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Essential Residues in the C Terminus of the Bacteriophage T7 Gene 2.5 Single-stranded DNA-binding Protein*

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Boriana Marintcheva[1], **Samir M. Hamdan**[2], **Seung-Joo Lee**[2], **and Charles C. Richardson**[3]

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115

Gene 2.5 of bacteriophage T7 encodes a single-stranded DNA (ssDNA)-binding protein (gp2.5) that is an essential component of the phage replisome. Similar to other prokaryotic ssDNA-binding proteins, gp2.5 has an acidic C terminus that is involved in protein-protein interactions at the replication fork and in modulation of the ssDNA binding properties of the molecule. We have used genetic and biochemical approaches to identify residues critical for the function of the C terminus of gp2.5. The presence of an aromatic residue in the C-terminal position is essential for gp2.5 function. Deletion of the C-terminal residue, phenylalanine, is detrimental to its function, as is the substitution of this residue with non-aromatic amino acids. Placing the C-terminal phenylalanine in the penultimate position also results in loss of function. Moderate shortening of the length of the acidic portion of the C terminus is tolerated when the aromatic nature of the C-terminal residue is preserved. Gradual removal of the acidic C terminus of gp2.5 results in a higher affinity for ssDNA and a decreased ability to interact with T7 DNA polymerase/thioredoxin. The replacement of the charged residues in the C terminus with neutral amino acids abolishes gp2.5 function. Our data show that both the C-terminal aromatic residue and the overall acidic charge of the C terminus of gp2.5 are critical for its function.

Essentially all DNA replication systems require a cognate single-stranded DNA (ssDNA)[4]-binding protein. The ssDNA-binding protein of bacteriophage T7 is encoded by an essential gene of the phage, gene 2.5 (1). The gene 2.5 protein (gp2.5) contains 232 residues and appears to exist in solution as a dimer (2). gp2.5 binds to ssDNA with low micromolar affinity (2–4). It also facilitates homologous base-pairing between two DNA strands (5). gp2.5 is one of the four proteins that comprise the T7 replisome. The other three proteins are the T7 gene 5 DNA polymerase, its processivity factor, *Escherichia coli* thioredoxin (trx), and the multifunctional gene 4 helicase-primase.

One role of gp2.5 *in vivo* is almost certain to be to remove secondary structures in ssDNA that are known to impede the progress of the otherwise processive T7 DNA polymerase/thioredoxin (T7 DNA pol/trx) complex (2). However, the host ssDNA-binding protein, *E. coli* SSB protein, is equally effective in accomplishing this role (6). The essential nature of gp2.5 protein may rather be related to its known physical interactions with the T7 DNA pol/trx and the T7 gene 4 helicase-primase (7, 8). Biochemical manifestations of these interactions are a slight stimulation of T7 DNA pol/trx activity on ssDNA templates and an increase in the utilization of primase recognition sites and the efficiency with which the primers are extended by T7 DNA pol/trx (24).

The crystal structure of a gp2.5 lacking the C-terminal 26 amino acids (gp2.5Δ26) revealed a signature oligosaccharide/oligonucleotide-binding core (OB-fold) (10). This OB-fold is essentially superimposable with the OB-fold found in the *E. coli* SSB protein and in the 70-kDa subunit of the human replication protein A (hRPA) (10). The crystallographic data also identified a dimer interface within the N-terminal one-third of the protein. Based on structural homology and amino acid sequence conservation of amino acids known to contact ssDNA in the *E. coli* SSB protein/ssDNA complex, a DNA binding cleft was proposed for gp2.5 as well (10). The functional significance of both the postulated DNA binding cleft and the dimer interface were confirmed by the biochemical properties of altered gp2.5 proteins purified from several point mutants selected in a screen for *in vivo* lethal mutations in gene 2.5 (3–5).

gp2.5 has an acidic C terminus, in which 15 of 21 residues have acidic side chains (Fig. 1). The presence of such an acidic C terminus is a conserved feature of all prokaryotic ssDNA-binding proteins despite the limited sequence homology (6, 11). The removal of the acidic C termini of these proteins is lethal and results in a higher affinity of the protein for ssDNA (12–15). In addition, protein-protein interactions have been mapped to the C termini of the T4 gene 32 ssDNA-binding protein and *E. coli* SSB protein (13, 16, 17). These C-terminal residues of T7 gp2.5 also define an important segment of gp2.5 because deletion of the sequence encoding these residues is lethal (7). The resulting protein, T7 gp2.5Δ26, binds more tightly to ssDNA and is defective in its interaction with T7 DNA pol/trx and gene 4 helicase-primase (4, 18). Recent studies have shown that the C terminus of gp2.5 binds to a highly positively charged segment

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[2] Both authors contributed equally to the work.

[3] To whom correspondence should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-1864; Fax: 617-432-3362; E-mail: ccr@hms.harvard.edu.

[4] The abbreviations used are: ssDNA, single-stranded DNA; wt, wild-type; trx, thioredoxin; SSB protein, single-stranded DNA-binding protein; hRPA, human replication protein A; cpm, counts per minute; DTT, dithiothreitol; SPR, surface plasmon resonance; RU, response units.

located in the thumb subdomain of the gene 5 T7 DNA polymerase (19). This unique fragment is also the site of binding of the processivity factor, *E. coli* thioredoxin and the C terminus of gene 4 helicase-primase. The multiple interactions of the C terminus of gp2.5 could thus function as switch to coordinate the multiple reactions occurring at the replication fork. We have previously shown that gp2.5 is critical for establishing coordinated leading and lagging strand DNA synthesis (20, 21). In one model the acidic C terminus of gp2.5 mimics the negatively charged phosphate backbone of ssDNA and can thus bind in the positively charged DNA-binding cleft found in the crystal structure. Upon binding to ssDNA the C terminus of gp2.5 would be displaced and become available for protein-protein interactions.

Unfortunately, the only available structure of gp2.5 protein is that of T7 gp2.5Δ26 lacking the C terminus. Attempts to crystallize the full-length protein were unsuccessful, suggesting that this domain is not stably folded and thus may interfere with crystal packing (10). The *in vivo* screen for lethal mutations in gene 2.5 did identify residues in the C terminus that are essential for T7 growth (3). Truncations of 12, 17, or 18 residues were lethal, as were a double (D212A/E222G) and quadruple (D212A/E222G/D227H/D232H) amino acid alteration. These deletions and amino acid substitutions all decreased the number of acidic residues within the C terminus. An intriguing lethal mutation was one that resulted in the replacement of the C-terminal phenylalanine with leucine (F232L). This altered protein, gp2.5-F232L, binds 3-fold more tightly to ssDNA and has a slightly reduced affinity for T7 DNA pol/trx when compared with wild-type gp2.5 (18). However, it has the unique property of promoting strand-displacement DNA synthesis by T7 DNA pol/trx in the absence of the gene 4 helicase.

Identification of the specific role of gp2.5 at the replication fork is elusive. Its multiple interactions with DNA and the other replication proteins have been difficult to dissect. In the present study we have identified critical determinants within the C terminus for gp2.5 function *in vivo* and *in vitro*. Not only is the acidic nature of the fragment important but surprisingly an aromatic residue at the C-terminal position is critical for a functional protein. In the accompanying article we show likewise that both of these chemical requirements must be manifested in the C terminus of the gene 4 helicase-primase (Lee *et al.*, Ref. 33). Not surprisingly, the C terminus of gene 4 protein likewise physically interacts with the highly basic thioredoxin binding domain of T7 DNA pol/trx (19).

