Mutational Analysis of the T4 Gp59 Helicase Loader Reveals Its Sites for Interaction with Helicase, Single-stranded Binding Protein, and DNA*

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Efficient DNA replication involves coordinated interactions among DNA polymerase, multiple factors, and the DNA. From bacteriophage T4 to eukaryotes, these factors include a helicase to unwind the DNA ahead of the replication fork, a single-stranded binding protein (SSB) to bind to the ssDNA on the lagging strand, and a helicase loader that associates with the fork, helicase, and SSB. The previously reported structure of the helicase loader in the T4 system, gene product (gp)59, has revealed an N-terminal domain, which shares structural homology with the high mobility group (HMG) proteins from eukaryotic organisms. Modeling of this structure with fork DNA has suggested that the HMG-like domain could bind to the duplex DNA ahead of the fork, whereas the C-terminal portion of gp59 would provide the docking sites for helicase (T4 gp41), SSB (T4 gp32), and the ssDNA fork arms. To test this model, we have used random and targeted mutagenesis to generate mutations throughout gp59. We have assayed the ability of the mutant proteins to bind to fork, primed fork, and ssDNAs, to interact with SSB, to stimulate helicase activity, and to function in leading and lagging strand DNA synthesis. Our results provide strong biochemical support for the role of the N-terminal gp59 HMG motif in fork binding and the interaction of the C-terminal portion of gp59 with helicase and SSB. Our results also suggest that processive replication may involve the switching of gp59 between interactions with helicase and the single-stranded binding protein.

The process of DNA replication requires cooperation and temporal regulation among multiple proteins. Bacteriophage T4 has provided a powerful model system for the study of this process. Coupled leading and lagging DNA synthesis can be reconstituted in vitro using a handful of proteins, allowing detailed biochemical analyses (reviewed in Refs. 1–3). In addition, the T4 replication proteins have functional homologues in many other organisms. Thus, mechanistic insights into their biochemistry combined with recent structural analyses provide a conceptual framework that can be applied across the kingdoms of life (4, 5).

The T4 replisome is composed of DNA polymerase, the gene product (gp)5 of gene 43, the clamp (gp45) that helps to couple the polymerase with the DNA template, and the replicative helicase (gp41). Both the clamp and the helicase are loaded onto the DNA substrate by specialized proteins. The accessory proteins (gp44/gp62) load the clamp. Gp59 loads the helicase by targeting fork structures that are formed when a single-stranded primer is annealed to the double-stranded DNA. These D- and R-loop forks are also targeted by the single-stranded binding protein (SSB, T4 gp32), which cooperatively binds to the single-stranded DNA displaced by the primer.

Initiation of the discontinuous, lagging strand synthesis requires both the helicase and a primase (gp61), which together synthesize the pentamer RNA primers. After the lagging strand DNA is synthesized from these primers, the RNA is excised by repair enzymes, and the adjacent fragments are joined by T4 DNA

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§ This article contains supplemental Fig. S1.
ligase. *In vitro* replication also requires the T4 type II topoisomerase (gp39, gp52, and gp60) when the template is a covalently closed circle.

Even though the gp59 helicase loader is small (26 kDa), it engages several partners. Previous work has shown that it interacts with SSB (6–9), helicase (7, 10), DNA polymerase (11), and primase (11). Gp59 also binds to single-stranded (ssDNA) and double-stranded DNAs, but its greatest affinity is for fork DNA that simulates a replication fork (12–14).

Polymerase can catalyze some leading strand synthesis in the absence of helicase. However, processive replication requires the action of the helicase to unwind the double-stranded DNA ahead of the fork. Furthermore, the presence of SSB inhibits helicase activity (15), and gp59 is required to overcome this inhibition (9, 16, 17). Thus, the interaction of gp59 with helicase, which stimulates helicase-mediated unwinding of DNA (9) and primer synthesis by the primase/helicase (18), is needed for efficient replication (9, 16). In addition, when gp59 is bound to the fork, it prevents the synthesis by DNA polymerase in the absence of helicase. Thus, gp59 has been termed a “gatekeeper,” as it coordinates coupled leading and lagging synthesis.

Although gp59 binds ssDNA, it is unlike other “typical” SSB proteins, such as gp32 SSB. SSB proteins contain a cleft that is responsible for binding ssDNA (19). The primarily α helical gp59 lacks this characteristic large peptide cleft. Furthermore, the protein sequence of gp59 has little homology to helicase loading proteins from distantly related organisms, like *Escherichia coli* DnaC (12). However, the gp59 crystal structure reveals a domain, which shares structural homology with the high mobility group (HMG) proteins from eukaryotic organisms (12) (*black region* in Fig. 1A). This HMG-like domain contains three α-helixes, denoted as H1, H2, and H3 (Fig. 1A), and is well conserved among the helicase loaders of various T4-like viruses (Fig. 1B).

