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Mutations of Bacteriophage T4 59 Helicase Loader Defective in Binding Fork DNA and in Interactions with T4 32 Single-stranded DNA-binding Protein*

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Bacteriophage T4 gene 59 protein greatly stimulates the loading of the T4 gene 41 helicase *in vitro* and is required for recombination and recombination-dependent DNA replication *in vivo*. 59 protein binds preferentially to forked DNA and interacts directly with the T4 41 helicase and gene 32 single-stranded DNA-binding protein. The helicase loader is an almost completely α-helical, two-domain protein, whose N-terminal domain has strong structural similarity to the DNA-binding domains of high mobility group proteins. We have previously speculated that this high mobility group-like region may bind the duplex ahead of the fork, with the C-terminal domain providing separate binding sites for the fork arms and at least part of the docking area for the helicase and 32 protein. Here, we characterize several mutants of 59 protein in an initial effort to test this model. We find that the I87A mutation, at the position where the fork arms would separate in the model, is defective in binding fork DNA. As a consequence, it is defective in stimulating both unwinding by the helicase and replication by the T4 system. 59 protein with a deletion of the two C-terminal residues, Lys^{216} and Tyr^{217}, binds fork DNA normally. In contrast to the wild type, the deletion protein fails to promote binding of 32 protein on short fork DNA. However, it binds 32 protein in the absence of DNA. The deletion is also somewhat defective in stimulating unwinding of fork DNA by the helicase and replication by the T4 system. We suggest that the absence of the two terminal residues may alter the configuration of the lagging strand fork arm on the surface of the C-terminal domain, so that it is a poorer docking site for the helicase and 32 protein.

Bacteriophage T4 DNA replication begins by synthesis from one of several origins in the early stage of infection, but replication from forks created on recombination intermediates becomes the predominant replication initiation mechanism at later times (1, 2). Recombination-dependent replication is made possible by the terminally redundant and circularly permuted arrangement of the 168-kb linear T4 DNA genome. Thus, the end of one molecule can invade the homologous internal region of another molecule. Phage T4 encodes the replication proteins that are needed for both modes of replication (reviewed in Refs. 3 and 4).

In the T4 replication system, gene 41 helicase unwinds the duplex ahead of the leading strand T4 DNA polymerase and associates with the gene 61 primase to enable it to make the pentamer primers that initiate each lagging strand fragment. *In vitro*, the helicase by itself loads slowly on replication forks, but its loading is greatly stimulated by the gene 59 helicase loading protein (5). *In vivo*, the helicase loading protein is essential for recombination and recombination-dependent replication (1, 2). Both 41 helicase and its 59 loader are needed for polar branch migration on joint molecules formed by the T4 UvsX recombinase and gene 32 single-stranded DNA-binding protein (6), and the helicase, 59 loader, and 32 protein are all necessary for branch migration on preformed deproteinized joint molecules (7). In contrast to T4 mutants in DNA polymerase, which are completely defective in replication, T4 gene 59 mutants do catalyze some early origin-dependent replication (8–10). Double mutants in gene 59 and the nonessential DNA helicase dda do not replicate T4 DNA, suggesting that the proteins catalyze redundant functions in replication initiation (11). Whether there are any T4 origins that require 59 helicase loader has not been established *in vivo*. 59 protein, *in vitro* strongly stimulates synthesis from a preformed R loop within the T4 UvsY origin (12).

59 helicase loader is a small (26 kDa) basic protein that binds DNA, 41 helicase, and 32 protein. It was initially characterized as a ss[1] and double-stranded DNA-binding protein (5, 13, 14) and has more recently been shown to have an increased affinity for forked DNA (15–17). It also binds and stimulates unwinding by 41 helicase of cruciforms and three-stranded recombination structures. There is evidence that the oligomerization of the helicase subunits to form a hexamer is increased by the presence of 59 protein and that higher oligomers of the monomeric 59 protein are formed when the helicase is present (18). However, the details of the assembly pathway and stoichiometry of the 41–59 protein complex remain to be established. 59 protein has an affinity for 32 protein, even in the absence of DNA (5, 13, 14, 19, 20), and the two proteins bind simultaneously to ssDNA. 32 protein has a much stronger affinity than 59 protein for ssDNA long enough for cooperative binding, but

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[1] The abbreviations used are: ss, single-stranded; TCEP, tris(2-carboxyethyl)-phosphine hydrochloride; AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride; HMG, high mobility group; C-domain, C-terminal domain; N-domain, N-terminal domain.

32 protein, unlike 59 protein, does not preferentially bind fork structures (21). In fact, 59 protein promotes binding of 32 protein on DNA forks with arms too short for cooperative 32 protein binding (21). Collectively, these findings have led to a proposal that in the assembly of the replication complex, 59 protein may bind first to the fork at an origin or recombination intermediate and then attract the helicase and 32 protein to the fork arm that will become the lagging strand template (17, 21). Alternatively, it has been proposed that the major role of 59 protein is to facilitate loading of the helicase on DNA coated with 32 protein (5, 22).

The interactions between the helicase, 59 helicase loader, and 32 protein have important consequences for how the replication complex functions. 32 protein is essential for leading strand synthesis when the helicase is loaded by 59 protein (21). In the absence of 59 protein, helicase is loaded on a much smaller fraction of the DNA molecules, but leading strand synthesis can occur without 32 protein (21, 23). We have recently suggested that 59 protein at the fork prevents the effective coupling of the leading strand polymerase and the helicase, unless the position of 59 protein is shifted by its association with 32 protein (21). Electron microscopic analysis of molecules replicated by the T4 replication system showed that the protein-covered ssDNA on the lagging strand is in a compact structure, different from 32 protein-covered ssDNA, and that this structure is less compact when the helicase is loaded without 59 protein (24).

The amino acid sequence of T4 59 protein has little similarity to those of other helicase loading proteins like *Escherichia coli* DnaC (15). The almost completely α-helical crystal structure of 59 protein is also novel (see Fig. 1) (15). It lacks a large DNA-binding cleft characteristic of other ssDNA-binding proteins, like T4 32 protein. The 217-residue protein is divided into two closely packed domains of similar size (called the N-domain and the C-domain), with a shallow central groove between these domains on the top surface of the protein. A region of the N-domain, residues 11–67 (*blue* in Fig. 1*A*) has strong structural similarity to the DNA-binding domain of the structures of several members of the high mobility group (HMG) family proteins, including rat HMG1A and the LEF-1 lymphoid enhancer-binding factor. Proteins with an HMG domain bind in the minor groove of duplex DNA, bending and partially unwinding the duplex. Like 59 protein, some HMG proteins bind cruciform DNA (reviewed in Refs. 25–28). The surface of 59 protein has a high density of hydrophobic and basic residues that may be involved in DNA and protein binding, but the binding sites are not obvious from the structure. We have previously speculated that the region of the 59 protein N-domain with structural similarity to the double-stranded DNA-binding domain of the HMG proteins may be the binding site for the duplex ahead of the fork arms. In this model, the C-domain of 59 protein would contain the binding sites for the fork arms and provide at least part of the docking area for the helicase and 32 protein (Fig. 1*B*) (15).