EXPERIMENTAL PROCEDURES

Bacterial Strains and Phages—*E. coli* DH5α was used in all cloning procedures. *E. coli* MRI 80 (22) and *E. coli* C600 were used for *in vivo* DNA complementation and DNA synthesis assays. *E. coli* BL21(DE3)pLysS (Novagen) was used for protein expression. Wild-type T7 and T7Δ2.5 phages were previously described (1).

Plasmids and Oligonucleotides—The parent plasmids encoding native and His-tagged versions of wild-type gp2.5 and gp2.5Δ26 lacking the 26 C-terminal residues, were provided by Lisa Rezende (Harvard Medical School) and are described in Ref. 3. All oligonucleotides were purchased from Integrated DNA Technologies Inc.

Construction of Plasmids Encoding Altered gp2.5 Proteins—Alterations of the sequence encoding wild-type gp2.5 were generated by PCR. All oligonucleotides used for mutagenesis contained a BamHI restriction site immediately following the stop codon of gene 2.5. An oligonucleotide encompassing nucleotides 256–287 from the gp2.5 sequence was used as a reverse primer. The generated PCR product was digested with BamHI and MluI restriction enzymes and inserted into BamHI/MluI-digested pET17bgp2.5-wt vector. The constructs expressing His-tagged versions of altered gp2.5 proteins were generated by subcloning NdeI-BamHI gp2.5-containing fragments from the corresponding pET17b constructs into pET19bPPS vector (3). The sequence of all constructs was confirmed by sequencing.

Gene Expression and Protein Purification—Native and histidine-tagged gp2.5 variants were purified from BL21(DE3)pLysS cells overexpressing their genes as previously described (3). T7 DNA pol/trx was purified as previously described (23).

In Vivo Complementation Assay—To test the ability of the gp2.5 variants to support the growth of T7 phage lacking gene 2.5 (T7Δgp2.5), pET17b plasmids containing genes encoding altered gp2.5 proteins were transformed in *E. coli* strains, maintaining high (DH5α) and low (MRI80) plasmid copy number. Serial dilutions of T7Δ2.5 phage were mixed with 0.5 ml of overnight culture of *E. coli* cells transformed with each plasmid, 3 ml of soft agar, and ampicillin. The mixtures were overlaid on TB plates and incubated at 37 °C for 4 h. The plaques were counted, and the plating efficiencies calculated as a ratio between the number of plaques observed on a specific strain transformed with plasmid encoding a gp2.5 variant and the number of plaques observed on the same strain transformed with pET17bgp2.5-wt.

In Vivo DNA Synthesis—*E. coli* MRI 80 cells were transformed with each plasmid of interest and infected with T7Δ2.5 phage. DNA synthesis was measured by [^{3}H]thymidine incorporation into acid-insoluble DNA as previously described (3). Relative incorporation was calculated as a ratio between the radioactivity incorporated for the specific gp2.5 variant and that incorporated for *E. coli* transformed with pET17b empty vector.

ssDNA Gel Shift Assay—5′-^{32}P-End-labeled oligodeoxynucleotides of different length were used as a substrate to compare the ssDNA binding abilities of wild-type and altered gp2.5 proteins. The 15-μl reactions included 0.3 nM ssDNA substrate, 10 mM $MgCl_2$, 5 mM DTT, 50 mM KCl, 10% glycerol, 0.01% bromphenol blue, and various concentrations gp2.5 variants diluted in a buffer containing 20 mM Tris (pH 7.5), 5 mM DTT, and 500 μg/ml bovine serum albumin. Reactions were incubated on ice for 10 min. Bound and free DNA species were resolved on a 10% TBE Ready Gel (Bio-Rad) using 0.5× Tris/glycine buffer (12.5 mM Tris base, 95 mM glycine, and 0.5 mM EDTA). Gels were run at 150 V at 4 °C until bromphenol blue reached two-thirds of the gel length. The gels were dried, exposed to a Fujix PhosphorImager plate, and the radioactivity was measured using ImageQuant software.

Surface Plasmon Resonance (SPR) Analysis—SPR was performed using a Biacore 3000 instrument. Wild-type and genet-

ically altered gp2.5 variants (150 response units (RU)) were immobilized on a CM-5 (carboxymethyl-5) chip using EDC/NHS chemistry. The immobilization was performed at a flow rate of 40 μl/min in 10 mM sodium acetate, pH 5.0, except for the gp2.5Δ6 protein, which was immobilized at pH 4.5. The binding studies were performed in a buffer containing 20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 250 mM potassium glutamate, 5 mM DTT, at a flow rate of 40 μl/min. A control flow cell was activated and blocked in absence of protein. The response units seen in the control cell were subtracted from the signal of the flow cells with immobilized protein to account for changes in the bulk refractive index. The chip surface was regenerated using 1 M NaCl at a 100 μl/min flow rate. The apparent binding constants were calculated under steady-state conditions and the data fitted using BIAEVAL 3.0.2 software (Biacore).

gp2.5 Stimulation of Strand Displacement DNA Synthesis—The reaction contained 4 nM M13 template DNA, 0.3 mM of all four dNTPs (0.1 μCi of [α-^{32}P]dGTP), 100 nM T7 DNA pol/trx, and 4 μM gp2.5. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of EDTA to a final concentration of 20 mM. An aliquot of the reaction mixture was spotted on DE-81 filter and washed three times with 0.3 M ammonium formate, pH 8.0, and one time with 95% ethanol. The filters were dried, and the retained radioactivity was determined by scintillation counting. The reaction products were also analyzed by electrophoresis through 1% alkaline agarose gel.

RESULTS

In Vivo Analysis of Alterations in the C Terminus of gp2.5—To determine the relative importance of the individual residues within the essential C terminus of the T7 gene 2.5 protein we have carried out extensive *in vitro* mutagenesis. As shown on Fig. 1*A* the acidic C terminus of gp2.5 contains 21 amino acids of which 15 have acidic side chain (8 glutamate (E) residues and 7 aspartate (D) residues). These acidic residues occur in blocks of varying lengths, separated mainly by non-polar amino acids such as alanine, serine, and glycine known to be associated with flexibility of the polypeptide chain. The C terminus ends with the aromatic residue phenylalanine (F) that by virtue of its free carboxyl group adds another negative charge. Fig. 1 summarizes all of the modifications of the C terminus used in the present study. Table 1 summarizes the ability of these same altered genes to complement the growth of T7 phage lacking gene 2.5. These constructs will be cited at the appropriate point in the text.