Among eukaryotic HMG proteins, similar “HMG boxes” mediate association with the minor groove of duplex DNA, bending the DNA, and partially unwinding the two strands (14, 20, 21). Because of the structural similarity of the N-terminal portion of gp59 to an HMG box, it has been suggested that the HMG-like region contributes to gp59 associations with DNA (14).

A model for gp59 action that incorporates its various biochemical functions was proposed based on the structure of the protein (14). In this model, the HMG-like portion binds to the duplex DNA ahead of the fork, whereas the C-terminal portion provides the docking sites for helicase, SSB, and the ssDNA fork arms. The tight interaction of gp59 with the replication fork then allows it to coordinate DNA synthesis through its sequential interactions with helicase/primase, SSB, and polymerase.

Here we have generated a panel of gp59 mutants and investigated how the mutations affect the various functions of gp59 and the interaction of the protein with different binding partners. Our results provide strong biochemical evidence that the N-terminal gp59 HMG motif is involved in both fork binding and associations with ssDNA, and suggest that the ssDNA arms emanating from the duplex fork follow a different trajectory across gp59 than previously modeled. In addition, we find that the C-terminal portion of gp59 interacts both with helicase and with SSB. Small angle x-ray scattering analysis of a gp59/SSB heterodimer agrees with this gp59/SSB contact (36). Finally, our results also suggest that processive replication may involve the switching of gp59 between interactions with the helicase and SSB.

**EXPERIMENTAL PROCEDURES**

*T4 Replication Proteins—Purification procedures for T4 DNA polymerase, gp44/gp62 clamp loader, gp45 clamp, gp41 helicase, gp61 primase (22), and gp32 (23) have been previously described.*

*Mutagenesis of T4 59 Helicase Loading Protein—Mutations in T4 gene 59 were made by site-directed mutagenesis of wild type (WT) gene 59 in the plasmid pNN2859 (24), using the method previously described by Kunkel et al. (25), modified by using T4 DNA polymerase, T4 gp44/gp62 clamp loader, and gp45 clamp to copy the ssDNA template. Primer sequences are available upon request.*

A combinatorial approach was also used to produce 59 mutations K27E (residue 159 Ala → Gly), F132S (residue 475 Thr → Cys), I147T (residue 520 Thr → Cys), and I170T (residue 589 Thr → Cys). The 59 plasmid pNN2859 was mutagenized *in vitro* using *Taq* polymerase (New England Biolabs) and low-fidelity PCR conditions: 95 °C for 2 min; 20 cycles of 95 °C for 30 s; 58 °C for 30 s; 72 °C for 2 min; 72 °C for 7 min. Plasmids harboring these mutations were used to transform SURE cells (Stratagene), and the resultant cell lines were used as plating hosts. All mutations were verified by DNA sequencing (CBR-DNA Sequencing Facility, UMBI Center for Biosystems Research, University of Maryland, College Park, MD).

*Purification of Gp59 Proteins— Cultures (5 ml) of E. coli BL21(DE3)/pLysE Gold (Stratagene) containing plasmids encoding WT or gp59 mutants K27E, F132S, I147T, or I170T or E. coli BL21(DE3)/pLysS Gold containing plasmids with gp59 mutants Q28A, K38A/Y39A, D47G, Y122A, Y138A/N139A, I156A, D179A, and Y193A were grown overnight at 37 °C in LB broth with 50 μg/ml of carbenicillin and 30 μg/ml of chloramphenicol. Cultures were diluted 1:100 into 200 ml of the same medium without chloramphenicol and grown to A*600* = 0.5. Isopropyl β-D-thiogalactopyranoside was added to a final concentration of 1 μM. After 2 h at 37 °C, the cells were harvested by centrifugation and stored at −80 °C. The gp59 mutant W183A was isolated from E. coli ArcticExpress (DE3) (Stratagene) cells containing the plasmid with the W183A mutation according to the manufacturer’s protocol and stored at −80 °C.*

Frozen cells were suspended in 10 ml of sonication buffer (50 mM Tris-Cl, pH 7.5, 0.2 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride (Pierce), and 1 complete 228 protease inhibitor tablet/50 ml (Roche Applied Science), broken by sonication, and centrifuged for 45 min at 100,000 × g. The supernatant was diluted with 2 volumes of PC buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, and 0.5 mM Tris(2-carboxyethyl)phosphine hydrochloride), and 1.5 ml of phosphocellulose resin, prepared as previously described (24), was then added. The suspension
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was stirred slowly at 4 °C for 1 h and centrifuged for 10 min at 8,000 × g. The supernatant was discarded and the pellet was then washed with 5 ml of PC buffer, containing 50 mM KCl, stirred for 15 min, and centrifuged at 6,000 × g for 10 min. This wash was repeated two more times. The pellet was suspended in 10 ml of PC buffer containing 50 mM KCl and transferred into a column. A step gradient of PC buffer (0.6 ml), containing 0.3, 0.5, and 1 M KCl was applied to the column. Fractions containing each of the mutant proteins or WT were collected between 0.5 and 1 M KCl; proteins appeared to be >95% pure by SDS-gel electrophoresis. Proteins were dialyzed against 50 mM Tris-HCl, pH 7.5, 0.1 M KCl, 10% glycerol, 1 mM EDTA. Incubation of the purified proteins with 5-32P-end-labeled ssDNA for 60 min at 37 °C showed them to be free of nuclease activity. Protein concentrations were determined by A280 and densitometry using purified KVP40 gp59 of a known concentration as a standard reference.

