Control of Helicase Loading in the Coupled DNA Replication and Recombination Systems of Bacteriophage T4

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Background: Helicase loading protein Gp59 coordinates recombination-dependent DNA replication in bacteriophage T4.

Results: A DNA binding-deficient mutant, Gp59-I87A, loads helicase normally onto ssDNA covered with the ssDNA-binding protein, Gp32.

Conclusion: Gp59-Gp32 interactions control helicase loading onto D-loops during recombination-dependent replication.

Significance: Helicase loading proteins work in concert with single-stranded DNA binding proteins to control the location and timing of helicase assembly in DNA replication, recombination, and repair.

The Gp59 protein of bacteriophage T4 promotes DNA replication by loading the replicative helicase, Gp41, onto replication forks and recombination intermediates. Gp59 also blocks DNA synthesis by Gp43 polymerase until Gp41 is loaded, ensuring that synthesis is tightly coupled to unwinding. The distinct polymerase blocking and helicase loading activities of Gp59 likely involve different binding interactions with DNA and protein partners. Here, we investigate how interactions of Gp59 with DNA and Gp32, the T4 single-stranded DNA (ssDNA)-binding protein, are related to these activities. A previously characterized mutant, Gp59-I87A, exhibits markedly reduced affinity for ssDNA and pseudo-fork DNA substrates. We demonstrate that on Gp32-covered ssDNA, the DNA binding defect of Gp59-I87A is not detrimental to helicase loading and translocation. In contrast, on pseudo-fork DNA the I87A mutation is detrimental to helicase loading and unwinding in the presence or absence of Gp32. Other results indicate that Gp32 binding to lagging strand ssDNA relieves the blockage of Gp43 polymerase activity by Gp59, whereas the inhibition of Gp43 exonuclease activity is maintained. Our findings suggest that Gp59-Gp32 and Gp59-DNA interactions perform separate but complementary roles in T4 DNA metabolism; Gp59-Gp32 interactions are needed to load Gp41 onto D-loops, and other nucleoprotein structures containing clusters of Gp32. Gp59-DNA interactions are needed to load Gp41 onto nascent or collapsed replication forks lacking clusters of Gp32 and to coordinate bidirectional replication from T4 origins. The dual functionalities of Gp59 allow it to promote the initiation or re-start of DNA replication from a wide variety of recombination and replication intermediates.

Bacteriophage T4, a well established model system for DNA replication and recombination, relies on carefully controlled functions of its replication proteins for the initiation of DNA synthesis (1–3). The T4 Gp59 protein plays critical roles in the assembly of the phage replisome and its coordination with homologous recombination (4, 5). Gp59 is a versatile mediator protein that loads the replicative DNA helicase, Gp41, onto replication forks and recombination intermediates that are generated during different stages of the T4 infection cycle in its host organism, Escherichia coli.

Bacteriophage T4 replicates its genome by two different processes, origin-dependent replication (ODR)3 and recombination-dependent replication (RDR) (3, 6). The ODR pathway functions during early infection and involves an R-loop initiation mechanism that is modulated by Gp59. Later in infection, ODR is suppressed, and RDR is activated, greatly amplifying the phage burst size. RDR initiates from strand invasion of a homologous duplex by a single-stranded chromosome end. The resulting “D-loop” is the intermediate structure on which the replisomal components assemble. T4 RDR is a model for highly conserved homology-directed repair pathways that carry out error-free repair of DNA double-strand breaks and stalled replication forks (1). Gp59 is a critical mediator protein in the coordination of T4 RDR, making it a protein of interest for the study of homology-directed repair.

The strict requirement for Gp59 in T4 RDR is a consequence of its primary function: helicase loading (7, 8). Across many species, helicase loading onto recombination intermediates appears to be a critical step for the coupling of recombination to DNA synthesis, as occurs in all homology-directed repair pathways (1). In T4 RDR, Gp59 is responsible for loading the replicative helicase, Gp41, onto the displaced strand of the D-loop. This event is what enables DNA unwinding to occur ahead of the polymerase during RDR leading strand synthesis. It is also what initiates RDR lagging strand synthesis, as Gp41 is an essential component of the primosome (9). Gp59 can also load Gp41 onto sites other than D-loops, such as at replication origins or four-way junctions (4).

In addition to helicase loading, Gp59 has been shown to perform a second function: polymerase blocking (10–12). Gp59

3 The abbreviations used are: ODR, origin-dependent replication; RDR, recombination-dependent replication; ssDNA, single-stranded DNA; HLC, helicase loading complex; PBC, polymerase blocking complex; ATPγS, adenosine-5′-(3-thio)-triphosphate.
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inhibits the DNA synthesis and exonuclease activities of the phage polymerase, Gp43, until the replisome is fully assembled. Helicase loading is the step that completes replisome assembly, which releases the polymerase block (10). The polymerase blocking activity of Gp59 prevents premature leading strand synthesis, which ensures that leading and lagging strand synthesis are coupled to each other and to the unwinding activity of Gp41 helicase (13). The polymerase blocking activity of Gp59 appears to have an important role in coordinating bidirectional DNA replication during T4 ODR (13).

The versatility of Gp59 function is clearly a critical factor in the coordination of both origin- and recombination-dependent replication. Questions remain about the dual functionality of Gp59, however. Specifically, the functional consequences of some of its binding interactions remain unclear. Gp59 interacts with several replisomal proteins, including Gp32, the T4 single-stranded DNA-binding protein, in addition to Gp41 and Gp43 (14–16). Gp59 also interacts with DNA in a structure-selective manner, binding to model substrates with the following relative affinities: pseudo-fork DNA > ssDNA > dsDNA (17). The large number of interaction partners for Gp59 presents the challenge of determining which interactions are necessary for the Gp59-mediated steps of T4 RDR and ODR. In this study, we investigate the correlation between Gp59-DNA interactions, helicase loading, and polymerase blocking activities.

Previous work suggested that the binding of Gp59 to fork DNA structures is important for its helicase loading function in the absence of Gp32 (18). However, under physiological conditions during RDR, Gp59 most likely co-occupies ssDNA with Gp32 to form a tripartite helicase loading complex (HLC) in which the DNA binding properties of both proteins are altered (19). Additionally, we recently provided evidence that Gp32 clusters on ssDNA may be the target for Gp59 binding rather than DNA itself and that Gp32 and Gp59 likely remain bound to each other throughout T4 RDR (20). Based on these observations, we hypothesize that helicase loading in RDR may depend more on Gp59-Gp32 interactions than on Gp59-DNA interactions.