The C-terminal Phenylalanine Is Essential for the Function of Gene 2.5 Protein—In earlier studies we have shown that gp2.5 proteins lacking the entire C terminus (gp2.5Δ21 and gp2.5Δ26) cannot complement for the growth of T7 phage lacking gene 2.5, T7Δ2.5 (3, 8). Inasmuch as we had attributed this phenotype to the removal of the acidic residues we were surprised to find that deletion of the C-terminal phenylalanine is as detrimental as deletion of the all C-terminal 26 residues (Table 1, A). In this initial experiment deletion mutants were constructed to progressively remove the charged residues by setting the boundaries of the deletions according to the positions

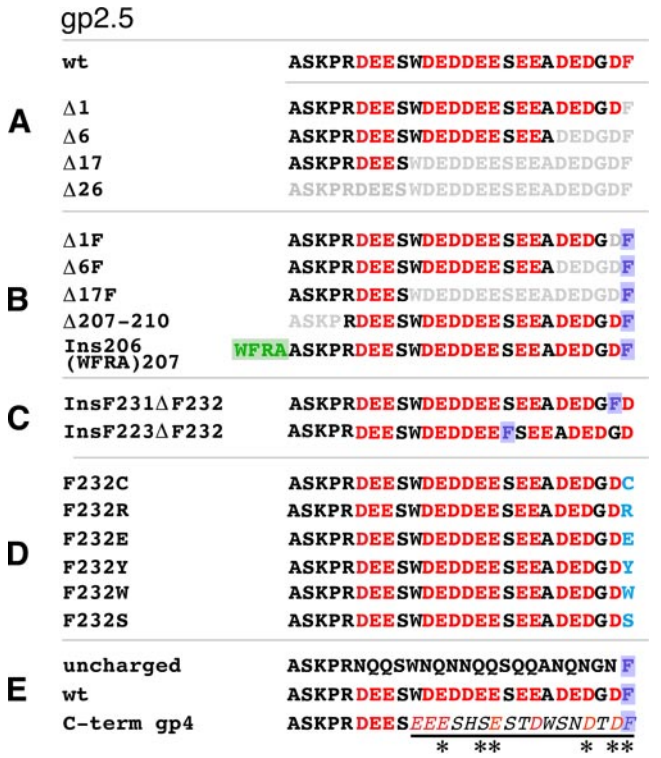

FIGURE 1. **Mutagenesis in the acidic C terminus of gp2.5.** The sequence of the C terminus of wild-type gp2.5 protein is depicted on the *top*. All acidic residues are colored in *red*. *A*, C-terminal deletions. Deleted residues are colored in *light gray*. *B*, internal deletions and insertions. Inserted residues are colored in *green*, and the C-terminal phenylalanine is colored and highlighted in *blue*. *C*, mutations varying the position of the phenylalanine. InsF231ΔF232 is a mutant in which the last two amino acids are swapped, *i.e.* the phenylalanine at position 232 is deleted and inserted in position 231. InsF222ΔF232 is a mutant, in which the C-terminal phenylalanine is deleted, and phenylalanine is inserted ten amino acids from the end. *D*, phenylalanine point mutations. The C-terminal phenylalanine was mutated to *R* (arginine), *C* (cysteine), *S* (serine), *E* (glutamic acid), *W* (tryptophan), *Y* (tyrosine). *E*, alignment of the C termini of gp2.5-wt and gp2.5-C-term-gp4 chimera. The acidic C terminus of gp4 within gp2.5-C-term-gp4 chimera is *underlined*, and the identical residues between gp2.5 and gp4 marked with a *star*. The sequence of the gp2.5 (uncharged) mutant, where all negatively charged residues were changed to uncharged residues is depicted on the *top*.

of non-acidic residues. Thus gp2.5Δ1 removes the C-terminal phenylalanine, gp2.5Δ6 removes 6 residues, gpΔ17 removes 17 residues, and gpΔ26 eliminates the entire C terminus of 26 residues (Fig. 1*A*). To eliminate possible effects of overexpression of gene 2.5 variants from the strong T7 promoter in the pET17b vector used for cloning, all complementation experiments were performed in *E. coli* strains maintaining either high (DH5α) or low (MRI 80) plasmid copy number. As shown in Table 1, gp2.5Δ1 lacking the C-terminal phenylalanine could not complement for the growth of T7Δ2.5. Not surprisingly, in view of the absence of the phenylalanine in the remaining deletion mutants, none of the other deletion mutants were able to complement for growth.

To determine if the inability of these deletion mutants to complement T7Δ2.5 arose from a defect in DNA synthesis, we measured the incorporation of [^{3}H]thymidine into DNA after T7Δ2.5 infection of cells harboring the altered gene 2.5 (Fig. 2). When wild-type gp2.5 is overexpressed from a plasmid, DNA synthesis peaks at ~35 min after phage infection. Little, if any,

TABLE 1

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

The ability of the gp2.5 variants to support the growth of T7Δgp2.5 phage was tested in *E. coli* strains maintaining high (DH5α) and low (MRI80) plasmid copy number. The plating efficiencies were calculated as a ratio between the number of plaques observed on a specific strain transformed with a pET17b plasmid encoding the gp2.5 variant and the number of plaques observed on the same strain transformed with pET17bgp2.5-wt.

	gp2.5	Plating efficiency	
		High copy (DH5α)	Low copy (MRI80)
A			
	Wt	1	1
	Δ1	0	0
	Δ6	0	0
	Δ17	0	0
	Δ26	0	0
B			
	Δ1F	0.7	0.7
	Δ6F	0.7	0.6
	Δ17F	0	0
	Δ207–210	1.2	1.2
	Ins206 (WFRA)	2	1.9
C			
	InsF231ΔF232	0	0
	InsF223ΔF232	0	0
D			
	F232C	0	0
	F232R	0	0
	F232E	0	0
	F232Y	1.2	1.3
	F232W	0.9	1.0
	F232S	0	0
E			
	Uncharged	0	0
	C-term gp4	1.7	1.7

DNA synthesis occurred in cells infected with each of the deletion mutants. gp2.5Δ1 is equally as defective as is gp2.5Δ26 (Fig. 2*B*).

An Aromatic Residue at the C Terminus of Gene 2.5 Protein Is Critical for Function—The inability of gene 2.5 protein lacking its C-terminal phenylalanine to function *in vivo* could reflect a requirement for a specific length of the acidic C terminus or a requirement for a terminal residue of a specific chemical nature. To examine the former possibility we generated a series of gene 2.5 proteins in which internal residues were deleted from the C terminus but preserving the C-terminal phenylalanine (Fig. 1*B*). gp2.5Δ1F lacks the aspartic acid normally present adjacent to the phenylalanine. gp2.5Δ6F and gp2.5Δ17F lack the same acidic residues as gp2.5Δ6 and gp2.5Δ17, whereas preserving the C-terminal phenylalanine. gp2.5Δ1F and gp2.5Δ6F complemented for the growth of T7Δ2.5 phage almost as well as did wild-type gp2.5 (Table 1). The *in vivo* DNA synthesis assay showed that gp2.5Δ1F and gp2.5Δ6F retain 70 and 30%, respectively, of wild type ability to synthesize DNA (Fig. 2*B*). The more extensive deletion, gp2.5Δ17, that removes a major portion of the negative charge of the C terminus is unable to complement regardless of presence or absence of C-terminal phenylalanine. We also shortened and lengthened the distance of the C-terminal phenylalanine from the OB-fold harboring the ssDNA binding site by inserting (gp2.5Ins206(WFRA)207) or deleting (gp2.5Δ207–210) four amino acids just before the beginning of the acidic C terminus at position 207. These altered gene 2.5 proteins having an intact C terminus functioned normally (Table 1, B and Fig. 2*A*). The finding that up to six residues can be deleted from the C terminus without a deleterious effect suggests strongly that a simple shortening of the fragment does not explain the defect arising from the deletion of the phenylalanine.