DNA Substrates—Sequences of the oligodeoxyribonucleotides and schematics of the fork, primed fork, and nicked circle DNAs are given in Table 1 and Fig. 2, respectively.

Fork DNA AB or CD consists of 12 complementary and 12 noncomplementary bases or 26 complementary and 30 noncomplementary bases, respectively. To prepare the fork DNAs, radiolabeled oligonucleotide A or C was mixed with unlabeled oligonucleotide B or D, respectively, at a ratio of 1:1.4 in annealing buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 0.2 M NaCl). The solution was heated at 95 °C for 3 min, 68 °C for 60 min, and then slowly cooled to 24 °C in about 3 h. Unincorporated [32P]ATP was removed from the DNA by filtration through mini-spin columns (Probe Quant 50, GE Life Science). Proper annealing of substrates was checked by gel electrophoresis.

To prepare the primed fork, the 56-base oligonucleotide C was radiolabeled and annealed with the 60-base and 25-base oligonucleotides D and E, respectively, as described above to form a DNA fork with a 25-base primer on the leading strand. The ratio of oligonucleotides in the annealing reaction was: 1:1.5:10 (C:D:E). Primed fork 32P-DNA was purified by electrophoresis in 1 × TBE at 4 °C through 16% polyacrylamide mini-gels (Invitrogen). DNA band(s) were excised from the gel, and the DNA was extracted from the polyacrylamide gel by electrolution. Eluted DNA was concentrated and washed with TE buffer in an Amicon Microcon centrifugal concentration device (3,000 M, cut-off).

pBSGless DNA, 456-bp circular replication substrate without G residues on the leading strand and no C residues on the lagging strand, has been described (26). Oligonucleotides (Sigma Genosys) were 5’-32P-end labeled using T4 polynucleotide kinase (Optikinase (U. S. Biochemical Corp.)) and [γ-32P]ATP.

Gel Mobility Shift Assays—Gel mobility shift assays were performed as previously described (12). Reaction mixtures contained 3 mM 5’-32P-end-labeled DNA substrate, 25 mM Tris acetate (pH 7.5), 60 mM potassium acetate, 6 mM magnesium acetate, 10 mM dithiothreitol, 100 μg/ml of BSA, and 2 mM ATP in a total volume of 5 μl. Protein concentrations are given with each figure. Detection of gp59 with an antibody was performed as described (12). All reactions were carried out at 30 °C, and unless otherwise noted, were incubated for 5 min. Loading buffer (2 μl of 15% glycerol with bromphenol blue) was added subsequent to incubation. Samples were electrophoresed on 6% native gels (NOVEX) at 12.5 V/cm in an X-cell electrophoresis device running in 0.5 × Tris borate/EDTA buffer at 4 °C. Gels were vacuum-dried on Whatman 3MM paper (Whatman) and autoradiographed on Bio-Max MR film (Eastman Kodak) or scanned on a Fuji FLA-3000 Image Analyzer. Quantitation of the scanned images was done using Multigague software, version 3.0, from Fuji Medical Systems.

Helicase Unwinding Assays—Helicase unwinding assays were carried out in a 40-μl reaction volume under the same conditions as described above. After a 1-min equilibration to 30 °C, SSB (when indicated) was added followed by a 5-min incubation. Gp59 was then added, followed by helicase to begin the reaction. Aliquots (5 μl) were removed at the indicated times, and reactions were quenched with the addition of 5 μl of 2× Proteinase K buffer (0.5 units of proteinase K (Roche Applied Science) in 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, and 1% SDS) for at least 5 min at 30 °C, followed by 5 μl of loading buffer (15% glycerol containing bromphenol blue). DNA products were separated on 8% Tris borate/EDTA gels (Invitrogen), which were electrophoresed at 18.75 V/cm in an X-cell electrophoresis device at room temperature in 1× Tris borate/EDTA buffer. Gels were vacuum-dried and imaged under the same conditions as with the gel mobility shift assays.

DNA Replication Assays—Replication reactions using pBSGless (26, 27) nicked template were carried out as previously described (23).

