Bacteriophage T4 32 Protein Is Required for Helicase-dependent Leading Strand Synthesis When the Helicase Is Loaded by the T4 59 Helicase-loading Protein*

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In the bacteriophage T4 DNA replication system, T4 gene 59 protein binds preferentially to fork DNA and accelerates the loading of the T4 41 helicase. 59 protein also binds the T4 32 single-stranded DNA-binding protein that coats the lagging strand template. Here we explore the function of the strong affinity between the 32 and 59 proteins at the replication fork. We show that, in contrast to the 59 helicase loader, 32 protein does not bind forked DNA more tightly than linear DNA. 59 protein displays a strong binding polarity on fork DNA, binding with much higher affinity to the 5′ single-stranded lagging strand template arm of a model fork, than to the 3′ single-stranded leading strand arm. 59 protein promotes the binding of 32 protein on forks too short for cooperative binding by 32 protein. We show that 32 protein is required for helicase-dependent leading strand DNA synthesis when the helicase is loaded by 59 protein. However, 32 protein is not required for leading strand synthesis when helicase is loaded, less efficiently, without 59 protein. Leading strand synthesis by wild type T4 polymerase is strongly inhibited when 59 protein is present without 32 protein. Because 59 protein can load the helicase on forks without 32 protein, our results are best explained by a model in which 59 helicase loader at the fork prevents the coupling of the leading strand polymerase and the helicase, unless the position of 59 protein is shifted by its association with 32 protein.

The bacteriophage T4 gene 59 helicase loading protein and gene 32 single-stranded DNA-binding protein are each part of the relatively simple multienzyme system used to replicate T4 DNA (reviewed in Refs. 1 and 2). In this system T4 DNA polymerase, which synthesizes both the leading and lagging strands, is held on the template by the circular T4 gene 45 clamp, which is loaded by the T4 gene 44/62 clamp loader. The hexameric gene 41 helicase moves on the lagging strand template, opening the duplex ahead of the leading strand polymerase and interacting with the gene 61 primase to enable it to make the pentameric RNA primers used to initiate lagging strand synthesis. Although 41 helicase can load on forked DNA templates by itself, its loading is much more rapid when the gene 59 helicase loading protein is present (3). On the lagging strand of the fork, 32 protein coats the single-stranded template strand and increases the rate of primer synthesis. 32 protein also increases the processivity of both the lagging strand polymerase and the T4 encoded 5′ to 3′ nuclease (RNase H) that removes the RNA primers and some adjacent DNA, before adjoining fragments are joined by DNA ligase (4).

Bacteriophage T4 replication begins at one of several replication origins. The products of this early origin-dependent replication invade each other to create forks for recombination-dependent replication, which is the predominant replication mode for this phage (reviewed in Refs. 5 and 6). Recombination-dependent replication requires the T4 replication proteins, as well as the phage uvsX and uvsY recombination proteins. In vitro, the 41 helicase has been shown to catalyze polar branch migration after 59 protein loads the helicase on joint molecules formed by the T4 uvsX recombinase and 32 protein (7). The helicase, 59 loader, and 32 protein are all needed for branch migration on deproteinized joint molecules (8). Mutants in T4 gene 59 are UV-sensitive and defective in recombination-dependent replication but capable of some early replication (9, 10). Although the 59 helicase loader strongly stimulates synthesis from an R-loop within the T4 uvsY origin in vitro (11), the role of 59 protein in synthesis from T4 origins in vivo is still not clear.

The 59 helicase loader is a small (26 kDa) basic protein that binds to both single- and double-stranded DNA but has a strong preference for binding forked DNA with either single- or double-stranded arms (12–14). Whereas 59 protein binds to forks with completely duplex arms, it loads the helicase only if there is a gap of at least 10 nucleotides on the lagging strand arm. 59 protein also binds and loads the helicase on model cruciforms and recombination structures, consistent with the role of 59 protein in recombination-initiated replication in vivo. The crystal structure of 59 protein is a two-domain, almost completely helical protein, with no similarity to the structures of other ssDNA1-binding proteins (12). A portion of the N-terminal domain shares strong structural similarity with several members of the high mobility group family proteins, which like 59 protein bind branched DNA structures.