Is the location of the phenylalanine as the C-terminal amino acid important or is there simply a requirement for a phenylalanine near the C terminus? To address this question we switched the two C-terminal residues such that aspartic acid is now the C-terminal residue and phenylalanine is adjacent as second residue (gp2.5InsF231ΔF232) (Fig. 1*C*). Likewise phenylalanine was removed from the C-terminal position and inserted ten residues distal to the C terminus (gp2.5InsF222ΔF232). Neither gene 2.5 construct could complement for the growth of T7Δ2.5 (Table 1, C) or support DNA synthesis (data not shown). Clearly the phenylalanine must occupy the C-terminal position.

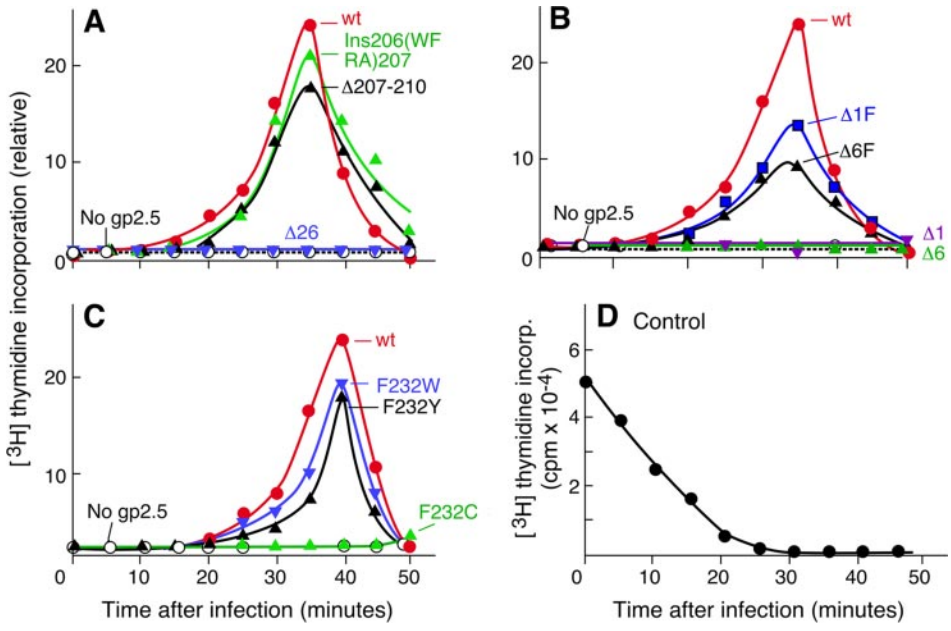

FIGURE 2. ***In vivo* DNA synthesis.** The ability of wild-type and mutant gp2.5 to support *in vivo* DNA synthesis was monitored by measuring the incorporation of [^{3}H]thymidine into acid-insoluble DNA. The amounts of radioactivity incorporated (y axis) are presented as a normalized ratio of the cpm measured for each specific sample divided by the cpm measured for the sample containing no gp2.5 (empty plasmid control). *A*, effect of internal deletions and insertions: *red*, gp2.5-wt; *blue*, gp2.5Δ26; *black*, gp2.5Δ207–210; *green*, gp2.5Ins 206(WFRA)207; *black*, *dashed line*, empty plasmid control. *B*, effect of the presence of phenylalanine at the end of the C terminus: *red*, gp2.5-wt; *purple*, gp2.5Δ1; *blue*, gp2.5Δ1F; *green*, gp2.5Δ6; *black*, gp2.5Δ6F; *black dashed line*, empty plasmid control. *C*, effect of point mutations at the C-terminal phenylalanine: *red*, gp2.5-wt; *blue*, gp2.5F232W; *black*, gp2.5F232Y; *green*, gp2.5F232C; *black dashed line*, empty plasmid control. All other non-complementing point mutants are undistinguishable from the gp2.5F232C mutant and omitted for clarity. *D*, plot of the actual CPM in the control reaction. *E. coli* MRI80 cells, transformed with pET17b empty vector, were infected with T7Δ2.5 phage. The steeply declining curve results from inhibition of host replication.

To gain insight into the properties of phenylalanine that gives rise to its essential role we constructed a series of gene 2.5 proteins in which various amino acids replaced the C-terminal phenylalanine (Fig. 1*D*). As seen in Table 1, D of the six amino acid substitutions only those proteins in which tyrosine (gp2.5-F232Y) and tryptophan (gp2.5-F232W) occupied the C-terminal position could function *in vivo*. gp2.5-F232Y and gp2.5F232W retain ~90% of the wild type ability to incorporate radioactive thymidine (Fig. 2*C*). In contrast, substitutions of positively charged (arginine), negatively charged (glutamic acid), small (serine), or sulfhydryl-containing (cysteine) residues resulted in loss of function. As mentioned earlier, gp2.5-F322L was selected in an *in vivo* screen for lethal mutations (3), and was characterized biochemically (18). These results imply that the aromatic nature of the C-terminal residue is critical for gp2.5 function.

Contribution of Acidic Residues—The essential nature of the phenylalanine does not rule out an equally important role for the acidic properties of the C terminus. The interaction between gp2.5 and T7 DNA pol/trx is salt-sensitive, suggesting involvement of charged residues (8). Indeed, recent studies on the binding of gp2.5 to the T7 DNA pol/trx show that at least a part of that interaction is mediated by charged residues in the thioredoxin-binding domain of the DNA polymerase (19). The loss of function for gp2.5Δ17F shown above also suggests that charge may be important but the decrease in length of the protein might also contribute to the loss. On the other hand, some of the charge is clearly dispensable in that gp2.5Δ6F lacking four of the acidic residues is still functional, although exhibiting diminished ability to synthesize DNA *in vivo*.

To separate the contribution of charge from that of length of the acidic C terminus we have maintained the length of the C terminus while varying the acidic content. The replacement of all acidic residues with uncharged residues, *i.e.* glutamate with glutamine and aspartate with asparagines (Fig. 1*E*), resulted in a nonfunctional protein (Table 1, E). We also replaced the C-terminal 17 residues of gp2.5 with the 17 C-terminal residues of the T7 gene 4 helicase-primase. The latter protein also has an acidic C terminus with a C-terminal phenylalanine (see Ref. 33 and Fig. 1*E*). However, the C-terminal tail of gene 4 protein is not as acidic as that of gp2.5, having five less acidic residues (Fig. 1*E*). This chimeric protein retained function as judged by its ability to complement the growth of T7Δ2.5 phage (Table 1, E) and to support wild type levels of DNA synthesis (data not shown). We conclude that the acidic properties of the C terminus are essential for function but that a modest reduction in charge can be tolerated.

Effect of the C-terminal Phenylalanine and Charge on Biochemical Properties of gp2.5—In an attempt to identify the biochemical roles of the the C-terminal phenylalanine and the acidic residues in the C terminus, we overexpressed and purified a subset of the altered proteins described above. In this section we present the biochemical properties of these proteins. The gene 2.5 proteins are designated as gp2.5Δ1, gp2.5Δ1F, gp2.5Δ6, gp2.5Δ6F, gp2.5InsF231ΔF232, gp2.5F232C, gp2.5F232Y, gp2.5F232W, gp2.5F232R, gp2.5F232C gp2.5-uncharged, and gp2.5C-term-gp4.