RESULTS

Experimental Rationale—The gp59 helicase loader of bacteriophage T4 is a multifunctional protein. It binds to single-stranded DNA, fork DNA, primed fork DNA, and duplex forks with a single-stranded gap of more than 5 nucleotides; it interacts with helicase and SSB protein in the presence and absence of DNA; it loads helicase onto the replication fork; and it stimulates both the unwinding and ATPase activities of helicase (6, 7, 9, 12, 13, 16, 17).

To determine the surfaces of gp59 that are needed for these disparate functions, we constructed a panel of gp59 mutants and determined the activities of the mutants using different DNA substrates (Fig. 1) and different assays. Fork DNAs and primed fork DNA (Table 1, Fig. 2) were used to mimic DNA substrates present at the replication fork, whereas ssDNA (Table 1) was used to mimic the lagging strand. The nicked circle DNA (Fig. 1) served to monitor replication reactions on both the leading and lagging strand.

The binding site sizes for gp59 and SSB are ~10 bp and 6–7 bp, respectively (28, 29); however, longer DNAs are needed for optimal binding (12, 23). Thus, stoichiometric levels of protein relative to the number of DNA binding sites do not result in complete binding of the DNA. The concentrations of gp59, helicase, and SSB relative to the DNA were based on previous studies (12, 23, 30) that indicated that these ratios of protein to DNA are needed to ensure complete binding.

All of the proteins either exhibited some activity in one or more of the various in vitro assays described below or were active in vivo (supplemental Fig. S1). Thus, it seems unlikely that the mutant proteins were grossly misfolded.
A shows the positions of mutated residues as spheres. Mutations that affected function in our assays are color coded as described below.

The HMG-like Motif of Gp59 Interacts with Fork DNA—Gp59 has a high affinity for fork DNAs, forming stable binding products that can be monitored by electrophoresis through native polyacrylamide gels (12). Given the role of HMG boxes in DNA binding, it was postulated that the HMG-like region of gp59 mediates its associations with DNA (14). To test this idea, we compared the binding of WT gp59 and various mutants using such a substrate that contains either two 30-nt single-stranded arms (Fig. 3A) or shorter (12 nt) arms (Fig. 3B, the reactions that lacked SSB).

Most of the gp59 mutants were not impaired in these assays; the positions of these mutations are indicated as spheres, which are not colored blue (Fig. 1A). However, four mutant proteins, which were within or near the HMG motif, were defective. The K27E mutant failed to bind either fork DNA (Fig. 3A and B); the reactions that lacked SSB). As WT gp59 was titrated into binding reactions, most of the substrate accumulated in a single, stable product band (12) (Fig. 3A). Higher molecular weight products also formed, but only after most of the substrate was bound by gp59. Most of the gp59 mutants were not impaired in these assays; the positions of these mutations are indicated as spheres, which are not colored blue (Fig. 1A). However, four mutant proteins, which were within or near the HMG motif, were defective. The K27E mutant failed to bind either fork DNA (Fig. 3A and B); the
Y193A and K38A/Y39A mutants were significantly impaired in binding to either substrate (Fig. 3, A and B); and the D47G mutant protein was impaired when using the 12-nt arm substrate (Fig. 3B).

Lys-27, Asp-47, and Lys-38/Tyr-39 are surface exposed residues within the HMG-like domain (Fig. 1A). The impairment of fork binding by mutations at these residues provides strong biochemical evidence that the HMG-like motif of gp59 constitutes a DNA binding domain needed for a stable interaction with fork DNA.

In addition, two other mutations affected binding, Y193A (Fig. 3, A and B) and the previously identified I87A (30). Both of
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these mutations are near the HMG-like domain. Ile-87 is surface exposed, whereas Tyr-193 is partially buried. We speculate that the DNA binding motif may extend to Ile-87 or that both I87A and Y193A impair binding by affecting local conformation.

The HMG-like Domain and Region Near the C Terminus of Gp59 Is Involved in Binding to ssDNA—In addition to fork DNA, gp59 also binds ssDNA, displaying a comparatively high affinity for ssDNA of 56 nt or greater length (12). It is thought that this binding is needed to “hold” the ssDNA arms at the replication fork. However, it has been unclear whether the interactions of gp59 with ssDNA are similar to its interactions with DNA forks.

To investigate which portion(s) of gp59 are involved in ssDNA binding, we assayed the binding of WT gp59 and the mutants to a 56-nt ssDNA substrate (Oligonucleotide C, Table 1). WT gp59 formed a single product with this ssDNA substrate. Again the K27E and K38A/Y39A mutants, which were defective for fork DNA binding, did not bind the ssDNA (Fig. 3C). These results suggest that the HMG-like domain binds to the ssDNA present at the fork.

Binding to ssDNA was also impaired by a V207G mutation, located near the C terminus of gp59 (Fig. 3C; labeled as a blue sphere in Fig. 1A). However, this mutation did not significantly affect fork DNA binding (Fig. 3, A and B). The position of this mutation, which is distant from the HMG-like domain, suggests that the region near Val-207 may be involved in binding to a ssDNA arm that extends from the fork, which is bound by the HMG-like motif.