Guided by the crystal structure, we have made several mutants of 59 protein as an initial step in identifying the binding sites for fork DNA, the helicase, and 32 protein. We find that the I87A mutation is defective in binding fork DNA. As a consequence, it is defective in stimulating both unwinding by the helicase and replication by the T4 system on model templates. 59 protein with a deletion of the two C-terminal residues, Lys^{216} and Tyr^{217}, binds fork DNA, as well as the wild type protein. It fails to promote binding of 32 protein on short fork DNA but binds 32 protein normally in the absence of DNA. This deletion protein is also somewhat defective in stimulating unwinding of fork DNA by the helicase and replication by the T4 system. We suggest that the absence of the two terminal residues may alter the configuration of the lagging strand fork arm on the surface of the C-domain, so that it is a poorer docking site for the helicase and 32 protein.

EXPERIMENTAL PROCEDURES

Proteins—Purification procedures for T4 DNA polymerase, 44/62 clamp loader, 45 clamp, 41 helicase, 61 primase, and 59 helicase loader (29) and 32 protein (21) have been described previously.

Site-directed Mutagenesis of 59 Protein—Mutations in T4 gene 59 were made by site-directed mutagenesis of wild type gene 59 in the plasmid pNN2859 (30), using the method of Kunkel *et al.* (31), modified by using T4 DNA polymerase, T4 44/62 clamp loader, and 45 clamp to copy the ssDNA template. The oligonucleotide primers used were (sequence complementary to mutation or stop codon underlined): 59 I37A, 5′-GCACCAATTATACTTAGCTACATCATACTTTCC; 59 W86A, GCGTCAGAGATGTCACCAATCGCAGCATCTTGGTTAGCAACC; 59 V142A, GCAGAAGTTTAAAAATATAACTTGATTGAGCTTTTGGATTATACTC; 59 F111A, GCGAATATCTTCTTCAAACTTAGCTTTAATTTGCTTTAAGCG; 59 ΔKY (ΔK216,Y217), CTGCATATCAATATTAGCAAGATTTCACAG; and 59 I87A, GCGTCAGAGATGTCACCAGCCCAAGCATCTTGGTTAGCAACC. The mutations were verified by DNA sequencing.

Induction and Purification of Mutant 59 Helicase Loader Proteins—Cultures (500 ml) of *E. coli* BL21(DE3)plysS Gold (Stratagene) containing plasmids encoding wild type 59 protein or the W86A, F111A, V142A, or C-terminal deletion (ΔKY) mutants were grown overnight in Luria broth with 50 μg/ml of carbenicillin (Invitrogen) and 30 μg/ml chloramphenicol at 37 °C, diluted 1:50 into 500 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 mM. After 2 h at 37 °C, the cells were harvested by centrifugation and stored at −80 °C. Cultures containing plasmids encoding 59 I37A or I87A were grown in the same way to A_{600} = 0.35, transferred to 25 °C, and grown to A_{600} = 0.4. 59 protein expression was then induced by adding isopropyl-β-D-thiogalactopyranoside to 0.4 mM for 9 h. All of the purification steps were at 4 °C. Frozen cells were suspended in 8 ml of low salt buffer (100 mM NH_4Cl, 50 mM Tris-Bis, pH 6.5, 0.5 mM TCEP-HCl (Pierce), 1 mM AEBSF, and 1 Complete 228 protease inhibitor tablet/50 ml (Roche Applied Science)), broken by sonication, and centrifuged for 30 min at 100,000 × *g*. A large fraction (50–70%) of 59 protein remained in the pellet under these conditions (15). The pellet was suspended in high salt buffer (buffer A (50 mM Tris-Bis, pH 6.5, 10 mM $MgCl_2$, 0.5 mM TCEP, and 1 mM AEBSF) with 750 mM NH_4Cl), left on ice with occasional stirring for 15 min, and then centrifuged as above. The high salt supernatants (8 ml) were diluted with 9.2 ml of buffer A to give a final NH_4Cl concentration of 350 mM and loaded on 0.5-ml columns of ceramic hydroxylapatite (Integrated Separation Systems) that had been equilibrated with buffer A with 350 mM NH_4Cl. The columns were then washed sequentially with 0.6 ml of buffer A with 350 mM NH_4Cl, 1.5 ml of buffer A with 450 mM NH_4Cl, and 1.5 ml of buffer A with 750 mM NH_4Cl. Approximately equal fractions of 59 protein for each mutant were found in the 350, 450, and 750 mM NH_4Cl eluates. The 350 mM NH_4Cl eluates were dialyzed against a buffer containing 50 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM EDTA, 0.5 mM TCEP, and 100 mM KCl and used for the experiments described here. Each of the mutant proteins appeared to be >95% pure by SDS gel electrophoresis (Fig. 2).

DNA Substrates—The following oligonucleotides, made and reverse phase-purified (24b) or gel-purified (56 and 60b) by Sigma-Genosys, Inc., were used to prepare fork DNA with 12b or 30b arms, as described (21). A, 5′-TAACGTATTCAAGATACCTCGTACTCTGTACAGGTTGCGATCCGACTGTCCTGCAT (56b); B, 5′-GATCATGCAGGACAGTCGGATCGCAACCTGATTTACTGTGTCATATAGTACGTGATTCAG (60b); C, 5′-GCAGTCCTAACTTTGAGGCAGACC (24b); and D, 5′-GGTCTGCCTCAAGACGGTAGTCAA (24b). Plasmid (pUCNICK) with a single recognition site for the N.BbvC IA nicking enzyme (New England Biolabs) was constructed by cloning the following sequence between the PstI and EcoRI sites of pUC19.

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5′     GTACCAATAACTCTTAATTAATCCTCA GCG     3′

3′ ACGTCATGGTTATTGAGAATTAATTAGGAGT|CGCTTAA 5′
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SEQUENCE 1

The N.BbvC IA recognition sequence on the bottom strand is underlined, and the position of the nick shown by a vertical line. The plasmid can be nicked on the top strand using the N.BbvC IB nicking enzyme.