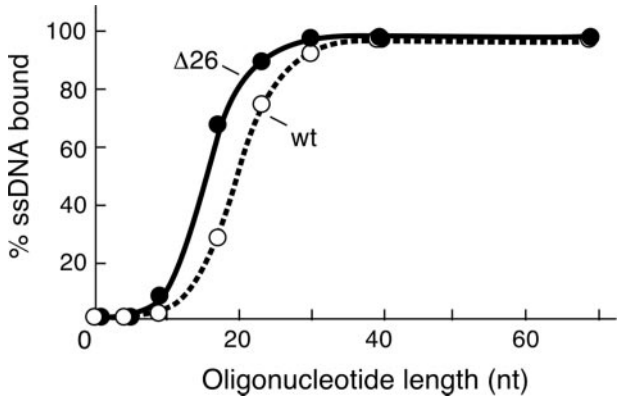

FIGURE 3. **ssDNA binding length dependence.** The ability of gp2.5 and gp2.5Δ26 to bind oligonucleotides with different length was examined by gel shift assay as described under "Experimental Procedures." Each reaction contained 10 μM protein and 0.3 nM ssDNA oligonucleotide. gp2.5-wt binding is depicted with *open circles* and *dashed line*; gp2.5Δ26 binding curve is depicted with *closed circles* and *solid line*. The experiment was performed in triplicate. The data from a representative experiment are shown.

Affinity for ssDNA—The affinity of the gp2.5 for ssDNA was examined using a gel-shift mobility assay. In earlier studies we used either a 39- or 70-nt oligonucleotide, whose lengths should contain 5 and 10 binding sites, respectively (3, 4, 18). In those studies both low and high mobility protein-DNA complexes were observed probably corresponding to heterogeneity in the number of protein molecules bound to the oligonucleotides. In order to simplify the interpretation of the data we have determined the minimal length of oligonucleotide suitable for gel-shift assays. Using oligonucleotides ranging from 4 to 70 nucleotides we examined the binding of wild-type gp2.5 using the gel-shift assay described under "Experimental Procedures" (Fig. 3). Four nucleotides were not sufficient for binding and only minimal binding occurs with oligonucleotides of 7 and 17 nucleotides as judged by the presence of smeary bands indicative of unstable binding. The shortest oligonucleotide that gave stable complex was the 23-mer. We also examined the binding of gp2.5Δ26 lacking the entire C terminus since previous studies had shown this altered protein to bind more tightly to ssDNA (4, 18). As shown in Fig. 3, the binding curve is essentially the same as that obtained with wild-type protein except that the curve is shifted left with significant binding now observed with the 17-mer. On the basis of these experiments we have used the 23-mer oligonucleotide in the gel-shift assays described here. With this oligonucleotide wild-type gp2.5 bound with K_D of 3 μM (Fig. 4). The K_D values calculated should be considered apparent K_D values rather than absolute K_D values because the oligonucleotide used in the mobility shift assay contains more than one binding site.

The C-terminal phenylalanine, essential for T7 function *in vivo*, does not appear to be involved in binding of the protein to ssDNA. Fig. 5*A* plots the reciprocal value of the K_D ($1/K_D$) so that the higher the bar, the higher the affinity of the protein for ssDNA. None of the amino acid substitutions for phenylalanine had any significant effect on the K_D for ssDNA nor did the switch of phenylalanine with its adjacent aspartic acid.

On the other hand, the negative charge of the C terminus is important in ssDNA binding. In the experiment shown on Fig. 5*B* progressive deletions were made in the C terminus resulting

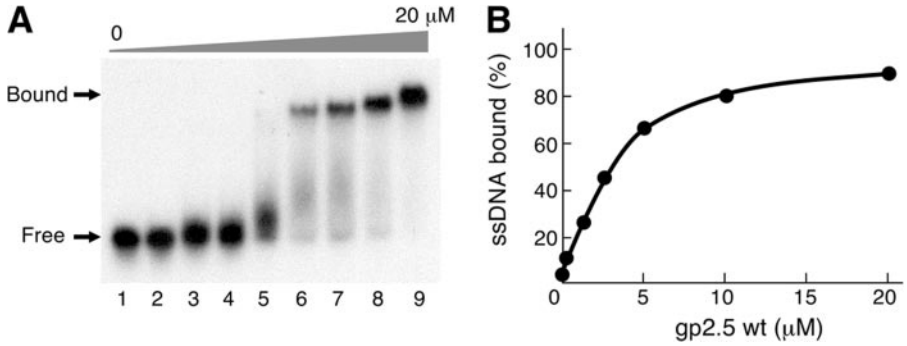

FIGURE 4. **gp2.5-wt binding to 23-mer ssDNA.** *A*, gel-shift with 23-mer ssDNA substrate. Increasing amounts of gp2.5-wt were incubated with 5′-^{32}P-labeled 23-mer as described under "Experimental Procedures," and DNA complexes were resolved on 10% Tris borate/EDTA acrylamide gel. The *arrows* point at free and bound ssDNA, respectively. *B*, K_D determination of gp2.5-wt binding to 23-mer. The amount of free and bound ssDNA was measured using a phosphorimager. Bound ssDNA was measured by counting the area of each lane between the band corresponding to the free ssDNA and the upper limit of the shifted band. The experiment was performed in triplicate. The data from a representative experiment are shown.

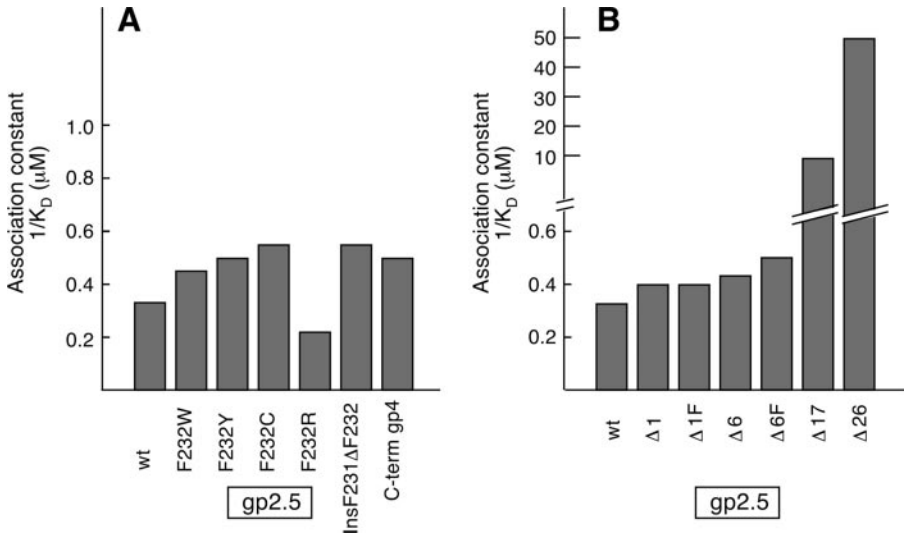

FIGURE 5. **ssDNA binding affinities of wild-type and altered gp2.5 proteins.** The K_D values for the binding of gp2.5 variants to the 23-mer oligonucleotide were determined as described in the legend of Fig. 4 for gp2.5-wt. *A*, effect of point mutations at position 232 on the ssDNA binding affinity. Association constants ($1/K_D$) are plotted to simplify discussion. *B*, progressive deletions of the gp2.5 C terminus result in increased affinity for ssDNA. The experiment was performed in triplicate. The data from a representative experiment are shown.

in a progressive loss of acidic residues. The removal of one to six residues had almost no effect on the ssDNA binding affinity (3 to 2.5 μM). The removal of 17 residues and hence 13 acidic residues results in a 30-fold increase in the affinity for the 23-mer oligonucleotide and the removal of all acidic residues (gp2.5Δ26) results in a 100-fold increase. Similar differences have been reported previously for the latter of the two altered proteins using longer oligonucleotides (4, 18). Again, the presence or absence of the C-terminal phenylalanine does not affect the increased affinity resulting from the loss of acidic residues as gp2.5Δ1F and gp5Δ6F are the same gp2.5Δ1 and gp2.5Δ6.