59 helicase loader and 32 protein have a strong affinity for each other, even in the absence of DNA. Barry and Alberts (3) initially purified 59 protein on a column of immobilized 32 protein, and Yonesaki (15) showed that immobilized 59 protein bound both 32 protein and the 41 helicase. Morrical and co-workers have shown (16) that the 32 and 59 proteins can bind
T4 32 Protein and 59 Helicase Loader at T4 Replication Fork

**Experimental Procedures**

**Proteins**—T4 DNA polymerase (wild type and exonuclease-defective D218A (33)), 4/62 clamp loader, 45 clamp, 41 helicase, 61 primase, and 59 protein were purified as described (34). T4 32 protein missing the C-terminal A-domain (32-A) (35) and 32 protein missing the N-terminal B-domain (32-B) (36) were a gift from David P. Giedroc, Texas A&M University. The polyclonal antibody against T4 59 protein was described by Jones et al. (13).

Full-length T4 32 protein was made from the plasmid pYS6 (37), which contains gene 32 under the control of the ρ_{E. coli} promoter, in *Escherichia coli* N4830 (38), which contains a temperature-sensitive cI857 A repressor. The culture was grown overnight in 450 ml with 50 μg/ml carbenicillin (Invitrogen) at 28 °C and used to inoculate 20 liters of the same media in a large scale fermenter (New Brunswick Scientific Bioflow 3000) at 28 °C. When the optical density (A_{600}) reached 0.6, the fermentation temperature was raised to 42 °C for 30 min and then lowered to 37 °C for 2 h to induce protein expression. Cells (31.5 g) were collected by centrifugation (8,000 × g, 10 min) and frozen in large pellets (−80 °C). The frozen cell pellets were added to 300 ml of lysis buffer (25 mM BisTris-HCl, pH 6.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.3 mg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (ICN), 1 mg of hen egg white lysozyme) at 21 °C with stirring for 20 min until thawed. The lysis mixture was placed on ice, sonicated (Brandson Sonifier 250, 100% power, 50% duty cycle, 2 min, with stirring), and centrifuged (100,000 × g, 4°C, 40 min). The supernatant was diluted 2-fold with 25 mM BisTris-HCl, pH 6.5, and loaded at 20 ml/min at 4 °C onto a Q-Sepharose Fast Flow column (50-ml bed volume XP26 column, Bioquest, Amersham Biosciences), which had been equilibrated with QS buffer (30 mM BisTris-HCl, pH 6.5, 50 mM NaCl, 1 mM DTT, 10% glycerol). Protein was eluted using a linear salt gradient (30 min, 20 ml/min, 0–100% QS buffer B (QS buffer A with 0.5 M NaCl)) with 32 protein eluting at about 300 ml QS buffer. Dilution buffer (10 mM BisTris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol) was added to the pooled 32 protein fraction to match the conductivity of HA buffer A (dilution buffer 100 mM NaCl). The diluted sample was loaded onto ceramic hydroxylapatite (10 ml/min, 4°C, 50-ml bed volume, Integrated Separation Systems) equilibrated with HA buffer A. Protein was eluted with a linear salt gradient (30 min, 10 ml/min, 4 °C, 0–100% HA buffer B (HA buffer A + 1 mM (NH₄)₂SO₄)) with 32 protein eluting at about 400 ml salt. Trace nuclease activity, which copurified with 32 protein, was eliminated using hydrophobic interaction chromatography. Solid ammonium sulfate was added to the pooled 32 HA fractions until the conductivity matched that of PE buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 600 mM (NH₄)₂SO₄). The sample was applied to a POROS 20 PE perfusion weak hydrophobic column (5-ml bed volume, 4 °C, equilibrated with PE buffer, Perspective Biosystems), which had been equilibrated with PE buffer A (dilution buffer 100 mM NaCl). The flow-through fraction (~250 ml) was pooled. The flow-through was loaded onto ceramic hydroxylapatite (10 ml/min, 4°C, 50-ml bed volume, Integrated Separation Systems) equilibrated with QS buffer B and eluted with a step to QS buffer B. The purified 32 protein (20 ml, 220 mg) was dialyzed against QS buffer B (overnight, 4 °C) and frozen in aliquots (4 ml each, −80 °C).