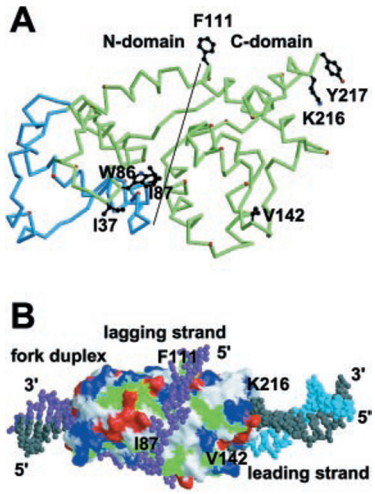

FIG. 1. **Speculative model for the position of fork DNA bound to T4 59 helicase loading protein and locations of residues altered in this study.** *A*, backbone of the crystal structure of 59 helicase loading protein (Protein Data Bank code 1C1K) with locations of the mutations shown in *black*. The region of 59 protein with structural similarity to the HMG family proteins is shown in *blue* (15). The *line* shows the position of the shallow groove between the N- and C-domains of the protein. *B*, speculative model of fork DNA bound on a space filling model of 59 protein. Positively charged residues are shown in *blue*, negatively charged residues are in *red*, and hydrophobic residues are in *green*. This model is based on the assumption that the region of 59 protein similar to the HMG proteins binds and unstacks the duplex ahead of the fork. The locations of mutations on the top surface of the protein are labeled. The Ile^{37} mutation is on the *bottom surface* and not visible on the figure (adapted from Ref. 15).

The inserted sequence also contains a recognition site for PacI endonuclease (TTAATTAA) and has a T4 primase recognition sequence (5′-GTT) on the bottom strand. *E. coli* XL10 (Stratagene) containing pUCNICK was grown overnight in 1 liter of Luria broth with 50 μg/ml of carbenicillin, and plasmid DNA was isolated using a Qiagen HiSpeed Plasmid Maxi Kit. pUCNICK plasmid DNA (72 μg) was nicked by incubating for 20 h at 37 °C in a reaction mixture (425 μl), containing 10 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol, and 25 units of BbvC1a nicking enzyme. The DNA was purified on DNA Clean and Concentrator 25 columns from Zymo Research, as described by the manufacturer.

DNA Binding and Helicase Assays—DNA gel retardation assays were carried out as described (16), in 5-μl reactions containing 3 nM DNA, 25 mM Tris acetate, pH 7.5, 60 mM potassium acetate, 6 mM magnesium acetate, 10 mM dithiothreitol, 2 mM ATP, and 20 μg/ml bovine serum albumin at 30 °C for 5 min, unless otherwise indicated. The protein concentrations are indicated in the figure legends. Helicase assays were carried out under the same reaction conditions for 5 min at 37 °C. When noted, the helicase reaction tubes were left in the 37 °C bath, and 1 μl of 10 mg/ml proteinase K (Roche Scientific) was added for 1 min; then 1 μl of 200 mM EDTA was added, followed by 3 μl of 15% glycerol (with bromphenol blue dye) and transfer of the sample to dry ice. After all of the samples were completed, the tubes were transferred from dry ice to a room temperature water bath for 5 min before loading onto the gel, as described in Ref. 32. Dried gels were autoradiographed on BioMax MR film (Eastman Kodak) or scanned on a Fuji FLA-3000 Image Analyzer. Quantitation of scanned images was done using Image Gauge software, version 3.12, from Fuji Medical Systems.

DNA Replication Reactions—The template for replication with T4 proteins was either 5 nM of the 2.9-kb nicked pUCNICK plasmid described above, or 1.6 nM of M13mp2 ssDNA annealed to 34 bases on the 3′ end of an 84b oligonucleotide (30), leaving a 50b tail. Reaction mixtures containing DNA, 2 mM ATP, 250 μM of each dNTP including [α-^{32}P]dCTP (~800 cpm/pmol), 250 μM CTP, GTP, and UTP, 25 mM Tris acetate, pH 7.5, 60 mM potassium acetate, 6 mM magnesium acetate, 10 mM dithiothreitol, 20 μg/ml bovine serum albumin, 32 ssDNA-binding protein (2 μM), 44/62 clamp loader (242 nM), 45 clamp (162 nM), and 59 helicase loading protein at the concentrations indicated in the figure legends were incubated for 2 min at 37 °C. Synthesis was then initiated by addition of a mixture of T4 DNA polymerase (30 nM), 61 primase (64 nM), and 41 helicase (328 nM monomer). At the times indicated, aliquots of the reaction mixtures were mixed with an equal volume of 0.2 M EDTA to stop the synthesis, and the products were analyzed by 0.6% alkaline agarose gel electrophoresis (33) and trichloroacetic acid precipitation (29).

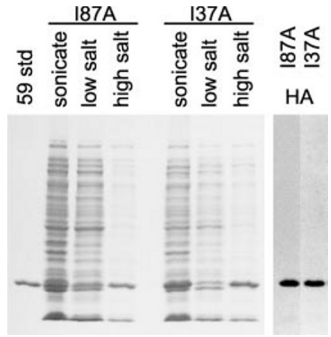

FIG. 2. **Purification of the I87A and I37A mutants of 59 helicase loading protein.** The proteins are shown on a 12% SDS-polyacrylamide gel. *HA* indicates fractions purified on hydroxylapatite, as described under "Experimental Procedures." Similar results were observed with each of the other mutant 59 proteins (not shown). *std*, 59 protein (15).

Interaction between 59 and 32 Proteins—Reaction mixtures containing 25 mM Tris acetate, pH 7.5, 60 mM potassium acetate, 6 mM magnesium acetate, 10 mM dithiothreitol, and 2 mM ATP were incubated for 1 min at 30 °C, before 59 and 32 proteins were added at the concentrations indicated in the figure legends (5 μl total). After 10 min, 1 μl of 15% glycerol with bromphenol blue dye was added to each sample, and the tubes were returned to ice. The samples were applied to wells in the center of a 9 × 13.2-cm 0.6% SeaKem ME agarose gel (1× Tris acetate/EDTA). Electrophoresis was carried out in a horizontal submarine electrophoresis unit (Aquebogue model 750) at 50 volts (constant) in 1× Tris acetate/EDTA buffer at 4 °C for 90 min. Under these conditions the positively charged 59 protein migrates toward the anode 32 protein toward the cathode, and complexes of 59 and 32 proteins migrate between the single proteins. Following electrophoresis, the gels were fixed in 10% methanol, 7% acetic acid for 30 min and then treated overnight with SYPRO Ruby protein gel stain (Molecular Probes, Inc.). After destaining in 10% methanol, 7% acetic acid for 30 min, the gels were photographed on a UV transilluminator with a Kodak DC120 camera with an ethidium bromide filter and a yellow SYPRO protein gel stain photographic filter (Molecular Probes catalog number S-6656), according to the protocol provided with the SYPRO Ruby stain.