Both the C-terminal Phenylalanine and the Acidic Residues Are Required for gp2.5 Interactions with T7 DNA pol/trx—It has been shown previously that T7 DNA pol/trx complex physically interacts with gp2.5. The interaction between gp2.5 and T7 DNA pol/trx complex is salt-sensitive (8). Furthermore, a deletion of the acidic C terminus (gp2.5Δ26) abolishes this interaction (8). Our results shown above raise the possibility that the presence of the C-terminal phenylalanine is an important determinant for the gp2.5 interaction with T7 DNA pol/trx. This possibility is especially interesting because the acidic termini of both gp2.5 and gene 4 helicase-primase contact T7 DNA pol/trx complex within the unique segment of the gene 5 T7 DNA polymerase to which thioredoxin binds (19). The acidic C termini of both gp2.5 and gene 4 protein have a C-terminal phenylalanine. However, as pointed out above, the C terminus of gp2.5 is more acidic than that of gene 4 protein. The altered gene 2.5 proteins used in the present study allow us to evaluate the relative contributions of the C-terminal residue and the charge of the C terminus on these interactions.

We have used SPR to measure the interactions of the gp2.5 proteins with T7 DNA pol/trx. In this study wild-type gp2.5 as well as the genetically altered gp2.5 proteins were immobilized on CM5 chips as described under "Experimental Procedures." Increasing concentrations of T7 DNA pol/trx were then flowed over the gp2.5 immobilized on the chip (Fig. 6*A*). The relevant binding constants were calculated using steady-state kinetics. Fig. 6*B* shows the binding curve used to calculate the K_D of 2.4 μM for the interaction of wild-type gp2.5 with T7 DNA pol/trx. The observed K_D is consistent with previously reported K_D for this interaction as determined by SPR experiments (3, 4, 18) or fluorescence anisotropy (8). A higher affinity (K_D of 0.13 μM) was measured for the interaction of wild-type gp2.5 with a T7 DNA pol/trx complex that was reconstituted from individually purified gene 5 protein and thioredoxin (19).

The presence of an aromatic residue at the C terminus of gp2.5 is essential for its interaction with T7 DNA pol/trx. The apparent association constants ($1/K_D$) of selected altered proteins are presented on Fig. 7. The removal of the phenylalanine (gp2.5Δ1) results in a greater than 10-fold reduction in the apparent K_D (34 μM). Again it is the absence of phenylalanine at the C terminus that is responsible and not the shortening of the fragment by one residue; gp2.5Δ1F in which the penultimate residue is deleted binds almost equally as well as does wild-type gp2.5. The phenylalanine must occupy the C-terminal position as illustrated by the switch of the two terminal residues (gp2.5InsF231ΔF232). Replacement of the phenylalanine with either tyrosine (gp2.5F232Y) or tryptophan (gp2.5F232W) yielded proteins that bound as well to T7 DNA pol/trx as did

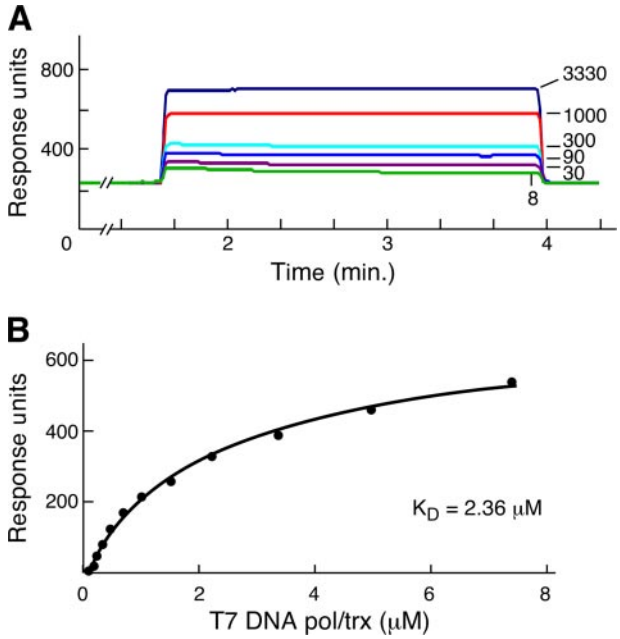

FIGURE 6. **Binding of T7 DNA pol/trx to gp2.5-wt.** 150 RU of gp2.5-wt were immobilized on a CM-5 chip, and increasing concentrations of T7 DNA pol/trx were flowed over the surface of the chip. *A*, sensograms of the binding of T7 DNA pol/trx (8–3300 nM) to gp2.5-wt. Only six of sixteen concentrations tested are shown for clarity. *B*, K_D determination of the binding of T7 DNA pol/trx to gp2.5-wt. 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 2.36 μM was calculated using steady-state fit model provided by BIAEVAL 3.0.2 software (Biacore). It should be noted that the calculated K_D is apparent rather than absolute since the immobilization of gp2.5 on the chip is random and some portion of the molecules could be in a conformation that does not support interaction with T7 DNA pol/trx. The experiment was performed in duplicate. The data from a representative experiment are shown.

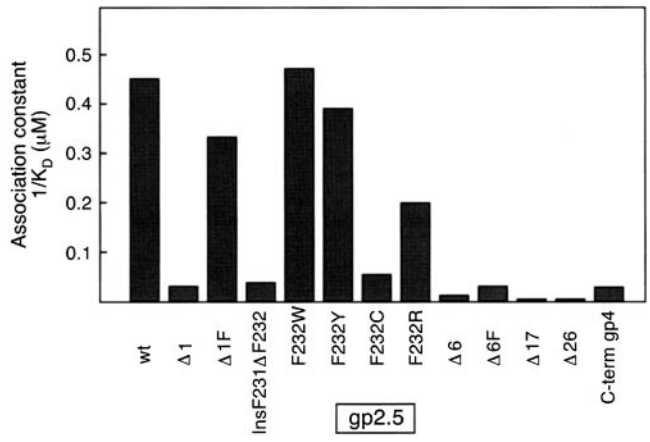

FIGURE 7. **Charge and aromatic nature of the C-terminal residue are critical for gp2.5-T7 DNA pol/trx interaction.** The K_D values for the binding of gp2.5 variants to T7 DNA pol/trx were determined as described in the legend of Fig. 6 for gp2.5-wt. Association constants ($1/K_D$) are plotted to simplify discussion. It should be noted that the calculated association constants are apparent rather than absolute constants, because the immobilization of the gp2.5 proteins on the chip is random and some portion of them could be in a conformation which does not support interaction with T7 DNA pol/trx. The experiment was performed in duplicate. The data from a representative experiment are shown.

wild-type gp2.5. Cysteine cannot substitute for phenylalanine although gp2.5 F232R bound surprisingly well in view of the inability of this mutant to complement for the growth of T7Δ2.5 phage.