**DNA Substrates**—The following oligonucleotides were made and reverse-phase-purified by Sigma-Genosys and were used to prepare DNA substrates. The wild-type cI857 plasmid DNA was nick-translated and then treated with S1 nuclease to produce blunt-ended DNA fragments (37). HD1 DNA substrates were prepared as described (3). The heated DNA was allowed to slowly cool to room temperature over a period of ~3 h and then purified by centrifugation through a Probe Quant G-50 spin column (Amersham Biosciences). The larger fork and partial duplexes were made by purifying 5'-32P-labeled oligonucleotide A on a G-50 spin column before annealing with the unlabeled oligonucleotide at a ratio of 1:1.5 under the same conditions.

In this paper, we explore the function of the strong affinity interaction between the 32 and 59 proteins. Recent electron microscopic studies show that the 32 protein-covered ssDNA on the lagging strand template is organized in a compact structure. This structure is significantly less compact in reactions where the helicase is loaded without the leading protein (29). Because 59 protein is still present on molecules with extensive replication, it is likely that both the 59 and 32 proteins are present on the DNA within the compact structure.

T4 32 protein has a nucleotide activity that is strongly stimulated by ssDNA, and inhibited when the DNA is covered with 32 protein. Addition of 59 protein increases the helicase-catalyzed hydrolysis of GTP or ATP on 32 protein-coated DNA. Thus, it has been proposed that a major function of 59 protein is to promote the loading of the helicase on 32 protein-coated DNA (3, 30). This would be of obvious physiological importance because the 32 protein concentration in T4-infected cells is 2-fold with 25 mM BisTris-HCl, pH 6.5, and loaded at 20 ml/min at 4 °C onto a Q-Sepharose Fast Flow column (50-ml bed volume XP26 column, Bioquest, Amersham Biosciences), which had been equilibrated with QS buffer (30 mM BisTris-HCl, pH 6.5, 50 mM NaCl, 1 mM DTT, 10% glycerol). Protein was eluted using a linear salt gradient (30 min, 20 ml/min, 0–100% QS buffer B (QS buffer A with 0.5 M NaCl)) with 32 protein eluting at about 300 ml QS buffer. Dilution buffer (10 mM BisTris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol) was added to the pooled 32 protein fraction to match the conductivity of HA buffer A (dilution buffer 100 mM NaCl). The diluted sample was loaded onto ceramic hydroxylapatite (10 ml/min, 4°C, 50-ml bed volume, Integrated Separation Systems) equilibrated with HA buffer A. Protein was eluted with a linear salt gradient (30 min, 10 ml/min, 4 °C, 0–100% HA buffer B (HA buffer A + 1 mM (NH₄)₂SO₄)) with 32 protein eluting at about 400 ml salt. Trace nuclease activity, which copurified with 32 protein, was eliminated using hydrophobic interaction chromatography. Solid ammonium sulfate was added to the pooled 32 HA fractions until the conductivity matched that of PE buffer (25 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 600 mM (NH₄)₂SO₄). The sample was applied to a POROS 20 PE perfusion weak hydrophobic column (5-ml bed volume, 4 °C, equilibrated with PE buffer, Perspective Biosystems), which had been equilibrated with PE buffer A (dilution buffer 100 mM NaCl). The flow-through fraction (~250 ml) was pooled. The flow-through was loaded onto ceramic hydroxylapatite (10 ml/min, 4°C, 50-ml bed volume, Integrated Separation Systems) equilibrated with QS buffer B and eluted with a step to QS buffer B. The purified 32 protein (20 ml, 220 mg) was dialyzed against QS buffer B (overnight, 4 °C) and frozen in aliquots (4 ml each, −80 °C).

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2 P. Chastain, E. Green, N. G. Nossal, and J. D. Griffith, unpublished experiments.
Similarly, flap DNAs were made by annealing 5′-32P-labeled oligonucleotide A with unlabelled oligonucleotides B and E (or F) (A:B:E or F, 1:1.5:10). Flap DNA substrates were further purified by electrophoresis from 6% polyacrylamide gels in 1X TBE (100 mM Tris, 90 mM boric acid, 1 mM EDTA) buffer, using an electrophoresis device (Owl Scientific, Inc.). Following electrophoresis, the purified DNA was concentrated in Microym-10 centrifuge filter devices (Millipore).