Mutations at several residues resulted in a complex with the ssDNA that migrated faster in the native acrylamide gel than the WT gp59-ssDNA complex: Tyr-138/Asn-139, Phe-132, Trp-183, Asp-47, and Gln-28, which are located along one side of the protein; Val-207, which is located on the opposite face; and Asp-179 and Y193A, which are buried within the interior of the protein.

It is unclear what this faster-migrating complex represents. However, previous work using electron microscopy and hydroxy radical footprinting has indicated that gp59 is able to oligomerize (27, 31). If this is the case here, the faster migrating species could arise from mutations that affect the ability of gp59 to oligomerize along the ssDNA.

Mutations at Gp59 Residues Phe-132, Tyr-138/Asn-139, and Tyr-216/Lys-217 Define a Face That Interacts with SSB on Fork DNA—Previous work has shown that once bound to fork DNA, gp59 can form ternary complexes with SSB (23, 30). These results suggest that Phe-132 and Tyr-138 and/or Asn-139, K27E, K38A/Y39A, D47G, and Y193A) efficiently bound the fork alone but was unable to form stable complexes with SSB on the same substrate. We conclude that this last class of mutants is deficient in the gp59 contacts with SSB needed to form the ternary gp59-SSB-fork complex.

These results suggest that Phe-132 and Tyr-138 and/or Asn-139, along with the previously described Lys-216 and Tyr-217 (30), define a face needed for the interaction of gp59 with SSB in the presence of fork DNA. These residues are shown as yellow (Tyr-138/Asn-139, Lys-216/Tyr-217) or orange (Phe-132) spheres in Fig. 1A.

Specific Contacts between SSB and Gp59 Are Needed for Gp59 to Interact with SSB-coated ssDNA—There might be a difference between the interaction of gp59 with SSB/fork DNA versus SSB/ssDNA because the activities of gp59 on the lagging strand are distinct from those on the leading strand. This is because the lagging strand is spooled out behind the already assembled replisome, so there is no need to load additional helicase. Moreover, unlike the leading strand, there should not be any DNA forks on the unpaired lagging strand. Thus, gp59 interactions with the lagging strand are more likely mediated by SSB-coated ssDNA rather than by SSB-coated fork DNA (23, 27). In addition, SSB shows a preference for binding lagging strand DNA compared with the leading strand in in vitro assays (23).

To investigate how gp59 interacts with the lagging strand with and without SSB, we employed the 56-nt ssDNA substrate (Oligonucleotide C, Table 1) as a lagging strand mimic. As shown in Fig. 4, in this reaction the WT gp59 bound to three regions of the ssDNA substrate (lane 2). The addition of an antibody to gp59 resulted in a supershift of the gp59-ssDNA complexes, producing very slowly migrating products just below the wells of the gel (Fig. 4, lane 3). In the presence of saturating SSB concentrations, all of the ssDNA substrate was bound by SSB (Fig. 4, lane 8). WT gp59 bound to this complex because all of it was supershifted with the gp59 antibody (Fig. 4, lane 11 versus 9).

To determine whether specific contacts between gp59 and SSB are needed for the gp59-SSB-ssDNA complex, we employed two of the gp59 mutants, K38A/Y39A and F132S. Our previous assays indicated that the K38A/Y39A mutant is defective for binding to either fork or ssDNA (Fig. 3, A–C). Thus, as expected, it also did not bind this naked ssDNA (Fig. 4, lanes 4 and 5). However, the K38A/Y39A mutant was able to bind SSB-coated ssDNA well (Fig. 4, lane 13). This result is consistent with the idea that protein-protein interactions between gp59 and SSB stabilize the gp59/SSB/ssDNA complex.
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Unlike the K38A/Y39A mutant, the F132S mutant bound the naked ssDNA substrate well. Most of the F132S/ssDNA products migrated similarly to those formed with WT gp59 (Fig. 4, lane 6). However, when SSB was present, the resultant products were poorly supershifted by the gp59 antibody (Fig. 4, lane 15).

Taken together, our results suggest that specific contacts with SSB are necessary for gp59 to interact with SSB-coated ssDNA just as they are needed for gp59 to interact with SSB/fork DNA (Fig. 3B) and that the mutation at Phe-132 identifies a region needed for interactions with SSB-covered ssDNA and fork DNA.

Mutations at Gp59 Residues Tyr-122 and Phe-132 Define a Face That Interacts with Helicase—Although the helicase can unwind DNA on its own, gp59 greatly stimulates this activity in vitro by loading helicase onto the DNA (9). Previous work has shown that gp59 mutation Y122A impairs helicase loading, suggesting that this region of gp59 is important for the gp59/helicase interaction (32) (red sphere in Fig. 1A).