RESULTS

Site-directed Mutagenesis of T4 59 Helicase Loading Protein—The T4 59 helicase loading protein binds preferentially to fork DNA and interacts directly with the T4 41 helicase and 32 ssDNA-binding protein (see the Introduction). Guided by the crystal structure of 59 protein (15), we have made a series of mutations in 59 protein in an effort to determine the DNA-binding and protein interaction sites on this protein. In the N-domain, these include I37A, on the "bottom" surface in the turn between helices 1 and 2, and I87A, on the "top" surface where the fork arms separate in the model, and the adjacent W86A. In the C-domain we made V142A, at the apex of a long turn between helices 8 and 9; F111A, a highly exposed hydrophobic residue at the top of the view shown in Fig. 1; and a deletion of the two C-terminal residues Lys^{216} and Tyr^{217}. The mutations were constructed by copying the ssDNA of the plasmid pNN2859 (30), encoding wild type gene 59, with T4 DNA polymerase, 44/62 clamp loader, and 45 clamp using an oligonucleotide primer with the desired mutation (see "Experimental Procedures"). The mutant proteins were purified by a rapid procedure based on the observation that 59 protein remains in the pellet containing cellular debris and DNA after sonication in low salt buffer and can be eluted from this pellet in buffer containing 750 mM NH_4CL (15). 59 protein in the high salt wash was then chromatographed on hydroxylapatite, yielding a protein >95% homogeneous, as judged by gel electrophoresis (Fig. 2; see "Experimental Procedures").

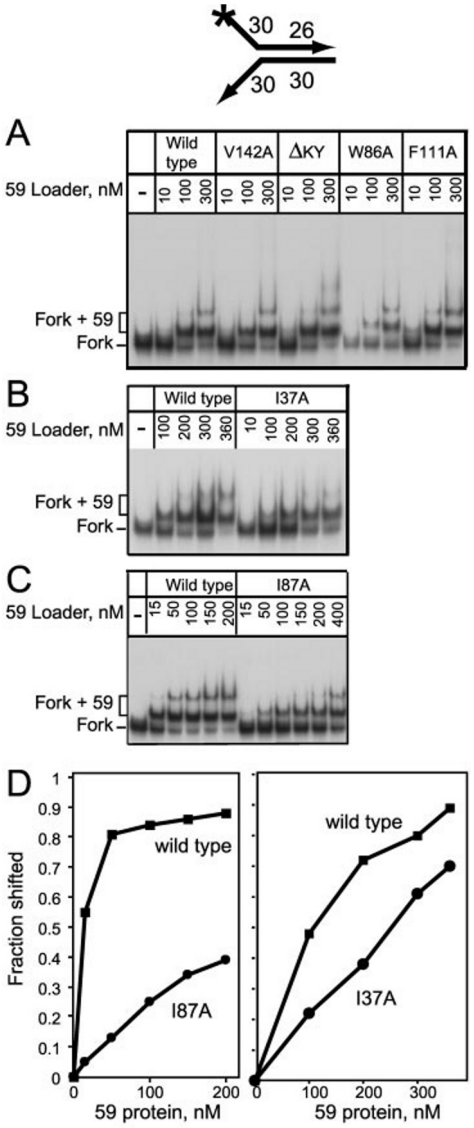

FIG. 3. **I87A and I37A 59 proteins have a reduced affinity for fork DNA.** *A–C*, gel mobility shift assays of fork DNA binding by wild type and mutant 59 helicase loading proteins. *D*, fraction of fork DNA shifted by wild type, I87A, and I37A 59 proteins. An *asterisk* indicates the 5′ ^{32}P label. The sequences of the fork and assay conditions are described under "Experimental Procedures."

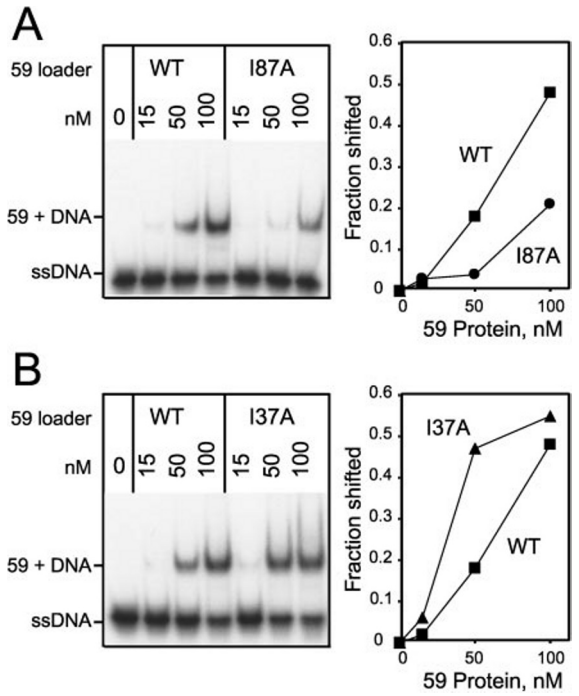

FIG. 4. **The I87A mutant 59 protein is defective in binding ssDNA.** Gel mobility shift assay of binding to a ssDNA 56-mer by wild type (*WT*) and I87A (*A*) and I37A (*B*) 59 helicase loading proteins. Each of the other mutant 59 proteins bound this ssDNA like the wild type. Sequence of the 56-mer and assay conditions are described under "Experimental Procedures."

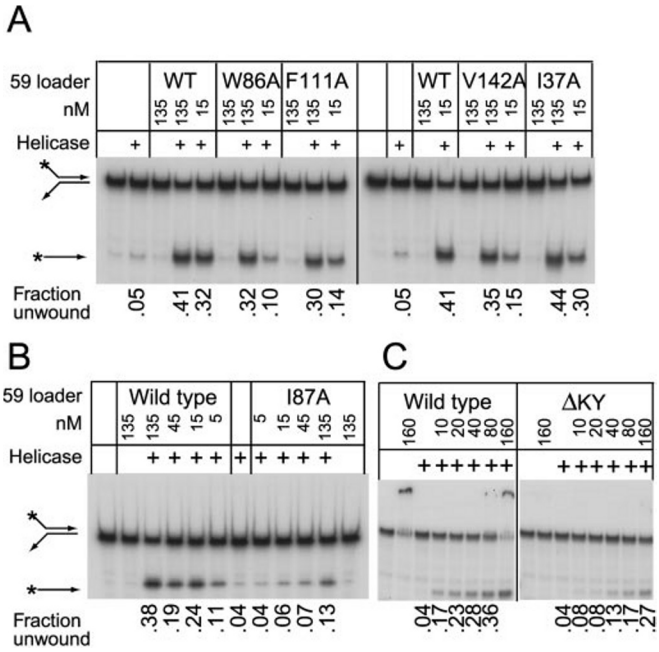

FIG. 5. **The I87A and C-terminal ΔKY mutants are defective in stimulating unwinding of fork DNA by the T4 41 helicase.** The sequences of the fork with 30b arms and assay conditions are described under "Experimental Procedures." Helicase was present at 90 nM (monomer) in *A* and *B* and at 80 nM in *C*. The protease K stop procedure was used in *B*. *WT*, wild type.