**Gel Retardation Assays**—Gel retardation assays were carried out as described (13) in 5-μl reactions containing 3 mM 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. The reaction time, temperature, and concentrations of 32 protein and the 59 helicase loader are indicated in the figure legends. Electrophoresis was carried out at 100 V in 1/2X TBE on 6% polyacrylamide DNA retardation gels (Invitrogen) that were precooled and run at 4 °C. Electrophoresis times were varied according to the DNA substrate: larger fork and flap DNA substrates for 120 min, the partial duplex DNA substrates or 56-base ssDNA for 85 min, and 12-base arm fork for 70 min. Gels were vacuum-dried on DE81 paper (Whatman), supported by Whatman 3MM paper, and autoradiographed on BioMax MR film (Eastman Kodak Co.). For quantitation, gels were exposed to Fujifilm BAS-MS imaging plates. Imaging plates were scanned on a Fuji FLA-3000 Image Analyzer (Fuji Medical Systems). Quantitation of scanned images was done, using Image Gauge software, version 3.12, from Fuji Medical Systems. The fraction of shifted density above the unshifted DNA in control reactions without 59 or 32 protein was subtracted as background from each lane.

**DNA Replication Reactions**—An 84-base oligonucleotide, complementary to positions 6198–6281 of M13mp19, was used to make the primer-template. When annealed to M13mp2 ssDNA, only the 3′ 34 bases are complementary, leaving a 60-base unpaired tail. The reaction mixtures contained 1.6 μM (circular molecules) of the primer-template, 2 mM ATP, 250 μM of each dNTP including [α-32P]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, and 20 μg/ml bovine serum albumin. Enzymes were diluted in a solution containing 50 mM Tris acetate, pH 7.5, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, 100 μg/ml bovine serum albumin, and 25% glycerol. The protein concentrations were 2 μM ssDNA-binding protein, 328 nm 59 helicase, 30 nm wild type, or D219A exonuclease-defective T4 DNA polymerase, 242 nm 44/62 clamp loader, 162 nm 45 clamp, 95 nm 59 helicase loading protein, and 64 nm 61 primase. The first stage reaction was carried out with polymerase, 44/62 clamp loader, and 45 clamp for 10 min at 37 °C. 41 helicase, 59 helicase loading protein, 61 primase, and 32 protein were added for the second stage. 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 (39) and trichloroacetic acid precipitation (34).

**RESULTS**

**Polarity of 32 Protein Binding to Fork DNA**—At the replication fork, 32 protein binds to the ssDNA unwound by the helicase. Although the parameters governing the binding of 32 protein to linear ssDNA have been studied extensively (22, 40, 41), the effect of fork structure on its binding has not been determined. We measured 32 protein binding to a fork with 30-b single-stranded arms (Fig. 1, right), a flap with a 5′-single-stranded lagging strand template arm, and a double-stranded leading strand template arm (a model for the replication fork) (Fig. 1, middle), and a flap with a 3′-single-stranded leading strand template arm (a model for the recombination intermediate formed when the invading strand is complementary to the lagging strand template) (Fig. 1, left). Titration with increasing concentrations of 32 protein (Fig. 1) showed that 32 protein bound with similar affinity to the fork DNA with two single-stranded arms (lanes 15–20) and to the flap with a single-stranded lagging strand template arm (lanes 8–13). This binding was comparable with 32 protein binding to single-stranded linear DNA under the same conditions (see Fig. 4, below). In contrast, 32 protein bound with much lower affinity to the flap with a single-stranded leading strand template arm (lanes 1–6). Thus, 32 protein displays a strong and unexpected polarity in its binding.

32 protein has a central core (residues 22–253) that contains the DNA-binding cleft, a short N-terminal B-domain (residues 1–21) that is thought to bind to the adjacent 32 protein when it is cooperatively bound to DNA, and an acidic C-terminal A-domain (residues 254–302) that has been implicated in binding to other T4 replication proteins, including the polymerase, primase, and helicase loading protein (see Introduction). To investigate which region of 32 protein was responsible for the observed polarity, we compared the binding of full-length wild type 32 protein, 32 protein without the A-domain (32-A), and the N-terminal deletion (32-B) to each flap DNA. As expected, because the A-domain has been shown to be essential for cooperative DNA binding (22), 32-B bound very poorly to both flaps (data not shown). Like full-length 32 protein, the truncated 32-A protein bound more tightly to the flap with the single-stranded lagging strand (Fig. 2, top) than to the flap with the single-stranded leading strand (Fig. 2, bottom). Although the binding polarity of 32-A was the same as that of full-length 32 protein, 32-A bound both flaps more tightly than the intact protein (Figs. 2 and 3A), in agreement with the increased affinity of 32-A for linear ssDNA (19, 22). The increased affinity of 32-A may be relevant to the function of 32 protein at the replication fork, because interaction of the A-domain with other proteins may move it away from the central core.