To investigate this idea further, we tested the ability of some of the gp59 mutants to stimulate helicase activity and to form a complex with helicase on primed fork DNA. A substrate that mimics the natural R-loop and D-loop substrates for helicase was performed in the presence of the nonhydrolyzable ATP analog ATPγS, which allows helicase to bind, but prevents its unwinding activity.

WT gp59 alone efficiently bound the primed fork, forming a series of concentration-dependent complexes, which migrated more slowly than the substrate alone on native polyacrylamide gels (Fig. 5, lanes 2–4). When the binding reactions also included ATPγS and helicase, a second, slowly migrating complex of helicase/gp59/primed fork DNA was observed (12) (Fig. 5, lanes 7 and 8). Helicase alone does not form complexes with the DNA under these conditions (12) (Fig. 5, lane 5).

We tested four of the gp59 mutants for their ability to form these complexes with helicase on the primed fork DNA: Y122A, which is known to be deficient in loading helicase (32), the DNA binding mutants, K38A/Y39A and K27E; and F132S, the mutant, which was defective in binding to SSB. The F132S mutant was chosen because Phe-132 is located close to Tyr-122 in the gp59 structure (Fig. 1A).

Although the K38A/Y39A mutant was unable to bind the fork DNA (Fig. 3, A and B), it did form a low level of complexes with the primed forks (Fig. 5, lanes 9–11), and it was fully competent to form a stable complex with helicase on this substrate in the presence of ATPγS (Fig. 5, lanes 13 and 14). This result suggests that although the helicase-primed fork and the gp59 mutant-primed fork complexes are weak or transient, they are stabilized when both helicase and gp59 are present. This is in stark contrast to the gp59 K27E mutant, which is essentially dead for primed fork binding, with or without helicase (Fig. 5, lanes 28–33).

Both Y122A and F132S mutants were able to form stable complexes with the primed fork DNA (Fig. 5, lanes 15–17 and 21–23). However, they failed to form significant amounts of the discrete gp59/helicase/DNA complexes (Fig. 5, lanes 18–20 and 24–26), although at the highest concentration of helicase, a trace of slowly migrating species was observed with F132S (lane 20). These results suggest that gp59 residues Tyr-122 and Phe-132, which are close within the structure (Fig. 1A), define a region of gp59 that is needed for a stable interaction with helicase on primed fork DNA.

The Y122A and F132S mutants were also tested in a helicase loading assay. We expect that this test is less stringent than the gel retardation assay in Fig. 5, because loading may be accomplished by a transient interaction between gp59 and helicase, even if a complex stable to electrophoresis cannot be achieved.

As seen in Fig. 6, A versus B (solid black symbols), WT gp59 greatly stimulated the ability of the helicase to unwind the primed fork DNA in the absence of SSB. Both the Y122A (Fig. 6A, lanes 21 and 22) and F132S (Fig. 6A, lanes 29 and 30) mutants were also tested in the helicase loading assay. Both mutants were unable to form a complex with the helicase, with or without ATPγS (Fig. 6A, lanes 23 and 24).

![Figure 4.](image4.png)

**Figure 4.** A mutation at gp59 residue Phe-132 inhibits binding to SSB on ssDNA. Radiolabeled ssDNA (56 bases) was present in the mobility shift reactions at 3 nM. When present, gp59 and SSB were 240 and 180 nM, respectively. Rabbit polyclonal antibody to gp59 (lanes 3, 5, 7, 9, 11, 13, and 15) was used as described (12).

![Figure 5.](image5.png)

**Figure 5.** Gp59 with a mutation at residues Tyr-122 or Phe-132 binds primed fork DNA, but does not stabilize helicase on the DNA. The mobility shift reactions contained 3 nM primed fork DNA (32P-labeled), along with 60, 180, or 360 nM gp59, as well as 360 nM helicase. ATPγS (2 mM) inhibited helicase unwinding of the primed fork substrate DNA during the reactions.
6C) and F132S (Fig. 6D) mutants were impaired compared with WT (Fig. 6A), but they did stimulate the helicase significantly. We conclude that even though interaction with helicase is impaired, the Y122A and F132S mutants are still able to interact with helicase in a manner that is sufficient to load it onto the DNA.

_Helicase and SSB May Compete for Overlapping or Close Sites on Gp59—_The ATPase and helicase activities of helicase are significantly inhibited by the presence of SSB, and gp59 overcomes this inhibition (9, 16, 17) (Fig. 6). To better define how SSB inhibits helicase, we examined how SSB affects interactions between gp59 and helicase on primed forks. We utilized a similar assay as that in Fig. 5 and monitored complexes formed between gp59 and helicase over time.

As seen in Fig. 7, gp59 and helicase rapidly formed a complex on the primed fork; an equilibrium was established within 30 s. However, when SSB was added to the binding reactions at 180 nM (half that of the helicase concentration), the fork complex containing helicase and gp59 quickly disappeared, and the gp59-SSB-DNA complex was now observed (Fig. 7). Therefore, SSB readily disrupts helicase-gp59-primed fork complexes.