Binding to Fork and Single-stranded DNA—The ability of each of the mutant 59 proteins to bind fork DNA was compared with that of the wild type by a gel mobility shift assay, as described previously (15, 16) (Fig. 3). With the exception of I87A and I37A, all of the mutant proteins had a fork DNA binding affinity that was similar to that of the wild type 59 loader. The binding affinity of the I87A mutant was decreased significantly relative to the wild type (Fig. 3, *C* and *D*, *left panel*). Fork DNA binding by the I37A mutant was decreased to a lesser extent (Fig. 3, *B* and *D*, *right panel*) and was more sensitive than wild type 59 protein to decreased concentrations of dithiothreitol in the reaction (data not shown). I87A was the only mutant 59 protein with a defect in binding ssDNA (Fig. 4*A*). The I37A 59 protein bound ssDNA with an affinity greater than that of the wild type (Fig. 4*B*). As shown previously (15, 16), the affinity of wild type 59 protein for the ssDNA 56-mer (Fig. 4) is significantly lower than its affinity for fork DNA that includes the same 56-mer (Fig. 3).

Stimulation of Helicase Activity—Current models for the loading of helicase by 59 protein posit that the helicase loader binds fork DNA in a fashion that makes the lagging strand accessible to the helicase and interacts directly with the helicase to promote the assembly of the helicase hexamer around the DNA strand (see the Introduction). The W86A, F11A, and V142A 59 proteins, which bound fork DNA normally and the I37A protein with somewhat reduced fork binding (Fig. 3),

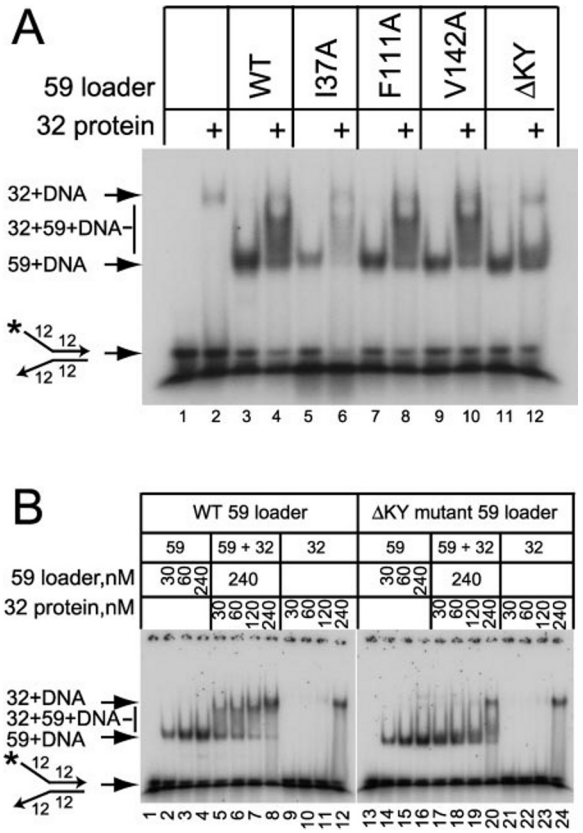

FIG. 6. **T4 59 helicase loader with a C-terminal deletion of K216 and Y217 (ΔKY) does not promote binding of T4 32 protein on DNA forks with single-strand arms too short for cooperative binding by T4 32 protein.** *A*, gel mobility shift assay for the binding of T4 32 protein and 59 helicase loader to forked DNA. 59 protein binds to forks with 12-base arms, but there is very little binding by 32 protein under these conditions. 32 protein binds to the short fork when wild type (*WT*) 59 protein is present, as shown by the complexes migrating behind 59 protein-fork complex (21). The ΔKY 59 protein bound this short fork DNA like the wild type but did not increase binding by 32 protein. The concentration of each 59 protein was 240 nM; the concentration of each 32 protein was 240 nM; and the concentration of each fork DNA was 3 nM. *B*, titration of wild type and ΔKY 59 protein and 32 protein in the fork binding assay.

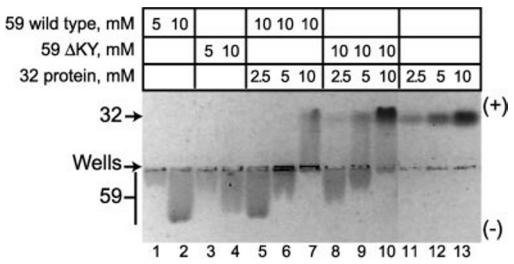

FIG. 7. **The T4 59 proteinΔKY deletion mutant forms stable complexes with 32 protein in the absence of DNA.** Complex formation between T4 59 helicase loader and 32 protein was measured in a neutral agarose gel with the wells in the center, as described under "Experimental Procedures." The (+) and (−) signs oriented at the *top* and *bottom* of the agarose gel show the relation of the gel to the cathode (+) and anode (−) during electrophoresis. Note that the WT and ΔKY 59 proteins migrated toward the anode, because of the very basic pI (9.37) of T4 59 protein, whereas 32 protein pI (4.82) migrates toward the cathode.

retained substantial helicase stimulation activity (Fig. 5*A*). As expected, the I87A mutant, which was defective in fork DNA binding, had a greatly reduced ability to stimulate the helicase (Fig. 5*B*). Helicase stimulation by 59 loader missing the C-terminal residues Lys^{216} and Tyr^{217} (ΔKY) was significantly decreased (Fig. 5*C*), although this mutant was not defective in DNA binding (Fig. 3).