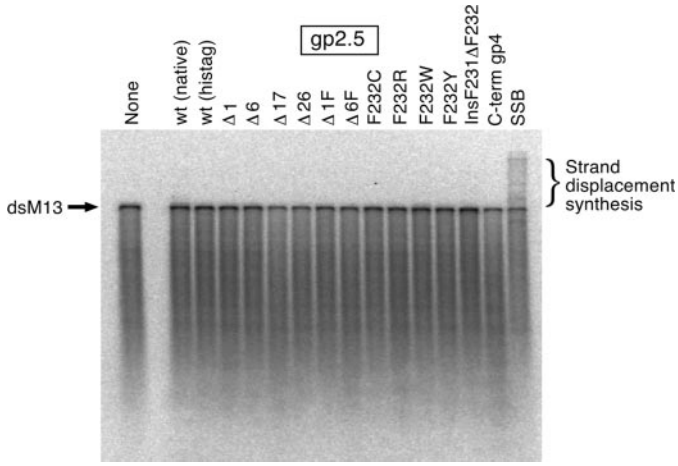

FIGURE 8. **Effect of gp2.5 variants on DNA synthesis catalyzed by T7 DNA pol/trx.** A template consisting of M13 ssDNA circle annealed to radioactively end-labeled 24-mer was used to evaluate the ability of gp2.5 variants to support strand displacement. The reaction contained 4 nM template, 100 nM T7 DNA pol/trx, 4 μM gp2.5, or *E. coli* SSB protein and proceeded at 37 °C for 20 min. The *arrow* depicts the band corresponding to full-length M13 dsDNA. The strand displacement products in the positive control with *E. coli* SSB protein are marked with *vertical bracket*. The counts, corresponding to the full-length M13 dsDNA, were used to compare the effect of gp2.5 variants on gp5 polymerization activity.

The presence of a negative charge in the C terminus is also important for binding of gp2.5 to T7 DNA pol/trx. Thus gp2.5Δ6F lacking 4 acidic residues but retaining the C-terminal phenylalanine binds ~10-fold less well as is the case for the chimeric gp2.5 bearing the C terminus of gene 4 protein (gp2.5-C-term-gp4). This latter protein has five less acidic residues in a C terminus of identical length and with a C-terminal phenylalanine. As expected gp2.5Δ26 displayed no detectable interaction with T7 DNA pol/trx.

Strand Displacement Activity—T7 DNA pol/trx and gene 4 helicase-primase are able to catalyze a strand displacement DNA synthesis on duplex DNA. Interestingly, *E. coli* SSB protein enables the T7 DNA pol/trx to catalyze strand displacement activity in the absence of helicase, whereas wild-type gp2.5 does not. However, we recently described a genetically altered gp2.5, gp2.5F232L, that could also enable T7 DNA pol/trx to catalyze strand displacement synthesis (18). This altered gp2.5 in which the C-terminal phenylalanine had been replaced by leucine came from a screen for essential amino acid residues in gp2.5. In view of this finding we have examined the ability of all altered gp2.5 proteins generated in this work to support strand displacement DNA synthesis by T7 DNA pol/trx. In the assays shown in Fig. 8 equal amounts of wild type and mutant gp2.5 variants were incubated at 37 °C for 20 min with a template consisting of a 5′ radioactively labeled 24-mer annealed to M13 ssDNA. Whereas it is clear that in the positive control reaction (Fig. 8, *lane 16*) with *E. coli* SSB protein strand displacement DNA synthesis is stimulated as judged by the appearance of high molecular weight products (marked with *vertical bracket*), none of the gp2.5 variants enables T7 DNA pol/trx to catalyze strand displacement DNA synthesis.

DISCUSSION

The C-terminal region of gp2.5 has been previously identified (8, 19) as a mediator for its interactions with the T7 DNA

pol/trx complex. In the current study we have carried out extensive genetic and biochemical analyses to identify critical determinants for the function of the C terminus of gp2.5. We find that both the negative charge of the C terminus and the aromatic nature of the C-terminal residue are critical for T7 growth *in vivo* and for the interaction of gp2.5 with T7 DNA pol/trx *in vitro*. Of these two critical determinants the most surprising and perhaps the most important is the requirement for an aromatic C-terminal residue. Only the substitution of tryptophan or tyrosine for the C-terminal phenylalanine preserves the function of gp2.5 *in vivo*, a finding that correlates with the ability of these aromatic residues to also preserve the interaction of gp2.5 with T7 DNA pol/trx. Furthermore, the position of the aromatic residue as the C-terminal amino acid is also critical. Positioning the phenylalanine penultimate to the C-terminal residue is not sufficient for the protein to function *in vivo* or to interact with T7 DNA pol/trx *in vitro*. Although the negative charge of the C-terminal tail of gp2.5 is clearly important, a moderate reduction in charge is tolerated as long as an aromatic residue is present as the C-terminal residue. Decreasing the charge of the C-terminal tail affects DNA binding as well, whereas the C-terminal phenylalanine is only critical for the interaction of gp2.5 with T7 DNA pol/trx.

Similarities of T7 gp2.5 with Other Prokaryotic ssDNA-binding Proteins—Acidic C termini and OB folds are features shared by all prokaryotic ssDNA-binding proteins thus far characterized. However, the importance of the C-terminal residue has not previously been observed. We have carried out a sequence homology search with each of the three well characterized prokaryotic ssDNA-binding proteins, *E. coli* SSB protein, T4 gene 32 protein (gp32), and T7 gp2.5. These three proteins, although having structural similarities, share minimal sequence homology. The C-terminal phenylalanine is well conserved among gp2.5 homologs as well as *E. coli* SSB protein homologs. In contrast, all gp32 homologs have leucine or isoleucine at the C-terminal position. A striking feature of the latter group is the presence of block(s) of serine within the C-terminal acidic tail (7 residues for gp32). All gp2.5 homologs have aspartate in the penultimate position, whereas all *E. coli* SSB protein homologs have proline. Interestingly, the replacement of the proline with serine results in a temperature sensitive phenotype (24). In the accompanying paper (33), we show that the T7 gene 4 helicase-primase also has a functionally important C-terminal phenylalanine. Consequently, we carried out a similar sequence analysis for all T7 gene 4 protein homologs. All T7-like phages encode a helicase-primase fusion protein with an acidic C terminus having phenylalanine as the C-terminal residue and aspartate in the penultimate position. In contrast, T4-like phages and *E. coli* encode separate helicase and primase proteins, neither of which possess acidic C termini. Thus, T7 whose replisome has only four proteins may have evolved a unique strategy for protein-protein interactions within the functional replisome.

Chimeric protein, in which the C terminus of gp2.5 was replaced with the C terminus of *E. coli* SSB protein was found to be functional. This result is not surprising because the C terminus of *E. coli* SSB protein has a similar length, charge distribution and a C-terminal phenylalanine. Chimeric protein, in which the C terminus of gp2.5 was replaced with the C terminus of phage T4 gp32 is partially functional, although its burst size is reduced ~10-fold (24). This finding is puzzling since gp32 has a leucine and not a phenylalanine in the C-terminal position. A gp2.5 mutant in which the C-terminal phenylalanine was replaced by leucine was selected previously in a screen for gp2.5 lethal mutations (3). The experiments with chimeric proteins suggest that although the C terminus of gp2.5 is indispensable for its function it does not confer specificity of the protein-protein interactions within the phage T7 replisome.