Our hypotheses regarding polymerase blocking are also based on previous results. Gp59 has been shown to form direct interactions with Gp43 (16). Gp59 has the ability to inhibit both 5’ → 3’ polymerase and 3’ → 5’ exonuclease activities of Gp43, the latter of which is used as a proofreading mechanism (21). Evidence suggests that there may be two mechanisms of polymerase blocking, that polymerase inhibition requires only Gp59-fork DNA interactions, whereas exonuclease inhibition requires a Gp43-Gp59 interaction (12). Therefore, we hypothesize that a DNA binding defect of Gp59 should result in decreased inhibition of DNA synthesis but should have no effect on the inhibition of exonuclease activity. We further hypothesize that Gp32 may affect polymerase blocking by Gp59, based on a previous report that Gp32 is required for leading strand synthesis driven by a Gp59-loaded helicase (22).

To test these hypotheses, our experimental strategy was to use a Gp59 mutant that is defective in DNA binding and directly compare it to Gp59 wild type in assays specific for each function. A logical choice was Gp59-I87A, which was reported to have reduced binding affinity for both ssDNA and pseudo-fork DNA (18). I87A also exhibited a reduced stimulation of helicase unwinding activity and a reduced ability to stimulate DNA synthesis in a reconstituted in vitro assay (18). Models of the Gp59-fork DNA structure suggest that Ile-87 occupies a position close to lagging strand ssDNA and near where the duplex and single-stranded arms of the fork separate (Fig. 1A) (18, 32). In this study we extend the characterization of the I87A mutant by employing additional functional assays. We provide evidence that 1) on Gp32-covered ssDNA a DNA binding defect of Gp59 is not detrimental to the formation of a helicase loading complex or to the processes of helicase loading or translocation, 2) on fork DNA a DNA binding defect of Gp59 is detrimental to helicase loading in the presence or absence of Gp32, and 3) Gp32 negatively affects the inhibition of Gp43 polymerase activity by Gp59 but not the inhibition of Gp43 exonuclease activity. These findings provide new insights into the mechanisms used to initiate T4 recombination- and origin-dependent DNA replication and repair processes.

EXPERIMENTAL PROCEDURES

Reagents and Enzymes—Concentrations of reagents and buffer components given in the text are final concentrations. γ-[32P]-Labeled ATP was purchased from PerkinElmer Life Sciences. Unlabeled ATP and deoxyribonucleotides were purchased from U. S. Biochemical Corp. T4 polynucleotide kinase was purchased from New England Biolabs. Analytical grade chemicals and reagents were purchased from Sigma unless otherwise indicated. 6-iodoacetamidofluorescein was purchased from Invitrogen. 6× protein loading dye was purchased from Promega. Buffers and solutions were all prepared using deionized water.

Nucleic Acids—All DNA concentrations given in the text are final concentrations. Unless otherwise indicated, DNA concentrations are reported in units of nucleotide residues. Circular M13mp18 ssDNA was purchased from New England Biolabs, and its concentration was provided by the manufacturer. All oligonucleotides were purchased from Operon Biotechnologies, Inc. Their sequences are listed in Table 1. The sequences of Oligos 4 and 6 were obtained from previously published work (18) in which the same two-stranded pseudo-fork substrate was used for functional assays with Gp59. The sequences of Oligos 7, 8, and 9 were also obtained from previously published work (10) in which the three-stranded replication fork mimic substrate was used for polymerase blocking assays. All oligonucleotides were HPLC-purified by the manufacturer. Oligonucleotide concentrations were determined spectrophotometrically by measuring the absorbance at 260 nm using the extinction coefficients provided by the manufacturer.

T4 Proteins—All protein concentrations given in the text are final concentrations. Gp32, Gp41, and Gp59 proteins were purified as described previously (14, 20). Gp43-D219A, an exonuclease-deficient form of the Gp43 T4 polymerase, was a gift from Dr. Linda Reha-Krantz (University of Alberta). Wild-type Gp43 was purchased from New England Biolabs. Nuclease contamination assays were based on a previously published protocol (8). These were carried out to verify the purity of all protein stock solutions. All proteins were also run on SDS-PAGE gels to verify that they were >95% pure.
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Site-directed Mutagenesis of Gp59—The Gp59-expressing custom IMPACT vector described in Ishmael et al. (14) was mutated to introduce the I87A substitution using the QuikChange method (Stratagene). Primer sequences used for this protocol are shown in Table 1 as Oligos 1 and 2. The presence of the I87A mutation was verified by DNA sequencing (Vermont Cancer Center DNA Sequencing Core Facility). Expression and purification protocols for the Gp59-I87A protein were identical to those used for wild-type Gp59.

Labeling of Gp32 with 6-Iodoacetamidofluorescein—Gp32 labeled with 6-iodeacetamidofluorescein (Gp32F) was produced as previously described (23). Labeling efficiency was calculated according to the manufacturer’s instructions using the following extinction coefficients: 41,300 M⁻¹ cm⁻¹ at 280 nm and 68,500 M⁻¹ cm⁻¹ at 494 nm (Invitrogen).

DNA Binding Assays—Electrophoretic mobility shift assays were carried out to compare the DNA binding affinities of Gp59 WT and I87A. Total reaction volumes were 10 μl, and the reaction buffer contained the following components: 25 mM Tris acetate, pH 7.8, 60 mM potassium acetate, 6 mM magnesium acetate, 2 mM DTT, 200 μg/ml BSA, and 2 mM ATP. A 60-mer oligonucleotide, Oligo 3, was 5'-32P-labeled using γ-32P[ATP and T4 polynucleotide kinase. First, 3 nM (molecules) of labeled Oligo 3 was added to each reaction sample. Next, Gp59 (WT or I87A) was added to each sample in the following concentrations: 0, 100, 200, 300, or 360 nM. Next, 2 μl of 6× loading dye were added. Samples were mixed and incubated for 3 min on ice before gel loading. Protein-DNA complexes were separated from unbound ssDNA by running on native 12% polyacrylamide gels, visualized by phosphorimaging using a Bio-Rad Molecular Imager FX, and analyzed by densitometry using Quantity One software (Bio-Rad).

Fluorescence Assays for Assembly and Helicase Loading Activity of Helicase Loading Complexes—Changes in Gp32F fluorescence were monitored to detect HLC formation on ssDNA and its remodeling upon helicase loading, as previously described (20). Reactions were carried out at room temperature. Using an excitation wavelength of 460 nm, the fluorescence emission was scanned over a range of wavelengths from 480 to 580 nm. The excitation and emission slit widths were set at 4 nm. Reaction buffer contained the following components: 25 mM Tris acetate, pH 7.8, 25 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT. First, the fluorescence emission of 100 nM Gp32F alone was scanned. Next, 700 nM (nucleotides) of either Oligo 4 or Oligo 5 was added to the cuvette and mixed followed by another scan of Gp32F fluorescence. 100 nM Gp59 (WT or I87A) was then added to the cuvette, and a final scan of Gp32F fluorescence was taken. Subsequently, the fluorescence emission of this complex at 519 nm was monitored as a function of time using an excitation wavelength of 460 nm. After acquisition of a stable signal, the scan was paused while 100 nM Gp41 and 1 mM ATP were added simultaneously. Data collection was resumed, and the fluorescence was monitored for up to 1000 s. Data were compared with control reactions containing Gp59 storage buffer in place of Gp59 WT or I87A protein.