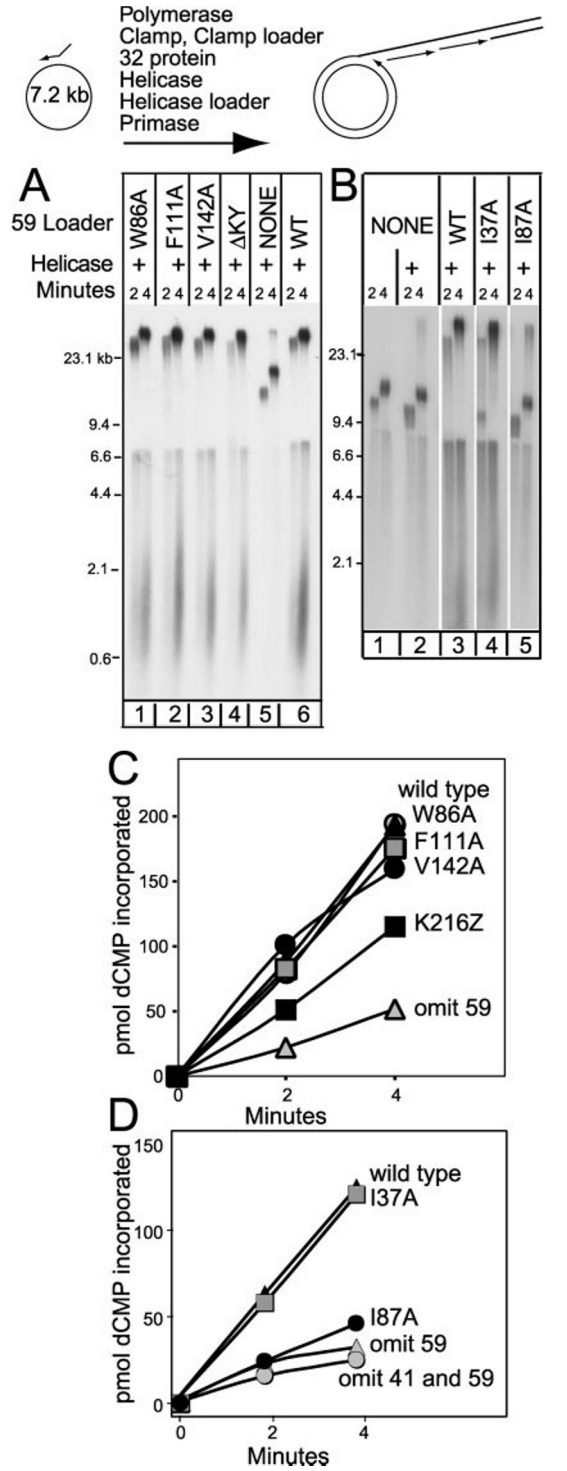

FIG. 8. **Replication of a primed M13 ssDNA template by the T4 replication system with wild type and mutant 59 helicase loading proteins.** The template is circular M13 ssDNA annealed to a 84b primer, which has 34 bases at the 3′ end that are complementary to the template. The assay conditions are described under "Experimental Procedures." *A* and *B*, replication products labeled with [^{32}P]dCTP are displayed on a 0.6% alkaline agarose gel. Products longer than the 7.2-kb template are from the leading strand, whereas most of the products shorter than 7.2 kb are from the lagging strand. *C* and *D*, quantitation of the total replication in *A* and *B*, respectively. *WT*, wild type.

The C Terminus of 59 Protein Has a Role in Stabilizing 32 Protein on DNA Forks—59 helicase loader binds directly to 32 protein in the absence of DNA (5, 13, 14, 20, 34) and has recently been shown to promote the binding of 32 protein on forks with arms too short for cooperative binding by 32 protein

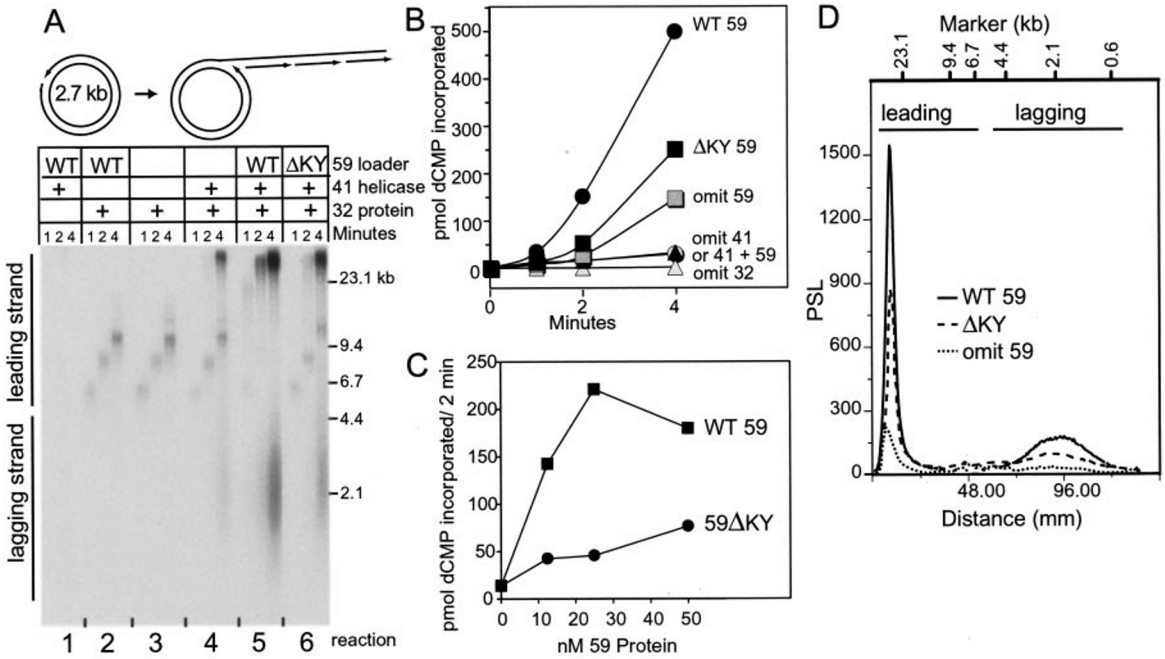

FIG. 9. **TheΔKY deletion 59 protein is defective in stimulating both leading and lagging strand synthesis.** The template is the 2.7-kb pUCNICK plasmid nicked at the single recognition site for the N-BbvC IA nicking enzyme, as described under "Experimental Procedures." Wild type (*WT*) or ΔKY mutant 59 protein was present at 25 nM. *A*, 0.6% alkaline agarose gel of the products. Products shorter than the 2.7-kb template are from the lagging strand. *B*, time course of the incorporation. *C*, titration of the wild type and ΔKY 59 proteins. Replication reactions were incubated for 2 min. *D*, PhosphorImager scan of gel lanes for 4-min reactions. The position of the λHindIII fragment size markers is shown at the *top*. *PSL* is the detected radiation in arbitrary units.

alone (21). The addition of antibody to 59 protein showed that it remained on this fork with 32 protein. The F111A, K142A, and ΔKY 59 mutants, which bound the fork DNA with 30b arms (Fig. 3), also bound to the fork DNA with the 12b arms (Fig. 6*A*, *lanes 3*, *7*, *9*, and *11*). The I37A protein showed reduced binding to the short fork (Fig. 6*A*, *lane 5*), as it had with the longer fork (Fig. 3, *B* and *D*). 32 protein by itself gave only a weak mobility shift band with this short fork (*lane 2*), as shown previously (21). The addition of 32 protein to 59 protein-DNA complexes resulted in 32 protein binding for all but one of the mutants tested (*lanes 4*, *6*, *8*, and *10*). The C-terminal deletion mutant, ΔKY, bound the fork DNA as well as the wild type (*lane 11*) but failed to form a slower migrating complex with 32 protein (*lane 12*).

Fig. 6*B* compares a titration of wild type 59 protein (*left panel*) with 59 missing Lys^{216} and Tyr^{217} (*right panel*) with regard to DNA binding and binding of 32 protein in the presence of DNA. At each concentration of 59 protein, the fork binding of the mutant (*lanes 14–16*) was similar to the wild type (*lanes 2–4*). The mutant showed no evidence of adding 32 protein to the complex, over a range of 32 protein concentrations (compare DNA with 32 protein and the mutant (*lanes 17–20*) or wild type 59 (*lanes 5–8*), with DNA and just 32 protein (*lanes 9–12* and *21–24*).