One possible explanation for the reduced binding of 32 protein to the flap DNA with a single-stranded leading strand is that duplex DNA at the 5′ end of a single-strand could alter the conformation of the 32 protein bound at the ss-ds junction in a way that disrupted the array of contiguous cooperatively bound 32 molecules. Alternatively, the first 32 protein might bind preferentially at a 5′ end of a single-strand, so that the array of 32 proteins assembled from the 5′ to the 3′ end of the single-strand. In either of these models, 32 protein would bind less tightly to a linear ssDNA with a duplex annealed at its 5′ end than to ssDNA with a duplex at the 3′ end. 32 protein does show weaker binding to linear ssDNA with a 5′ duplex (Fig. 4A) than to ssDNA with a 3′ duplex (Fig. 4B) or to ssDNA (Fig. 4C).

32-A also bound less tightly to ssDNA with a 5′ duplex than a 3′ duplex, but the distinction was not as great as with the full-length protein (Figs. 3 and 4). Note that the range of 32-A concentrations tested in Fig. 4 (1–40 nM) was lower than that of
FIG. 2. Removal of the A-domain from T4 32 protein does not alter the polarity of binding to flap DNA observed with the full-length protein. Like full-length 32 protein, 32 protein without the A-domain (32-A) binds with higher affinity to the flap DNA with a single-stranded lagging strand (top gels), compared with flap DNA with a single-stranded leading strand (bottom gels). However, the binding affinity of 32-A is higher than that of the full-length protein. Binding reactions were for 5 min at 25°C. DNA flap substrates are described in the Fig. 1 legend.

FIG. 3. Quantitation of the binding of 32 and 32-A proteins to DNA flaps and linear partial duplexes. A, binding to flaps was determined as shown in Fig. 2 B, binding to linear ssDNA and partial duplexes was determined as shown in Fig. 4. The fraction of DNA shifted was measured as described under “Experimental Procedures.”

FIG. 4. Binding of 32 and 32-A proteins to single-stranded linear DNA is inhibited by a duplex region at the 5′ end. Each DNA substrate was titrated with 32-A protein at a lower range of protein concentrations (1–40 nM) than the corresponding titrations with full-length 32 (10–160 nM). Binding reactions were for 5 min at 25°C. The DNA substrates in A–C were made with oligonucleotides A + D, A + L, and A, respectively.
molecules (40). On forks with arms of 30 b (Fig. 6A) and single-strands of 56 b (Fig. 6B), the complexes formed in reactions with both 59 and 32 proteins (lanes 13–15) had mobilities similar to the complexes formed in reactions with 32 protein alone (lanes 19–21). However, the quantitative supershift of these complexes by antibody to 59 protein showed clearly that 59 protein was present with the cooperatively bound 32 protein on both the longer fork (Fig. 6A, lanes 16–18) and the linear ssDNA (Fig. 6B, lanes 16–18). These results are in agreement with studies from the Morrical laboratory (17) that showed that the 59 and 32 proteins could bind simultaneously to ssDNA cellulose.

The Morrical laboratory also showed (16) that binding of the truncated 32-A protein to ssDNA impedes the binding of 59 protein to the DNA. We corroborated this result by showing that antibody to 59 protein did not quantitatively shift complexes formed in reactions of the 56-b single-stranded oligonucleotide with the 32-A and 59 proteins (Fig. 6B, compare lanes 3–5 without antibody to lanes 6–8 with antibody against 59 protein), under conditions where the complex with 59 protein alone (lane 24) and the complex with 59 and full-length 32 (lanes 16–18) were shifted. However, we found that 59 protein remained bound on most of the fork DNA in the presence of 32-A protein (Fig. 6A, compare 59 alone (lane 24) with 59 and 32-A (lanes 6–8) and 59 and 32 (lanes 16–18), consistent with the stronger binding of 59 protein to fork than linear ssDNA.