Similar assays were performed to monitor the formation and remodeling of HLC on a DNA pseudo-fork substrate, Substrate C (Fig. 1B). The pseudo-fork was constructed by annealing Oligo 4 (60-mer) to the partially complementary Oligo 6 (56-mer) as described (24). Reactions were carried out at room temperature. Reaction buffer contained the following components: 25 mM Tris acetate, pH 7.8, 90 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT. Reaction components were added in the order 100 nM Gp32F, 14.3 nM (molecules) Substrate C, and 100 nM Gp59 (WT or I87A); a fluorescence spectrum was obtained after each incubation. The fluorescence of this complex was monitored at 519 nm. 100 nM Gp41 and 500 μM ATP were then added simultaneously, and the fluorescence was continually monitored for 500 s after this addition. Controls were carried out in which the ATP was replaced with an equal volume of water.

ATPase Assays—ATPase assays were based on previously published spectrophotometric assays with Gp59 and Gp41 (8). For all ATPase assays, a Varian Cary Bio 50 UV-visible spectrophotometer was used. The Varian Cary Single Cell Peltier was used to maintain the temperature at 37 °C during the experiments. The following buffer was used for ATPase reactions: 25 mM Tris acetate, pH 7.8, 90 mM potassium acetate, 10 mM magnesium acetate, and 1 mM DTT. The following components were incubated in a cuvette in a total volume of 700 μl for 5 min before initiating the reaction: 10 μM (nucleotides) M13mp18 ssDNA, 500 nM Gp32, 500 nM Gp59 (WT or I87A), and 250 nM Gp41. Each reaction was initiated by the addition of 1 mM ATP. Absorbance was monitored at 380 nm over a time course of 15 min after ATP addition. The rate of ATP hydrolysis was determined by converting the absorbance to concentration of NADH and plotting it versus time using an extinction coefficient of NADH at 380 nm of 1.3 mm⁻¹ cm⁻¹. Rates of ATP hydrolysis were determined by calculating the slopes at the linear portions of each absorbance scan. The decrease in concentration of NADH (nmol min⁻¹) is equivalent to the concentration of ATP hydrolyzed by Gp41 (nmol min⁻¹).

Helicase Unwinding Assays—Oligo 6 was 5'-32P-labeled using γ-32P[ATP and T4 polynucleotide kinase. The labeled Oligo 6 was then annealed to Oligo 4 to generate a labeled version of Substrate C, the pseudo-fork DNA substrate. Helicase unwinding reactions were carried out at room temperature in reaction buffer containing 25 mM Tris acetate, pH 7.8, 60 mM potassium acetate, 6 mM magnesium acetate, 2 mM DTT, 200 μg/ml BSA, and 2 mM ATP. Reagents were added in the following order in a total reaction volume of 15 μl: 30 nM (molecules) labeled Substrate C, 300 nM Gp32, and 300 nM Gp59 (WT or I87A). Reactions were initiated by the simultaneous addition of 300 nM Gp41 and 120 nM DNA trap (unlabeled Oligo 6). Reactions were allowed to proceed for 1 min, then stopped by adding 15 μl of the following stopping solution: 100 mM EDTA, 2.6% SDS, and 2× loading dye. Reactions were carried out both in the presence and absence of Gp32. Controls were performed in which Gp32, Gp59, and Gp41 were individually replaced with their respective storage buffers. Products were separated on native 12% polyacrylamide gels. Gels were visualized by exposure on a phosphorimaging K-Screen by a Molecular Imager FX (Bio-Rad) and analyzed by densitometry using Quantity One.
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RESULTS

Comparison of DNA Binding Activities of Gp59 WT and I87A—Fig. 1A shows the position of Gp59 residue Ile-87 in the context of the structural model for Gp59 bound to pseudo-fork DNA and the ssDNA binding domain of Gp32 (32). Ile-87 is located in the N-terminal HMG homology domain of Gp59, close to the 5′ ssDNA arm of the pseudo-fork (which corresponds to the lagging strand ssDNA of a replication fork, where Gp59 would load Gp41 helicase). The DNA binding defect of Gp59-I87A was previously reported (18). We verified this defect by performing electrophoretic mobility shift assays (Fig. 2). Gp59 wild-type (WT) binds to a single-stranded 60-mer (Oligo 3, Table 1), whereas Gp59-I87A exhibits little or no binding to the same oligo (Fig. 2, A and B). Identical results were obtained using Oligo 6 (data not shown), a single-stranded 56-mer with a different sequence, demonstrating that the ssDNA binding deficiency of I87A does not depend on a particular DNA sequence. Similarly, the binding of I87A to a 56/60-mer DNA pseudo-fork (Substrate C; Fig. 1B) is greatly reduced compared with WT (Fig. 2, C and D). Note that Gp59 WT appears to bind with higher affinity to the pseudo-fork compared with ssDNA alone (Fig. 2, B and D). These results reproduce the findings of Jones et al. (18). Therefore, the results in Fig. 2 confirm that the I87A mutation markedly decreases the affinity of Gp59 for fork DNA and severely decreases its affinity for ssDNA.