The interaction between the C-terminal deletion mutant (ΔKY) of 59 protein and 32 protein was further investigated in reactions carried out in the absence of DNA. Because T4 59 protein is very basic (pI 9.37), it will not enter a vertical native acrylamide gel at neutral pH. For this reason, we looked for a 59–32 protein complex using a horizontal agarose gel with centrally located loading wells that permitted migrating proteins to enter the agarose gel in either the positive (cathode) or negative (anode) direction relative to the applied electric field. Fig. 7 shows typical results of protein-protein interaction assays carried out with the same reaction and running buffers used in the mobility shift assays with proteins and DNA. The gel in the figure is oriented with the cathode at the *top*. As expected, 32 protein (pI 4.82) migrated toward the cathode (*lanes 11–13*), whereas 59 wild type (*lanes 1–2*) and the ΔKY C-terminal deletion (pI 9.33) (*lanes 3–4*) proteins migrated toward the anode. The faster migration of the higher concentration of 59 protein would be consistent with a multimer of 59, with a higher surface charge. In lanes containing either wt 59 protein (*lanes 5–7*), or the ΔKY mutant (*lanes 8–10*) in the presence of increasing amounts of 32 protein, there was a shift in the direction of migration from the anode toward the cathode, as expected for a 59–32 complex with a net charge between that of the two separate proteins. The formation of protein-protein complexes between ΔKY and 32 protein was surprising in light of the lack of interaction between these proteins in the gel mobility shift assays with the fork DNA substrate. One possibility is that the C-terminal residues are required to hold the fork arm in a configuration that is more accessible to 32 protein (see "Discussion").

Both the C-terminal ΔKY and I87A 59 Mutant Proteins Are Defective in Replication—At the replication fork, 59 protein loads the helicase and must also interact with 32 protein, which we have recently shown is required for leading strand synthesis when the helicase is loaded by 59 protein (21) (see "Discussion"). We initially used M13 circular ssDNA primed with a forked primer as a template to test the ability of the mutant 59 helicase loaders to carry out these reactions (Fig. 8). Polymerase copies the ss circle by elongating the primer and then begins strand displacement leading strand synthesis. The displaced leading strand serves as a template for the priming and elongation of the shorter lagging strand fragments. When helicase is added without the loading protein (Fig. 8*A*, *reaction 5*), there is a small amount of leading strand >23 kb at 4 min, showing that helicase has loaded by itself on a fraction of the molecules. The shorter leading strand products (between the 9- and 23-kb markers) are made by a reaction that requires only T4 DNA polymerase, 44/62 clamp loader, 45 clamp, and 32 protein (Ref. 33 and data not shown). When 59 protein loads the helicase (Fig. 8*A*, *reaction 6*), the longer leading strand

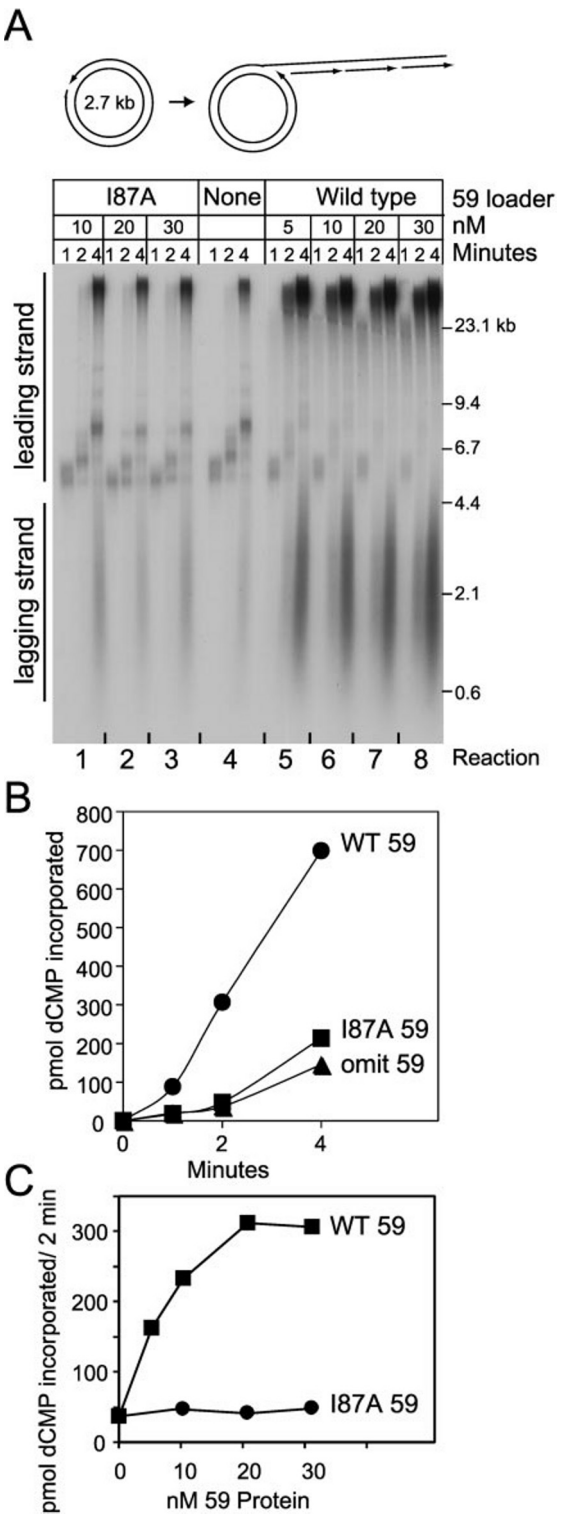

FIG. 10. **Replication of a nicked circular template by the T4 replication system with the wild type or I87A 59 helicase loading protein.** The template is the 2.7-kb nicked circle described in Fig. 9. *A*, 0.6% alkaline agarose gel of the products. Products shorter than the 2.7-kb template are from the lagging strand. *B*, time course of the incorporation with 30 nM wild type (*WT*) or I87A mutant 59 protein. *C*, titration of the wild type and I87A 59 proteins. The replication reactions were incubated for 2 min.

products and the shorter lagging strand fragments are evident earlier and greatly increased. The W86A, F111A, V142A (Fig. 8, *A* and *C*), and I37A (Fig. 8, *B* and *D*) mutant loading proteins were indistinguishable from the wild type in this reaction. 59 protein C-terminal deletion (ΔKY) stimulated leading and lagging strand synthesis on the primed M13 template, but only at a rate of about 50% that of the wild type helicase loader (Fig. 8, *A* and *C*). There was almost no synthesis above that observed without 59 protein with the I87A mutant (Fig. 8, *B* and *D*).

Further analysis of replication stimulation by the ΔKY 59 protein using the singly nicked pUCNICK plasmid DNA (Fig. 9*A*) showed that molecules elongating at the slow rate that does not require helicase (*reactions 2* and *3*) persisted throughout the reaction with the ΔKY mutant helicase loader (*reaction 6*). Synthesis of the long leading strand and shorter lagging strand fragments were each affected to a similar extent when the ΔKY mutant replaced the wild type (Fig. 9*D*). A small increase in the lagging fragment size range was also noted with this mutant. The ΔKY helicase loader binds fork DNA as well as the wild type. Thus, its poor replication relative to the wild type must result from its defects in interaction with the helicase and 32 protein.