Our finding would be consistent with the idea that the binding of gp59 to helicase or SSB is mutually exclusive. This idea is particularly attractive because the surfaces of gp59 that interact with SSB and helicase are physically close (Fig. 1A).

To test this idea, we investigated the ability of the F132S and Y122A mutants to relieve SSB inhibition of helicase. Although Phe-132 and Tyr-122 are close in the gp59 structure, our assays indicated that the F132S mutant is impaired in its interaction with either SSB or helicase (Figs. 3B and 5), whereas the Y122A mutant is only defective in its interaction with helicase (Fig. 5).
We found that the F132S mutant was able to stimulate helicase in the presence of SSB (Fig. 6D). In contrast, the Y122A mutant was unable to do so (Fig. 6C). Our results support a model in which SSB and helicase engage close or overlapping sites on gp59. In the presence of both SSB and helicase, the Y122A mutant, with its normal binding to SSB, but weak binding to helicase, remains bound to SSB. In contrast, the F132S mutant, with its weak binding to both SSB and helicase, can cycle between each partner, like WT gp59.

FIGURE 8. Mutations in gp59, which alter interactions with SSB or DNA replication fork, result in a lengthening of Okazaki fragments. The chart above the agarose gel shows the DNA synthesis in PSL units from a PhosPhor Image scan of the dried gel; quantitation was performed using Multi Gauge software (Fuji Medical). In vitro DNA synthesis was carried out with T4 DNA replication enzymes on the 456-base pair pBSGless nicked DNA template, described under “Experimental Procedures.” Complete DNA replication reactions contained gp59, SSB, T4 RNase H, gp43 polymerase, gp61 primase, gp44/62 clamp-loader, gp45 clamp, and helicase. When present, gp59 was 50 nM in the 10⁻⁶M reactions. The 4-min replication reactions (37 °C) were stopped by addition of EDTA. Gp59 and helicase were omitted in reactions 1 and 2; gp59 was absent in reactions 3 and 4. Mutated versions of gp59 were substituted for WT in reactions 7–10. Replication products were separated on a 0.6% alkaline-agarose gel. See text for further details.
A Gp59 Mutant Defective in Either Fork Binding or Protein-Protein Interactions Gives Aberrant Leading and Lagging Strand DNA Synthesis—Our results suggested that during replication gp59 alternately interacts with SSB and helicase, facilitating efficient DNA synthesis. To examine this idea, we investigated how some of the gp59 mutations affect leading and lagging strand synthesis. We employed a 456-bp circular replication substrate with no G on the leading strand and no C on the lagging strand (Fig. 2). Thus, leading and lagging strand DNA synthesis can be monitored separately by altering the radioactive nucleotides used in replication reactions, [α-32P]dGTP versus [α-32P]dCTP. Furthermore, because the reactions lacked DNA ligase, it is possible to compare the size of lagging strand fragments made in the various reactions.

As expected, little leading strand synthesis and no lagging strand synthesis were observed when replication reactions lacked both gp59 and helicase (Fig. 8, lanes 1 and 2, respectively). Leading strand synthesis increased and lagging strands were made upon addition of helicase (Fig. 8, lanes 3 and 4), but the levels of both leading and lagging strand synthesis remained much lower than that observed when gp59 loaded the helicase (Fig. 8, lanes 5 and 6).

Leading strand synthesis was reduced about 2-fold when using either the F132S mutant, which is defective in interacting with SSB or helicase, or the K38A/Y39A mutant, which is defective in binding fork DNA (Fig. 8, lanes 7 and 9, respectively, versus lane 5). This modest effect was somewhat surprising given how defective these mutants are in individual assays. However, the binding of the K38A/Y39A mutant to DNA is greatly improved by the presence of SSB (Fig. 4) or helicase (Fig. 5), and the ability of the F132S mutant to stimulate helicase is improved by the presence of SSB (Fig. 6). We conclude that protein-protein interactions among the full complement of replication factors can suppress defects of the individual mutants. In fact, T4 containing the K27E mutation, which was totally inactive in our in vitro assays, or the Y122A mutation, which was unable to load helicase in the presence of SSB (Fig. 6C), produced phage in vivo (supplemental Fig. S1).

The overall level of lagging strand synthesis was not affected when using the K38A/Y39A mutant (Fig. 8, lane 10 versus 6) and was modestly affected when using the F132S mutant (Fig. 8, lane 8 versus 6). However, the average size of the lagging strand products made in the presence of these gp59 mutants was about 3 times larger than those made in the reactions with WT gp59 (Fig. 8). This is the same size range as was seen when the gp59 protein was missing from the reaction (Fig. 8, lane 4).