Effect of I87A Mutation on Formation of Gp59-Gp32-ssDNA Helicase Loading Complex—It was previously shown that Gp32F, a conjugate of Gp32 and fluorescein, displays similar ssDNA binding properties compared with unmodified Gp32 (23). We have also shown that the fluorescence intensity of Gp32F is increased when it is ssDNA-bound and further increased when it is in a helicase loading complex with ssDNA and Gp59 (20, 23). This Gp32F fluorescence assay was used to determine the effects of the Gp59-I87A mutation on HLC formation (Fig. 3). The experiments were carried out using two ssDNA oligonucleotides with different lengths and sequences, either Oligo 4 (60-mer, Fig. 3A) or Oligo 5 (70-mer, Fig. 3B), with identical results. The average fluorescence increase of Gp32F resulting from the addition of either oligo was greater than 2-fold. As expected, the addition of Gp59 WT to the ssDNA-bound Gp32F resulted in an additional fluorescence enhancement of ~30% (Fig. 3, A and B). An identical result was obtained when Gp59-I87A was added instead of WT (Fig. 3, A and B). These results indicate that Gp59-I87A retains the ability to form a helicase loading complex on Gp32-covered ssDNA despite its ssDNA binding defect. HLC formation by both WT
FIGURE 1. A, model of Gp59 (cyan) bound to pseudo-fork DNA and the ssDNA binding domain of Gp32 (green). The positions of point mutations that affect DNA binding are shown in blue. Residue Ile-87, site of the I87A point mutation that attenuates DNA binding, is located in the N-terminal HMG homology domain of Gp59 and close to the 5’ ssDNA arm of pseudo-fork DNA. The 5’ arm corresponds to the lagging strand ssDNA of a replication fork, the site where Gp59 loads Gp41 helicase. This research was originally published in Hinerman et al. (32). B, representation of the fork DNA substrates used for experiments. Substrates were either fork mimics (Substrates A and B) or pseudo-fork (Substrate C) structures. Sequences of the oligonucleotides used in their construction are shown in Table 1. Asterisks denote the positions of 5’-32P labels.

FIGURE 2. Electrophoretic mobility shift assays for Gp59-DNA interactions; comparisons of I87A to WT. DNA binding assays were performed as described under “Experimental Procedures.” A, binding to 60-mer ssDNA (Oligo 3). The concentration of 60-mer in each lane is 3 nM (molecules). Lanes 1–10 contain 0–360 nM Gp59 WT as indicated. Lanes 6–10 contain 0–360 nM Gp59-I87A as indicated. B, quantified results from panel A. Plot shows the fraction ssDNA bound versus Gp59 concentration for Gp59 WT (solid line) or I87A (dotted line). C, binding to pseudo-fork DNA (Substrate C). The concentration of Substrate C in each lane is 3 nM (molecules). Protein concentrations are the same as in A. D, quantified results from panel C. The plot shows the fraction pseudo-fork DNA bound versus Gp59 concentration for Gp59 WT (solid line) or I87A (dotted line). Error bars in panels B and D represent S.D. from three separate experiments.
Effects of I87A Mutation on HLC Remodeling during Helicase Loading on ssDNA—It was previously shown through Gp32F fluorescence assays that the loading of Gp41 helicase remodels the HLC and that this remodeling is dependent on ATP binding (but not hydrolysis) by Gp41 (20) (Fig. 4A). Gp59-I87A was used to form HLC on the 70-mer (Oligo 5) to determine whether the mutation affects the remodeling process. Fig. 4B shows that when Gp41 is added to HLC containing WT Gp59 in the presence of ATP, Gp32F fluorescence decreases by ~10% over a time course of 4–5 min, consistent with previous results (20). This fluorescence change is the signature of HLC remodeling. Gp32F fluorescence changes at a similar rate and to a similar extent (~13%) when the I87A mutant replaces WT Gp59 in the reaction (Fig. 4B). The observed Gp32F fluorescence change was Gp59-dependent (Fig. 4B) as well as ATP-, Gp41-, and ssDNA-dependent (data not shown). The results shown in Fig. 4 demonstrate that despite the ssDNA binding defect of Gp59-I87A, helicase loading complexes formed with this mutant retain the ability to recruit Gp41 helicase onto Gp32-covered ssDNA and to be remodeled by Gp41-ATP.

Gp59-I87A Promotes Gp41 Translocation on Gp32-covered ssDNA—Previous studies established that the ATPase-coupled translocation of Gp41 helicase on Gp32-covered ssDNA requires Gp59 (8, 20). Spectrophotometric ATPase assays (data not shown) were used to quantitatively compare the ability of Gp59 WT versus I87A to promote Gp41 translocation on long circular ssDNA molecules complexed with Gp32. In the presence of Gp32-ssDNA, Gp41 hydrolyzes ATP at a steady-state rate of 0.4 ± 0.0 nmol min⁻¹ when no Gp59 was added. When Gp59 WT was added the rate improved to 15.4 ± 0.5 nmol min⁻¹. Alternatively, when Gp59-I87A was added the rate was 17.7 ± 2.1 nmol min⁻¹. Thus both WT and I87A forms of Gp59 increase the ATPase activity of Gp41 by ~40-fold in the presence of Gp32-ssDNA. Gp59 lowers the apparent Km of Gp41 for ATP without greatly affecting Vmax (8). Therefore, under the experimental conditions (ATP ≈ apparent Km), the addition of Gp59 would directly stimulate the ssDNA-dependent ATPase activity of Gp41. The data suggest that Gp59-I87A is just as
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**FIGURE 4. Effects of Gp59-I87A mutation on helicase loading activity.** Changes in Gp32F fluorescence were used to monitor the loading of Gp41 helicase onto 70mer ssDNA (Oligo 5) as described under “Experimental Procedures.” The components of the helicase loading complex were added in the following order and preincubated: 100 nM Gp32F, 700 nM ssDNA, and 100 nM Gp59 (WT or I87A). Helicase loading reactions were initiated by the simultaneous addition of 100 nM Gp41 and 1 mM ATP. A, reaction schematic for helicase loading assays. Green circles, Gp32F; solid black line, ssDNA; red triangles, Gp59; purple triangles, Gp41 monomers (hexamerizes upon assembly onto ssDNA). B, relative fluorescence of the helicase loading complex versus time after Gp41/ATP addition in the presence of Gp59 WT (red), Gp59 I87A (black), or in the absence of Gp59 (purple).

effective as wild type at this stimulation. ATP hydrolysis by Gp41 is strongly coupled to its translocation on ssDNA, which is initiated by the helicase loading activity of Gp59 when the ssDNA is covered with Gp32. Therefore, the observed stimulation of Gp41 ATPase by Gp59 WT and I87A likely involves a combination of increased loading of the Gp41 onto Gp32-ssDNA (as confirmed by fluorescence experiments in Fig. 4) and direct activation of Gp41 enzymatic activity. These results demonstrate that Gp59-I87A and WT have similar capacities to promote helicase translocation activity on Gp32-covered ssDNA.

**Effect of the I87A Mutation on Gp41 DNA Unwinding Activity—** It was previously reported that Gp59-I87A has a weakened ability to stimulate DNA unwinding by Gp41 (18). In this study we added Gp32 to the unwinding reactions to observe whether Gp59-I87A would behave similarly to WT in its presence. The assay employed pseudo-fork Substrate C; the design of the unwinding assay is shown in Fig. 5A. Reactions were first run in the absence of Gp32 (Fig. 5B, lanes 2 and 3), the results of which confirm that I87A has weaker helicase stimulating activity than WT, consistent with published results (18). Specifically, the unwinding reaction in the presence of I87A resulted in the formation of ~57% of the product formed in the presence of Gp59 WT (Fig. 5C). The addition of Gp32 lowered the amounts of unwinding observed in both reactions; however, it did not change the result that I87A is weaker than WT at stimulating helicase unwinding (Fig. 5, B and C). Reactions containing I87A and Gp32 yielded ~64% of the unwound product seen in reactions containing WT and Gp32. The observation that Gp59-I87A has a weaker helicase stimulating activity than WT under these conditions suggests that Gp59-DNA interactions play a more important role in the unwinding of fork DNA by Gp41 than they do in the loading and translocation of Gp41 on Gp32-covered ssDNA.