Synthesis on the nicked plasmid template with the I87A mutant was barely above that observed in the absence of any 59 loader, even at 59 protein concentrations that were saturating for the wild type helicase loader (Fig. 10). The replication defect of the I87A mutant protein is consistent with its decreased affinity for fork DNA and weak helicase stimulation.

DISCUSSION

T4 gene 59 helicase loading protein, which is essential for recombination-directed replication *in vivo* and greatly stimulates DNA synthesis *in vitro*, associates with a remarkable number of other T4 replication proteins. It was initially shown to accelerate the loading of 41 helicase (5). There is now evidence that 59 remains on the replication fork after loading the helicase (16, 35)[2] affects the compact structure of 32 protein covered ssDNA on the lagging strand (24), stimulates primer synthesis by the primase-helicase (36), and plays a role in coordinating leading and lagging strand synthesis (12, 21, 37). 59 protein has been shown to bind or cross-link to T4 41 helicase (14, 18), 32 ssDNA-binding protein (5, 13, 14, 34), DNA polymerase (35), and 61 primase (cited in Ref. 35). 59 protein binds tightly to both ssDNA and double-stranded DNA and has its highest affinity for forked DNA with either ss or double-stranded arms (5, 14–16, 19). The crystal structure of the monomeric 26-kDa protein showed that the N-terminal domain has strong structural similarity to the DNA-binding domain of the HMG family of proteins, making the N-domain a plausible binding site for the duplex region of fork DNA (Fig. 1) (15). The surface of 59 protein structure had a high density of hydrophobic and basic residues but provided few clues to the location of the binding sites for the arms of the replication fork or the other replication proteins. We have characterized a series of mutations in 59 protein, based on this structure, in an effort to identify these interaction sites.

The I87A 59 protein mutant bound fork DNA poorly, compared with the wild type, consistent with our speculative model for DNA binding (Fig. 1), in which Ile^{87} is close to the position at which the fork arms would separate on the protein. As a result, the I87A mutant 59 protein was defective in stimulating unwinding of fork DNA by 41 helicase and gave almost no increase in replication by the T4 proteins. In the HMG proteins, several residues in the turn between helix H1 and H2 make contact with the minor groove of the DNA. The I37A mutation, within this turn in 59 protein, had somewhat reduced binding to fork DNA but stimulated unwinding by the helicase and replication by the T4 system like the wild type. Fork DNA binding by the I37A mutant was more sensitive than

[2] P. D. Chastain, E. Green, N. G. Nossal, and J. D. Griffith, unpublished experiments.

the wild type to the concentration of dithiothreitol in the binding reaction. It is possible that the neighboring Cys^{42} is more sensitive to oxidation in the I37A mutant protein. Cysteine 42 is not essential because the C42A and C42S mutants were indistinguishable from the wild type in DNA binding, helicase, and replication reactions.[3]

The physical interaction between the T4 59 and 32 proteins has been extensively characterized. 32 protein has a central core with a DNA-binding site, an N-terminal B-domain needed for cooperative binding by adjacent 32 protein monomers on ssDNA, and a C-terminal A-domain needed for interactions with other replication proteins (reviewed in Ref. 38). Immobilized full-length 32 protein (5) or the A-domain alone (13) has been shown to bind 59 protein, and immobilized 59 protein binds 32 protein and 41 helicase (14). 59 protein inhibited proteolysis of the link between the core and the A-domain but not the link between the core and the B-domain. 59 protein had a similar affinity for the isolated A-domain or a truncated protein with the core and A-domain (called 32-B). Although these studies (13, 19) suggest that 59 protein contacts 32 protein through its A-domain, there is also evidence for binding to the core. Cross-linking with thio-reactive reagents demonstrated a link between Cys^{166} in the core of 32 protein and Cys^{42} in the N-domain of 59 protein but not to Cys^{215} near the C terminus of 59 protein (34). Fluorescence anisotrophy experiments suggested that the shape of 59 protein is elongated on its longest axis when bound to 32 protein (20).

In this study we have shown that deletion of the two C-terminal residues of 59 protein (Lys^{216} and Tyr^{217}) does not decrease its ability to bind 32 protein in the absence of DNA, consistent with the lack of cross-linking between 32 protein and the adjacent 59 residue Cys^{215}. Full-length 59 protein promotes the binding of 32 protein on forks with arms too short for cooperative binding by 32 protein alone (21). The C-terminal deletion mutation (ΔKY) is unable to carry out this reaction (Fig. 6). 59 ΔKY binds fork DNA as well as the full-length protein but stimulated unwinding by 41 helicase at about half the rate of wild type 59 protein (Fig. 8). Thus, the C-terminal deletion of 59 protein decreases loading of 32 protein on fork DNA in the absence of helicase and loading of the helicase in the absence of 32 protein. It is possible that deletion of the two terminal residues alters the configuration of the lagging strand fork arm on the surface of the C-domain, so that it is a poorer docking site for both the helicase and 32 protein.

Ishmael *et al.* (18) have shown that 41 helicase can be cross-linked to Cys^{215} of 59 protein in the absence, but not in the presence, of ssDNA. The site of this cross-linking on 41 helicase was not determined. An aryl azide positioned on either the C or N terminus of 41 helicase could be cross-linked to undetermined sites on 59 protein, and ssDNA did not interfere with these reactions. An interpretation of these cross-linking studies that is consistent with our results is that ss lagging strand DNA, bound on the C-domain of 59 protein, prevents close contact between the helicase and the C terminus of the loading protein.

The ΔKY deletion significantly decreased the ability of 59 protein to stimulate both leading and lagging strand synthesis (Fig. 9). In is unclear whether this results from its defect in loading the helicase or 32 protein, or both proteins, because interactions between the helicase loader and both the helicase and 32 protein are needed for leading and lagging strand synthesis. Loading the helicase is needed to open the duplex ahead of the leading strand polymerase and for the synthesis of primers on the lagging strand. Primer synthesis is highest in reactions with saturating levels of both 59 and 32 proteins, in addition to primase and helicase (36). Although 32 protein is not necessary for leading strand synthesis when the helicase loads (inefficiently) by itself (23), there is no leading strand synthesis in the absence of 32 protein when the helicase is loaded by 59 protein (21). We have previously suggested that 59 protein bound at the fork may keep the leading strand polymerase from following behind the helicase, unless the position of 59 protein is altered by its association with 32 protein. Clearly, further mutagenesis and structural studies are needed to further clarify how 59 protein binds the replication fork DNA and each of these other proteins.

[3] R. Blalock and N. G. Nossal, unpublished experiments.

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