Interaction between 32 Protein and the 59 Helicase Loading Protein Is Needed to Stimulate Helicase-dependent Replication—The 59 helicase loading protein has been shown to increase helicase-dependent replication in vitro (3, 42). To determine whether 32 protein also plays a role in this reaction, we have used rolling circle replication beginning with a tailed primer annealed to M13 single-stranded DNA (Fig. 7). This reaction was carried out in two stages. In the first stage, the single-stranded 7.2-kb template was copied by extending the primer in a 10-min incubation with T4 polymerase, clamp, and clamp loader (Fig. 7, lane 1). Strand displacement synthesis from the tailed duplex circle and lagging strand synthesis on the displaced strand were then initiated by addition of 41 helicase, 61 primase, 59 helicase loader, and 32 protein. In the
leading strand products, showing that helicase has now loaded on a fraction of the molecules. The small amount of helicase that is loaded in the absence of 59 protein (Fig. 7A, Reaction 2) is also loaded in the absence of both 59 loader and 32 protein (Reaction 4). Thus 32 protein is not required for the very rapid leading strand synthesis that occurs when the helicase is loaded in the absence of the 59 helicase loading protein. In their characterization of the T4 replication system before the role of 59 protein was appreciated, Cha and Alberts (43) also noted a small amount of very rapid helicase-dependent synthesis in the absence of 32 protein. More recently, Delagoutte and von Hippel (44) showed rapid synthesis on a small fraction of the DNA molecules with only T4 polymerase and helicase when these proteins were loaded by molecular crowding with polyethylene glycol. However, 32 protein is required for the rapid helicase-dependent leading strand synthesis that occurs when 59 protein is present (compare Fig. 7A, Reaction 1 with both 32 protein and 59 loader to Reaction 3 with the 59 loader but no 32 protein). There is considerably less synthesis in Reaction 3, which contains both helicase and the loading protein but no 32 protein, than in Reaction 4, which has helicase but is missing both 32 protein and the 59 helicase loader. Because 32 protein is not required for 59 protein to load the helicase on forked substrates (13, 32), 59 protein appears to be inhibiting leading strand synthesis when 32 protein is not present. The most likely explanation for this inhibition by 59 protein (Reaction 3) is that 59 protein bound at the fork prevents the effective coupling of the polymerase extending the leading strand and the helicase unwinding the duplex ahead. In this model, the strong interaction between 32 protein and the 59 loader moves the 59 protein at the fork, so it no longer keeps the polymerase and helicase apart (see "Discussion").

Wild type T4 DNA polymerase has a strong associated 3' to 5' editing exonuclease. When it reaches an obstacle such as 59 protein at the fork, it would be expected to catalyze an idling reaction on the nascent leading strand, alternately moving backward with the exonuclease and forward with its polymerase activity. Indeed, we have shown, with a tailed nicked plasmid in which the 26 nucleotides nearest the nick were labeled, that polymerase rapidly hydrolyzed at least these 26 nucleotides when 32 protein was omitted from an otherwise complete reaction. This backward movement could prevent effective interaction between the polymerase and the helicase. Reaching the helicase should be less of a problem for an exonuclease-defective T4 polymerase, which will synthesize as far as it can and then wait for an opportunity to resume synthesis, due to movement of the annealed strand or another protein. Fig. 8 shows that there is much more leading strand synthesis with the exonuclease-defective (D219A) T4 polymerase in the absence of 32 protein than with wild type polymerase (compare Reactions 1 and 3 in Figs. 8 and 7). With wild type T4 DNA polymerase, it is clear that 32 protein is essential for leading strand synthesis when the helicase has been loaded by 59 protein.