Gp59-I87A Forms Helicase Loading Complexes on Pseudo-fork DNA—Pseudo-fork Substrate C (Fig. 6A) was used to compare the HLC formation and remodeling behaviors of Gp59 WT and I87A. The fluorescence enhancement corresponding to HLC formation on pseudo-fork DNA occurs with Gp59 WT, consistent with previous results (20). When an equal concentration of I87A replaces WT in the reaction, the identical fluorescence enhancement occurs, indicating that the HLC still forms on the pseudo-fork in the presence of the Gp59 mutant (Fig. 6B).

The HLC formed with Gp59 WT on pseudo-fork DNA undergoes a sharp fluorescence drop upon the addition of Gp41 and ATPγS (Fig. 6C), consistent with the rapid remodeling of the HLC as previously reported (20). When ATPγS is left out, no fluorescence drop occurs (Fig. 6C), indicating that the remodeling effect on the pseudo-fork HLC is dependent on active loading of Gp41, similar to the result on ssDNA (Fig. 4C). When Gp59 WT is replaced by I87A in the reaction, the fluorescence change occurs as a gradual decrease rather than a sharp drop (Fig. 6C), resembling the results seen with these assays on single-stranded oligos. This slow fluorescence change does not occur in the absence of ATPγS, however, confirming that it is also dependent on Gp41 loading (Fig. 6C). Therefore, although Gp59-I87A and WT form qualitatively similar helicase loading complexes on fork DNA, the kinetics of HLC remodeling upon helicase recruitment are much slower for the mutant. The kinetic effect of Gp59-I87A is consistent with slower loading or activation of Gp41 on pseudo-fork DNA by the altered HLC, which would explain the weaker stimulation of helicase activity by I87A compared with WT.

**Effects of the Gp59-I87A Mutation on Polymerase Blocking Activity—** The abilities of Gp59 WT and I87A to interfere with the 5′ → 3′ strand displacement DNA synthesis and 3′ → 5′ exonuclease functions of T4 DNA polymerase were compared. These assays employed the replication fork mimic Substrate A. A schematic of the DNA synthesis assay is shown in Fig. 7A, and the corresponding data are shown in Fig. 7, C and E. These assays also employed the exonuclease-deficient D219A mutant of Gp43 polymerase to avoid primer degradation that would interfere with the measurement of DNA synthesis (10). As
shown in Fig. 7, C and E, Gp59 WT and I87A exhibit equal blockage of polymerase activity under the conditions of these assays. Both inhibit primer extension by ~45%.

A schematic of the exonuclease assay is shown in Fig. 7B, and the corresponding data are shown in Fig. 7, D and F. These assays employed the exonuclease-proficient, wild-type Gp43 polymerase in the absence of deoxyribonucleotide substrates, so that the extent of primer degradation would not be masked by new DNA synthesis (10). As shown in Fig. 7, D and F, Gp59 WT and I87A exhibit equal blockage of polymerase 3' → 5' exonuclease activity under the conditions of these assays. Both inhibit exonuclease activity by ~75%. The observation that Gp59-I87A inhibits polymerase activities to the same extent as WT, despite its DNA binding defect, suggests that DNA binding affinity is not a critical aspect of Gp59 polymerase blocking function.

**Effects of Gp32 on Polymerase Blocking Activity of Gp59**—HLC formation by Gp59 and Gp32 on lagging strand ssDNA could affect the polymerase blocking functions of Gp59. To test this possibility, we measured Gp59 polymerase blocking activity in the presence and absence of Gp32. The effect of lagging strand ssDNA length was also tested because this parameter could affect the efficiency of HLC formation. Fig. 8 shows DNA synthesis and exonuclease reactions of Gp43 polymerase in the presence and absence of Gp32 and/or Gp59. Assays were conducted using two different fork mimic DNA constructs: one
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FIGURE 6. Effects of Gp59-I87A mutation on HLC formation and helicase loading on pseudo-fork DNA. A, unlabeled pseudo-fork DNA (Substrate C) contained 56-mer (Oligo 6) annealed to 60-mer (Oligo 4). B, Gp32F fluorescence assays for HLC formation on pseudo-fork DNA were carried out as described under “Experimental Procedures.” Shown are fluorescence emission spectra of 100 nM Gp32F (green), 100 nM Gp32F + 14.3 nM (molecules) Substrate C (blue), 100 nM Gp32F + 14.3 nM Substrate C + 100 nM Gp59 WT (red), and 100 nM Gp32F + 14.3 nM Substrate C + 100 nM Gp59-I87A (black). C, changes in Gp32F fluorescence were used to monitor the loading of Gp41 helicase onto pseudo-fork DNA, as described under “Experimental Procedures.” 100 nM Gp32F was preincubated with 14.3 nM Substrate C followed by the addition of 100 nM concentrations of either Gp59 WT or I87A and a stable signal was attained. 100 nM Gp41 was then added either alone or in combination with 500 μM ATPγS, and the change in fluorescence was monitored versus time. Individual traces represent reactions containing Gp59 WT (gray), Gp59 I87A (green), Gp59 WT + ATPγS (red), or Gp59 I87A + ATPγS (black).

containing a 7-base 5’ ssDNA flap (Substrate A, Fig. 8A) and the other containing a 30-base 5’ ssDNA flap (Substrate B, Fig. 8B). When Gp32 was present in the absence of Gp59, we observed slightly more efficient DNA synthesis by T4 polymerase using Substrate A with the 7-base flap (Fig. 8C, lanes 1 and 3). This effect was more pronounced using Substrate B with the 30-base flap (Fig. 8D, lanes 1 and 3), which is consistent with the cooperative ssDNA binding properties of Gp32. When Gp59 WT was added to reactions with Substrate A, it was able to block DNA synthesis with equal efficiency in the presence and absence of Gp32 (Fig. 8C, lanes 2 and 4; results are quantified in Fig. 8G). In contrast, when Gp59 WT was added to reactions with Substrate B, its blockage of DNA synthesis was decreased significantly in the presence of Gp32 (Fig. 8D, lanes 2 and 4; results are quantified in Fig. 8G). These results are consistent with the notion that HLC formation by Gp59 and Gp32 occurs efficiently only on the longer ssDNA segment, which is consistent with previously published results (20). The data suggest that HLC formation reorganizes Gp59 at the fork so that it is less likely to interfere with the DNA synthesis activity of Gp43.