Our results argue that the timing of Okazaki fragment synthesis is altered when gp59 does not bind normally to the fork (K38A/Y39A) or when it does not interact normally with SSB and helicase (F132S). Indeed, a similar lengthening of Okazaki fragments has been reported when using a truncated SSB that is unable to interact with gp59 (34).

Based on our finding that F132S is defective in interacting with SSB-bound ssDNA (Figs. 3B and 4), our results suggest that gp59 must interact with both SSB and ssDNA to facilitate normal lagging strand synthesis. This finding is consistent with the model of Lefebvre et al. (31) that specialized gp59-SSB-ssDNA complexes form structures that are competent for loading helicase, and we speculate that such structures are involved in the assembly of helicase on the lagging strand.

DISCUSSION

The T4 gp59 helicase loader plays a pivotal role in directing both leading and lagging strand synthesis through its interactions with helicase, SSB, primase, DNA polymerase, and the replication fork DNA. Although helicase is able to access a replication fork and promote processive replication at physiological rates, gp59 is needed to load helicase efficiently, thus effectively increasing the overall level of DNA synthesis (discussed in Ref. 13) (Fig. 8). This is vitally important in the context of the full complement of replication proteins, because helicase cannot load itself onto SSB-covered DNA. Thus, without gp59, helicase is forced to “catch” the newly exposed ssDNA from the growing fork before it can be covered by SSB.

The sequence of T4 gp59 lacks homologs outside of the T4-like phage family. Consequently, there are no immediately obvious locations for the positions of fork DNA, leading and lagging strands, and the various protein partners known to interact with gp59. However, the N-terminal domain of gp59 shares structural similarity with eukaryotic HMG proteins, suggesting that this region of gp59 could be involved in interactions with fork DNA (14). Based on this homology, a speculative model for gp59 function was proposed. In this model, the N terminus of gp59 bound the double-stranded DNA, whereas the C-terminal portion provided docking sites for helicase, SSB, and the ssDNA fork arms (14). Specifically, the HMG-like domain was predicted to function as the binding site for the fork, whereas a shallow cleft was predicted to serve as the interaction site for the lagging strand DNA.

Our work has now allowed us to definitely identify portions of gp59 that are involved in various protein-protein and protein-DNA interactions, providing solid biochemical support for the assignment of the N-terminal HMG-like domain as the gp59 fork binding region (Fig. 1A). Our work also indicates that crucial interactions between gp59 and helicase or SSB are mediated by the C-terminal region of gp59, which is located distal to...
the HMG-like domain (Figs. 1A and 9). Small angle x-ray scattering analysis of a gp59/SSB heterodimer agrees with the gp59/SSB contact (36). It should be noted that single molecule photoxidation experiments have suggested that the active form of gp59, like that of helicase, is a hexamer (35). Thus, it may be that each monomer of gp59 makes contact with individual monomers of helicase and/or SSB.

Contrary to the proposed model (14), we did not isolate mutations located within the shallow cleft that impaired binding to ssDNA. Instead, we found that mutations within the HMG-like domain, which also decreased binding to the fork, as well as the mutation V207G impaired binding to ssDNA. The location of Val-207 is not compatible with a ssDNA trajectory through the previously predicted lagging strand binding cleft. Our results suggest that ssDNA either follows a path that includes Val-207 or that the V207G substitution affects the conformation of gp59, which in turn alters the unidentified ssDNA binding region. Clearly, more work is needed to determine the exact location of ssDNA along the surface of gp59.

Previous work has established that a protein-protein interaction between gp59 and SSB facilitates the loading of helicase onto SSB-coated DNA (6), and indicated that the SSB-gp59-DNA complex creates a structure that is competent for loading helicase (31). Our results suggest that the locations of the helicase and SSB contact sites physically overlap (Fig. 9). In addition, our results with the F132S and Y122A mutants are consistent with the idea that gp59 cannot simultaneously bind tightly to both helicase and SSB. In particular, we find that F132S, which is impaired in its interaction with both helicase and SSB, is still able to load helicase in the presence of SSB, whereas Y122A, which is only impaired in its interaction with helicase, is not.

Our results agree with a model in which the loading of helicase involves the switching of gp59 between its interactions with SSB and helicase. However, other work has shown that a SSB affinity column binds helicase only in the presence of gp59, suggesting that both SSB and helicase interact with gp59 simultaneously (16). These results may be reconciled if gp59 is unable to sustain tight interactions with both helicase or SSB, but may still be able to interact with the proteins at the same time by using portions of its interaction sites. Indeed, suboptimal binding might be useful during the transfer of gp59 from SSB to helicase and vice versa. However, we cannot eliminate the possibility that gp59 interacts with SSB and helicase differently in the absence or presence of DNA. If so, the interpretation of our mutational analysis would not be as straightforward. Future structural work of gp59 with its various protein partners will be needed to determine exactly how gp59 functions in its interactions with helicase, SSB, and other protein partners.

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