**DISCUSSION**

Bacteriophage T4 41 helicase plays pivotal roles in both leading and lagging strand synthesis. It opens the duplex ahead of the leading strand polymerase and associates with the primase to allow it to make the pentanucleotide RNA primers that initiate lagging strand fragments (reviewed in Refs. 1, 2, 45, and 46). T4 59 protein, which accelerates the loading of this helicase, has been shown to bind to forked DNA (12–14) and the T4 32 single-stranded DNA-binding protein (3, 15–17, 30), in addition to binding to the helicase. These studies were directed
predominantly on the lagging strand. The presence of 32 increases the binding of the 44/62 clamp loader, thus facilitating the loading of the 45 clamp and polymerase on primers on both the leading and lagging strands. On the lagging strand, the clamp must be loaded repeatedly to extend the RNA primer initiating each new fragment. In addition, 32 protein covers the single-stranded lagging strand template unwound by the helicase. As described below, recent electron microscopic studies (29) show that this single-stranded DNA is organized into a compact protein-covered structure that includes both 32 and 59 proteins. Adding 32 protein to the lagging strand template increases the rate of elongation by polymerase and the rate of degradation of the primers and adjacent DNA by T4 RNase H, thus controlling how much DNA adjacent to the primers is removed before the adjacent fragments are joined (4). 32 protein has also been shown to bind directly to the 61 primase and 59 helicase loading protein, increasing the rate of primer synthesis (47).

Consistent with the need for 32 protein on the lagging strand, we find that 32 protein has a strong preference for binding to the arm of a fork that corresponds to the lagging strand template, and that it binds with higher affinity to a single-strand with a duplex at its 3′ end than to a single-strand with a 5′ duplex. The strength of binding to different DNAs is as follows: linear single-strand >> linear single-strand with duplex at 3′ end >> fork with a single-stranded lagging strand arm > linear single-strand with duplex at the 5′ end >> fork with only single-stranded leading strand arm. This order of binding strength to different DNAs would be explained if new 32 protein monomers are added more easily at the 3′ DNA end of a contiguous array of 32 monomers, and if this array is disrupted by duplex DNA at the 5′ end. During replication, 32 monomers binding to lagging strand DNA unwound by the helicase will be added at the 3′ end of the 32 cluster, and monomers will be removed from the 3′ end of an array as the discontinuous fragment is elongated by polymerase. The observation that the binding of 32 protein to linear ssDNA with a duplex at the 5′ end is significantly greater than its binding to a fork with only a single-stranded leading strand arm suggests that the duplex lagging strand arm of the fork interferes with binding of 32 protein on the leading strand arm.

59 Helicase Loader Promotes the Loading of 32 Protein on Short DNA Forks—The tight binding of 32 protein on single-stranded DNA is highly cooperative, depending on interactions between adjoining 32 protein monomers (40). Thus 32 protein binds poorly on forks with arms (12 b) too short for this cooperative binding. 59 protein, in contrast, binds tightly to forks with arms at least 9 b long (12) and allows simultaneous binding of 32 protein at concentrations where 32 protein itself does not bind (Fig. 5). These results demonstrated that the presence of 59 protein on the shorter-armed fork DNA compensated for the decreased cooperative binding of 32 protein on the limited amount of ssDNA available, enabling 32 protein to join the complex at concentrations well below that required for 32 binding to these DNA substrates in the absence of 59 protein. The 32 and 59 proteins also bond together on longer forks (30-b arms) where 32 protein can bind cooperatively. These results are in agreement with earlier studies (17) showing that the 32 and 59 proteins bound simultaneously and stoichiometrically to ssDNA cellulose.

The finding that 32 protein promotes the binding of 32 protein on short forks may be relevant to the point in the lagging strand cycle at which the helicase begins to unwind DNA behind a new primer. 32 protein by itself would be expected to bind poorly to this short region of ssDNA with a duplex at its 5′
end. Recent electron microscopic studies show that after loading the helicase, 59 protein remains with the complex of replication proteins at the fork on molecules with extensive replication. Thus, 59 protein bound at the fork would be in a position to attract 32 protein to a short stretch of ssDNA behind the new primer and could affect how 32 bound to this DNA. This proposal is supported by our finding that the 32 protein-covered ssDNA on the lagging strand of molecules being replicated by the T4 protein in vitro was in a much more compact structure than 32 protein-covered ssDNA, and that this structure was significantly looser on replicating molecules where the helicase was loaded without 59 protein (29). We have suggested previously (48) that this compact structure may play a role in determining when primase recognition sequences are accessible to the primase.