In contrast to its effects on DNA synthesis, the blockage of Gp43 exonuclease activity by Gp59 WT was found to be independent of both Gp32 and flap length under the conditions of our experiments (Fig. 8, E and F; results are quantified in Fig. 8H). This suggests that the reorganization of Gp59-DNA interactions that occurs upon HLC formation can relieve the blockage to DNA synthesis without exposing the primer to unwanted degradation.

**DISCUSSION**

The Versatility of Gp59 Function Is Driven by Its Broad Spectrum of Protein-Protein and Protein-DNA Interactions—Gp59 differs from other helicase loading proteins in its versatility of function. Gp59 has the ability to load Gp41 onto replication origins, nascent replication forks, stalled replication forks, D-loops, R-loops, and Holliday junctions (4). In other systems, distinct helicase loading proteins are required to load the correct helicases onto these different DNA structures. For example, in *E. coli*, DnaC is required to load the DnaB helicase onto replication origins in the presence of a third, initiator protein, DnaA (25). A separate helicase loading protein, PriA, loads DnaB onto D-loops, R-loops, and stalled replication forks (26, 27). Meanwhile, RuvA recruits the DNA motor protein, RuvB, to Holliday junctions to promote branch migration (28). The mechanisms of these helicase loading proteins are more specialized than Gp59, which is the only protein of its type in the T4 system and is responsible for many of the helicase loading events involving the replicative helicase, Gp41. The versatility of Gp59 function is increased further by its polymerase blocking activities.

The functional versatility of Gp59 is the result of its ability to recognize different types of nucleoprotein structures. The helicase loading and polymerase blocking activities of Gp59 are controlled not only by its structure-selective DNA binding activity but also by specific interactions with Gp32, Gp41, and Gp43 (10–12, 17, 18, 20, 22). The strong interaction of Gp59 with Gp32 has emerged as a major factor in the loading of Gp41 helicase onto Gp32-covered ssDNA (19, 20, 29–31). Direct interactions of Gp59 with Gp41 and Gp43 are essential for helicase loading and for inhibition of exonuclease activity, respectively (12). Structural biology has provided insights into the Gp59 ability to recognize different DNA structures, from simple ssDNA oligos to replication fork mimics (24, 32). The N-terminal domain is structurally homologous to the HMG (high mobility group) proteins, which are known to recognize the
minor groove of duplex DNA (33–35). Docking models suggest that the N-terminal domain of Gp59 binds to the duplex portion of the replication fork, whereas the C-terminal domain contains binding sites for the single-stranded arms of the replication fork (24). Residue Ile-87 is located near the region of Gp59 where the fork DNA arms should separate (Fig. 1A) (24, 32). As reported here (Fig. 2) and by others (18), Gp59-I87A is defective in fork DNA binding and severely defective in ssDNA binding. Therefore, Ile-87 appears to be directly involved in recognizing fork DNA and is critical for the binding of Gp59 to ssDNA. These features make Gp59-I87A an attractive reagent for testing the dependence of helicase loading and polymerase blocking functions on Gp59-DNA interactions.

**Gp59-ssDNA Interactions Are Not Required for Loading or Translocation of Gp41 Helicase on Gp32-ssDNA**—Previously, we demonstrated using Gp32F fluorescence assays that Gp59 targets Gp41 loading specifically onto Gp32-ssDNA clusters, even in the presence of excess free ssDNA (20). This finding led to our hypothesis that Gp59-Gp32 protein-protein interactions control the trafficking of helicase during recombination-determining events. In this study, we investigated the role of Gp59-ssDNA interactions in helicase trafficking using a Gp32F-FRET assay. We observed that Gp41 loading on Gp32-ssDNA is not affected by the presence of wild-type or mutant Gp59. These results suggest that Gp59-ssDNA interactions are not required for helicase loading, and that helicase recruitment is primarily determined by protein-protein interactions between Gp59 and Gp32.

**Figure 7. Effects of I87A mutation on the polymerase blocking functions of Gp59.** Assays for the inhibition of Gp43 DNA synthesis and exonuclease activities were carried out as described under “Experimental Procedures.” Strand displacement DNA synthesis reaction mixtures contained 150 μM each of dATP, dTTP, dGTP, and dCTP. A, reaction scheme for DNA synthesis assays. The replication fork mimic, Substrate A, containing a labeled 34-mer primer strand (Oligo 7), is shown in red. Extension of the primer to a run-off length of 62 bases is measured by denaturing gel electrophoresis and phosphorimaging. B, reaction scheme for exonuclease assays. Substrate A contained the same labeled 34-mer primer strand (Oligo 7), shown in red. Degradation of the primer is measured by denaturing gel electrophoresis and phosphorimaging. C, visualization of DNA synthesis reactions by denaturing PAGE. Lane 1, 50 nM Substrate A with no proteins added; lane 2, 250 nM Gp43 D219A; lane 3, 250 nM Gp43-D219A + 50 nM Gp59 WT. D, visualization of exonuclease reactions by denaturing PAGE. Lane 1, 50 nM Substrate A with no proteins added; lane 2, 250 nM Gp43 WT; lane 3, 250 nM Gp43 WT + 50 nM Gp59 WT. E, quantification of DNA synthesis data from panel C. The histogram shows the fraction of primer that is extended in the presence of Gp43-D219A (white), Gp43 WT (red), or Gp43 WT + Gp59 WT (black). F, quantification of exonuclease data from panel D. The histogram shows the fraction of primer that is degraded in the presence of Gp43 WT (white), Gp43 WT + Gp59 WT (red), or Gp43 WT + Gp59-I87A (black). Error bars represent S.D. from three separate experiments.
pendent replication. The findings of our current study support this hypothesis. Fluorescence and ATPase data demonstrate that despite its severe defect in ssDNA binding (Fig. 2A), Gp59-I87A forms normal helicase loading complexes on Gp32-ssDNA (Fig. 3) and promotes Gp41 helicase loading and translocation on Gp32-ssDNA with activity that is indistinguishable from wild type (Fig. 4 and data not shown). Therefore, the assembly and activation of Gp41 helicase on Gp32-covered ssDNA is driven by Gp59-Gp32 interactions, not Gp59-DNA interactions.