Modulation of the ssDNA Binding Properties of gp2.5 by the C Terminus— gp2.5 binds ssDNA with micromolar affinity in a nonspecific manner. Similar to other ssDNA-binding proteins, it is thought that ssDNA binds within the cleft of the oligonucleotide/oligosaccharide binding fold (OB-fold) via contacts with positively charged and aromatic residues (10). Proteolytic fragments of gp32 of phage T4 and *E. coli* SSB protein, lacking the C-terminal portions of these proteins were reported to bind ssDNA with higher affinity than the full-length protein (12–14,25). A similar phenomenon has been described for HMG (High Mobility Group) proteins found in chromatin (26). HMG proteins bind dsDNA nonspecifically and are abundant and well conserved among eukaryotes. They facilitate the assembly of nucleoprotein complexes within the chromatin. Interestingly, HMG proteins have long negatively charged C termini, that contact the DNA binding region of the proteins. Similarly to prokaryotic ssDNA-binding proteins, the removal of the acidic C terminus results in higher affinity for ssDNA. In contrast, the phosphorylation of the C terminus, which increases the negative charge, results in lower affinity for DNA. It appears that the modulation of the DNA binding activity by a flexible acidic tail is not unique to prokaryotic ssDNA-binding proteins but rather associated with proteins exhibiting nonspecific DNA binding. The C-terminal phenylalanine does not play a role in the modulation of the ssDNA binding properties of gp2.5.

Significance of Unstructured C Terminus and an Aromatic C-terminal Residue in Protein-Protein Interactions—Not only do both gp2.5 and gp4 helicase-primase have acidic C termini and a C-terminal phenylalanine but their C termini are also thought to be unstructured. The C terminus of gp4 is disordered as seen by x-ray (27, 28) and the presence of the C terminus of gp2.5 prevented crystallization (10), suggesting that these regions are highly flexible. NMR experiments have revealed that peptides corresponding to the acidic C terminus of gp2.5 and gp4 helicase-primase are unstructured in solution.[5] Recently, it was reported that the C terminus of *E. coli* SSB protein is unstructured even when the protein is in complex with DNA (29).

Unstructured flexible regions are not unique for ssDNA-binding proteins. It is generally thought that regions which are involved in protein-protein interactions with multiple partners are flexible and unstructured in order to accommodate different requirements of the interacting partner. For example, histones have unstructured N termini and C termini that are known to interact with multiple proteins. Similarly, when the C

[5] B. Marintcheva, A. Marintchev, G. Wagner, and C. Richardson, unpublished data.

terminus of phage T4 gp32, which is also unstructured, was immobilized on an affinity column multiple members of replication and recombinational pathways, were found to bind the column (12).

An interesting example of a protein with flexible acidic C terminus ending with a C-terminal phenylalanine is Stradα. Stradα is a member of the MO25α/Stradα/LKB1 signal transduction pathway, mutations within which result in Peutz-Jeghers syndrome (30). Stradα has a short acidic C terminus ending with a WEF (tryptophan-glutamate-phenylalanine) motif. Stradα alone has low affinity for LKB1, a protein kinase. However the complex Stradα/MO25α, where MO25α is a scaffolding protein, binds LKB1 with higher affinity and stimulates its kinase activity up to 50-fold, triggering downstream signaling (31). Stradα/MO25α interaction is mediated by the C terminus of Stradα. The peptide corresponding to the C terminus of Stradα is unstructured in solution and the C-terminal aromatic residues are indispensable for binding to MO25α (30). From the above examples it is evident that the aromatic residue(s) within flexible unstructured regions offer multiple possibilities for protein-protein interactions and their modulation.

Possible Mechanistic Role of the C Terminus of gp2.5—Whereas the mechanistic details about the role of the flexible acidic C terminus and the C-terminal phenylalanine of gp2.5 protein remain to be understood, it is clear that gp2.5 plays a central role at the T7 replication fork. It has been hypothesized previously, that in absence of DNA the C terminus of gp2.5 occupies the DNA binding cleft of the protein (10). Because such interaction is likely to be weak, the C-terminal tail may "oscillate" between bound and free position, being easily displaced when ssDNA is present. According to this model, when the C terminus is shortened its interactions with the DNA-binding cleft is weakened. Correspondingly, less energy is required for the C terminus to be displaced from the cleft by ssDNA, thus explaining the increased binding affinity for ssDNA observed with the C-terminal deletion mutants of gp2.5. A similar model has been proposed previously to explain the ssDNA-binding properties of phage T4 gp32 and its proteolytic fragments (reviewed in Ref. 6).

One can visualize the C terminus as a mimic of ssDNA. The high acidic content resembles the negatively charged phosphate backbone of DNA, whereas the C-terminal phenylalanine and the tryptophan in position 216 resemble the aromatic moiety of the bases. In the absence of ssDNA the negatively charged C terminus binds in the DNA binding cleft. In addition to a role of the C terminus of gp2.5 in binding to T7 DNA pol/trx, its binding to the positively charged DNA binding cleft could be a simple mechanism to protect the binding site from random interaction with negatively charged surfaces of other molecules in the cell and thus ensure fast and efficient coverage of arising ssDNA. The C terminus is a good candidate for a molecular switch, coordinating the ssDNA binding of gp2.5 and its involvement in protein-protein interactions. One possible scenario predicts that after the DNA binding cleft is occupied, the C terminus is displaced and becomes readily available for contacts with other proteins. If the C terminus-mediated interaction results in stable complex this complex can serve as a platform for recruitment of multiprotein complexes such as the replisome, the recombination machinery or DNA repair machinery. ssDNA-binding proteins, including the T7 gp2.5, have been shown to play role in all of the above processes involving DNA transactions. Such a mechanism employing a single surface as a target of multiple interacting partners has been proposed for hRPA32, a subunit of the human SSB protein. Proteins involved in 3 different repair pathways bind the same surface of hRPA32 and promote the recruitment and the assembly of the repair complex relevant to the type of the DNA damage (32).

It is striking that all T7-like phages encode a helicase-primase fusion protein and ssDNA-binding protein, both of which have an acidic C terminus and a C-terminal phenylalanine. In addition, the C termini of gp2.5 and gp4 contact T7 DNA polymerase at the same surface, the thioredoxin binding domain (19). Our working model postulates that the negatively charged C termini make initial contact with gp5 via electrostatic interactions with the positively charged residues within the trx binding loop. Most likely, the role of the phenylalanine is to "fine-tune" or "validate" the electrostatic interaction. Obviously, neither the acidic charge nor the phenylalanine could ensure the specificity of the T7 DNA pol/trx interaction with gp2.5 and gp4 helicase-primase. It is tempting to speculate that the acidic flexible tail in combination with the C-terminal aromatic residue, leads to a weak initial binding to T7 DNA pol/trx complex. The length of the negatively charged region and the net weight of the charge may then guide the polymerase toward a conformational change facilitating second site interaction, outside the boundaries of the acidic C terminus. A two-site interaction is supported by the fact that the overall affinity of T7 DNA pol/trx interactions with gp2.5 and gp4 helicase-primase is different, despite the similarities in their acidic C termini. In addition, while the acidic C terminus of gp2.5 is essential for the interaction with T7 DNA pol/trx in both the presence and absence of DNA, the acidic C terminus of gp4 helicase-primase is essential only in the absence of DNA and dispensable in the presence of a primer/template (19). The parallel between the mode of gp2.5 and gp4 helicase-primase interactions with T7 DNA pol/trx complex is intriguing and points to a well regulated network of dynamic protein-protein interactions within the phage T7 replisome.

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