32 Protein Is Required for Helicase-dependent Leading Strand Synthesis When the Helicase Is Loaded by 59 Protein—Once T4 polymerase and helicase are loaded, no other proteins are needed for rapid strand displacement leading strand synthesis. Indeed, when these two proteins are loaded (inefficiently) by molecular crowding with polyethylene glycol, the rate of elongation on the small fraction of molecules that does have the two proteins is similar to the elongation rate in reactions with the complete replication system (49). T4 polymerase can even be replaced by the processive T7 DNA polymerase thioredoxin complex (44, 50). In the absence of 59 loader, helicase loads by itself at a reduced rate on a fraction of the molecules (3, 42) (Fig. 7). The helicase-dependent, rapid synthesis observed in the absence of 59 protein is also found when both 59 and 32 proteins are omitted (Fig. 7). However, if 32 protein is omitted when the helicase and helicase loader are both present, there is no rapid leading strand synthesis. There is significantly less synthesis when 32 is omitted in the presence of 59 protein than when both the 59 and 32 proteins are absent (Fig. 7). Thus, 32 protein is required for leading strand synthesis when the helicase is loaded by 59 protein, and in the absence of 32 protein, leading strand synthesis is strongly inhibited by 59 protein.

Why is 32 protein needed for leading strand synthesis when 59 protein is present? 32 protein is not required with 59 protein to load the helicase on forked substrates (13) and does not stimulate or inhibit unwinding of forked DNA by the helicase, although it did stimulate unwinding of a partial duplex (32). Furthermore, 32 protein is not required for leading strand synthesis without 59 protein (Fig. 7). Rapid leading strand synthesis depends on a close coupling between the polymerase extending the strand and the helicase unwinding the duplex. The elongation rate for leading strand synthesis is much faster than the rate of strand displacement synthesis in the absence of the helicase or the rate of DNA unwinding by the helicase in the absence of synthesis (reviewed in Ref. 44). It is likely that, in the absence of 32 protein, 59 protein bound at the fork prevents the effective coupling of the leading strand polymerase and the helicase. In this model, the strong interaction between 32 protein and the 59 loader moves the 59 protein at the fork so it no longer keeps the polymerase and helicase apart (Fig. 9). In support of this model, we have shown that there is more leading strand in the absence of 32 protein when wild type T4 DNA polymerase is replaced by an exonuclease-defective (D219A) mutant (Fig. 8). This would be expected because when synthesis is blocked, wild type polymerase will retreat using its 3' to 5' exonuclease, whereas the mutant will remain at the fork, waiting for an opportunity to resume synthesis. With both the wild type and mutant polymerases, the fraction of molecules with helicase-dependent leading strand synthesis is highest in reactions containing both 32 protein and the helicase loader (Figs. 7 and 8). Using the same mutant polymerase, the Benkovic laboratory initially reported that leading strand synthesis was unchanged in the absence of 32 protein (51). However, their most recent studies (52) show that leading strand synthesis decreases as the 32 protein concentration is reduced, in agreement with our experiments. The notion that 59 protein at the fork is in a position to block polymerase is supported by the finding that the exonuclease-defective polymerase and 59 protein bound on a small primed fork DNA were cross-linked to each other under conditions where polymerase did not cross-link to the helicase or primase present in the same reaction (53). The interaction between polymerase and 59 protein on forked DNA was also observed by fluorescence resonance energy transfer (53).

Replication blockage by the 59 helicase loader was also detected in our studies of synthesis from the T4 ori(uvsY) origin (11). In this system, the leading strand is synthesized by extension of the RNA in an R-loop at the origin. Rapid leading strand synthesis depends on the helicase and is stimulated by the 59 protein. There is slow leading strand synthesis when both helicase and 59 protein are omitted. 59 protein strongly blocked leading strand synthesis from this origin when helicase was not present. 32 protein was present in all these reactions. Barry and Alberts (3) observed that 59 protein inhibited strand displacement synthesis from a nicked plasmid in reactions without the helicase. Finally, the Kreuzer laboratory has reported evidence that 59 protein inhibits leading strand synthesis until the helicase is loaded in vivo (cited in Ref. 54). Taken together, these studies suggest that 59 helicase loader must associate with both 32 protein and the 41 helicase to configure the fork so that the helicase and polymerase can function together for rapid leading strand synthesis. 41 helicase and 32 protein are essential for priming and synthesis on the lagging strand. By blocking leading strand synthesis when helicase or 32 protein is missing, 59 protein helps to coordinate synthesis on the two strands.

4 C. E. Jones, unpublished experiments.
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