Gp59-DNA Interactions Are Important for Helicase Loading and Unwinding on Fork DNA Structures—The mechanisms of Gp59-dependent helicase loading on pseudo-fork DNA versus ssDNA substrates appear to be distinct. Although Gp59-I87A loads Gp41 normally onto Gp32-bound ssDNA substrates, results from helicase unwinding assays indicate that I87A is weak in stimulating Gp41 loading/unwinding of pseudo-fork substrates even in the presence of stoichiometric concentrations of Gp32 (Figs. 5 and 6). The unwinding defect appears to correlate directly with the low affinity of I87A for pseudo-fork DNA (Fig. 2), and it is not masked by Gp59-Gp32 interactions (Fig. 5). This is the opposite of the behavior observed for helicase loading onto Gp32-ssDNA, which is independent of Gp59 ssDNA binding affinity (Figs. 3 and 4). Fluorescence data show that Gp59-I87A forms normal helicase loading complexes on pseudo-fork DNA in the presence of Gp32; however, the kinetics of helicase loading appear to be slower for the mutant HLC than for the wild-type complex (Fig. 6). This result suggests that the formation of the mutant HLC on fork DNA causes either

![FIGURE 8. Effects of Gp32 on the polymerase blocking functions of Gp59. Assays for the inhibition of Gp43 DNA synthesis and exonuclease activities were carried out as described under “Experimental Procedures.” Strand displacement DNA synthesis reaction mixtures contained 150 μM each of dATP, dTTP, dGTP, and dCTP. A, schematic representation of the replication fork mimic, Substrate A, containing a 7-base ssDNA flap. The 34-mer primer is radiolabeled. B, schematic representation of the replication fork mimic, Substrate B, containing a 30-base ssDNA flap. The 27-mer primer is radiolabeled. C, visualization of DNA synthesis reactions by denaturing PAGE with Substrate A in the presence/absence of Gp59 and Gp32. All reactions contained 50 nM Substrate A and were initiated by the addition of 250 nM Gp43-D219A. Lane 1, no Gp59 or Gp32 added; lane 2, 1 μM Gp32; lane 3, 1 μM Gp32; lane 4, 1 μM Gp32 + 1 μM Gp59. D, visualization of DNA synthesis reactions by denaturing PAGE, with Substrate B in the presence/absence of Gp59 and Gp32. All reactions contained 50 nM Substrate B and were initiated by the addition of 250 nM Gp43-D219A. Lanes 1–4 are the same as in panel C. E, visualization of exonuclease reactions by denaturing PAGE, with Substrate A in the presence/absence of Gp59 and Gp32. All reactions contained 50 nM Substrate A and were initiated by the addition of 45 nM Gp43 WT. Lanes 1–4 are the same as in panel C. F, visualization of exonuclease reactions by denaturing PAGE with Substrate B in the presence/absence of Gp59 and Gp32. All reactions contained 50 nM Substrate B and were initiated by the addition of 45 nM Gp43 WT. Lanes 1–4 are the same as in panel C. G, quantification of DNA synthesis data from panels C (Substrate A) and D (Substrate B). The histogram shows the fraction of primer that is degraded in the presence of Gp43 WT alone (black) or with Gp32 (red), Gp59 (green), or Gp32 + Gp59 (blue). H, quantification of exonuclease data from panels E (Substrate A) and F (Substrate B). The histogram shows the fraction of primer that is degraded in the presence of Gp43 WT alone (black) or with Gp32 (red), Gp59 (green), or Gp32 + Gp59 (blue). Error bars in panels G and H represent S.D. from three separate experiments. p values shown for adjacent columns in the panels G and H are based on Student’s t test. Asterisks in panels A and B denote the positions of 5′-32P labels.
the initial recruitment of helicase or a conformational change needed to activate it to become rate-limiting in the unwinding reaction.

Interactions Controlling the Inhibition of DNA Polymerase by Gp59—Gp59-I87A inhibits the exonuclease activity of Gp43 polymerase to the same extent as wild-type Gp59 (Fig. 7), which supports the hypothesis that high affinity Gp59-fork DNA interactions are not important for this inhibition. This finding is consistent with the report that Gp59-Y122A, a mutant that binds to DNA but not to Gp43, does not inhibit Gp43 exonuclease activity (12). The data support the latter study conclusion that exonuclease inhibition requires Gp59-Gp43, not Gp59-DNA interactions. In contrast, our finding that Gp59-I87A inhibits strand displacement DNA synthesis appears contrary to the hypothesis that Gp59-DNA interactions are important for this activity. This finding appears to conflict with the report that Gp59-Y122A inhibits DNA synthesis independent of Gp59-Gp43 interactions (12). One possible explanation for the unexpected behavior of Gp59-I87A is that this mutation does not completely eliminate fork DNA binding activity (Fig. 2). The reduced affinity of I87A for fork DNA may still be sufficient to interfere with DNA synthesis, especially if I87A-DNA interactions are stabilized via interactions with Gp43.

The inhibition of strand displacement synthesis by Gp59 is partially relieved by Gp32, but only when the lagging strand ssDNA is sufficiently long to support the formation of Gp32 clusters (Fig. 8G). Stable Gp32 clusters are a prerequisite for HLC formation (20). We interpret this to mean that Gp32 cluster formation triggers a remodeling of Gp59 at the fork, perhaps removing a steric block to polymerase activity (Fig. 9A). The data indicate that there are two alternative mechanisms to unlock the DNA synthesis activity of Gp43 when it is inhibited by Gp59. As others have shown, the direct loading of Gp41 onto fork structures by Gp59 unlocks polymerase activity (10). In addition, our data show that in the absence of Gp41, formation of the Gp59-Gp32 helicase loading complex on lagging strand ssDNA is sufficient to partially unlock polymerase activity (Fig. 8G). Both mechanisms presumably involve the remodeling of Gp59-fork DNA interactions to allow the passage of DNA polymerase.

In contrast, HLC formation does not relieve the inhibition of Gp43 exonuclease activity by Gp59, as inhibition is independent of both Gp32 and ssDNA flap length (Fig. 8H). This suggests that the remodeling of Gp59-fork DNA interactions that occurs upon HLC formation can relieve the blockage to DNA synthesis without exposing the primer to unwanted degradation. The mechanism presumably involves the retention of Gp59-Gp43 interactions upon formation of the HLC (Fig. 9A). The fact that HLC formation does not fully restore polymerase activity (Fig. 8G) suggests that it could protect the primer by simultaneously inhibiting exonuclease activity and allowing a slow rate of DNA synthesis before helicase acquisition by the replication fork.

