. Mol. Biol.* **386**, 612–625
50. Lu, D., Windsor, M. A., Gellman, S. H., and Keck, J. L. (2009) *Biochemistry* **48**, 6764–6771