Gp59 Protein-Protein Interactions Promote Recombination-dependent Replication—The stable, cooperative binding of Gp32 to ssDNA requires a minimum cluster size of approximately three Gp32 protomers, each occupying approximately seven nucleotide residues of ssDNA (20, 36). Gp59 requires a stable cluster of ssDNA-bound Gp32 to form a helicase loading complex (20). D-loop formation by the T4 recombination system generates relatively long tracts of ssDNA that are rapidly sequestered by Gp32 (37), an enriched target for Gp59-dependent helicase loading (Fig. 9B, Steps 1 and 2). Because of the processive, 5’ → 3’ translocation of Gp41, helicase loaded anywhere along the displaced strand would extend the heteroduplex portion of the D-loop to incorporate the 3’ end of the invading strand, thus generating the primer/template for RDR (Fig. 9B, Step 3). The same translocation of Gp41 along the Gp32-bound displaced strand positions the helicase to engage the new replication fork (Fig. 9B, Steps 3 and 4). Simultaneously, Gp41 assembly is restricted from the invading ssDNA due to Gp32 exclusion from the presynaptic filament (Fig. 9B, Step 1), thus protecting the D-loop from Gp41-catalyzed anti-recombination (1, 2). The D-loop may be further stabilized by Gp59 through its interaction with Gp43 and inhibition of 3’ → 5’ exonuclease activity, which would protect the 3’ end of the invading strand/heteroduplex from degradation (Fig. 9B, Step 3). Therefore, Gp59 protein-protein interactions promote the efficient initiation of recombination-dependent replication by delivering Gp41 helicase selectively onto what will become the lagging strand of the template and by preventing degradation of the invading strand that will become the primer for leading strand synthesis.

“Gatekeeper” Function of Gp59 in Bidirectional Replication from Origins—Bacteriophage T4 initiates bidirectional DNA replication from origins by an R-loop mechanism (3, 13). A promoter upstream of a DNA unwinding element (DUE) generates an RNA transcript that remains associated with its template DNA strand, forming a stable R-loop. The ODR pathway is active at early infection times; later in infection it is suppressed, and recombination-dependent replication is activated. Gp41 and Gp32 are essential for ODR; Gp59 is non-essential for ODR, but 59− mutants exhibit defects in the coordination of bidirectional replication (13). Based on the results of two-dimensional gel electrophoresis studies, Gp59 was proposed to act as a gatekeeper during ODR by blocking leading strand synthesis of the retrograde fork until primosome assembly occurs, thus ensuring that both forks originating from the origin proceed with coupled leading/lagging strand synthesis (13).

The biochemical properties of Gp59 revealed in this study and elsewhere (12, 17) support the gatekeeper model of Dudas and Kreuzer (13) and suggest a mechanism for the process (Fig. 9C). The R-loop mechanism of ODR resembles the D-loop mechanism of RDR, with the exception that two divergent replication forks originate at the site of the R-loop. The R-loop structure is asymmetric, with a 3’ margin and a 5’ margin as defined by the orientation of the annealed transcript. Different priming mechanisms are used at the 3’ and 5’ margins; at the 3’ margin, leading strand synthesis is primed by the RNA transcript to generate an “initial fork.” At the 5’ margin, lagging strand synthesis from the initial fork produces an Okazaki fragment that primes leading strand synthesis in the “retrograde fork.” We propose that Gp59 is arranged asymmetrically at the R-loop, forming an HLC for the initial fork and a PBC that regulates the retrograde fork (Fig. 9C). The formation of the RNA/DNA hybrid at the origin displaces the opposite strand, which is sequestered by Gp32 and, therefore, becomes a target
FIGURE 9. Models for Gp59 action during bacteriophage T4 DNA replication and recombination. See “Discussion.” A, at a replication fork, Gp59 may exist in either of two complexes depending on the Gp32-bound status of lagging strand ssDNA. In the PBC, oligomerized Gp59 is tightly associated with the fork DNA, blocking DNA synthesis activity by the leading strand polymerase (Pol) through a combination of steric effects and protein-protein interactions. The exonuclease (Exo) activity of Gp43 is inhibited. The assembly of a cooperative cluster of Gp32 on lagging strand DNA triggers the reorganization of Gp59 into a HLC, the formation and activity of which depend primarily on Gp59-Gp32 interactions. HLC formation unlocks the DNA synthesis activity of Gp43 by removing the steric block. The exonuclease activity of Gp43 is still inhibited via interactions with Gp59 in the HLC. The exact structures of the PBC and HLC are unknown; the schematic merely emphasizes that they are different. B, in recombination-dependent replication or RDR, presynaptic filaments of UvsX recombinase and UvsY recombination mediator protein form on ssDNA, excluding Gp32. Step 1, UvsX/UvsY-promoted strand invasion generates a D-loop, the displaced strand of which is rapidly coated by Gp32. Gp32 recruits Gp59 to the displaced ssDNA to form an HLC. The red T denotes the exclusion of Gp59 from the invading ssDNA that is covered with UvsX/UvsY. Step 2, Gp59 loads Gp41 helicase onto the displaced strand of the D-loop. Step 3, Gp41 is now correctly positioned to drive 5' → 3' branch migration. This results in the incorporation of the 3' end of the invading strand into the D-loop heteroduplex. Gp59 may stabilize the 3' end by inhibiting the exonuclease activity of Gp43 polymerase. Step 4, the 3' end of the heteroduplex primes leading strand DNA synthesis by Gp43, coupled to template unwinding by Gp41. Step 5, Gp41 recruits Gp61 primase to reconstitute lagging strand synthesis. C, at the R-loop 3' margin, Gp59 loads helicase and unlocks polymerase to generate the retrograde fork. The first Okazaki fragment of the initial fork primes leading strand synthesis in the retrograde fork. Step 4, primase is recruited to reconstitute lagging strand synthesis in the retrograde fork. The dual functions of Gp59 allow it to act as a gatekeeper in ODR, ensuring that both initial and retrograde replication forks proceed with coupled leading/lagging strand synthesis (13).
for Gp59-promoted helicase assembly to reconstitute the initial fork (Fig. 9C, Step 1). This process is likely to involve similar biochemical steps as described for RDR, i.e. HLC formation on the displaced strand and unlocking of polymerase to extend the transcript/primer while maintaining inhibition of Gp43 exonuclease activity until helicase is loaded. Helicase loading and primase recruitment reconstitute lagging strand synthesis, resulting in an Okazaki fragment that elongates toward the 5′ margin of the R-loop, where the PBC blocks further synthesis by the polymerase (Fig. 9C, Step 2). There, helicase loading by Gp59 unlocks the polymerase to generate the retrograde fork (Fig. 9C, Step 3). Lagging strand synthesis ensues with the recruitment of Gp61 (Fig. 9C, Step 4). Thus two divergent replication forks with coupled leading/lagging strand synthesis are generated from a single R-loop.

The models for Gp59 activity in RDR and ODR explain key aspects of the roles of this protein in T4 replication but also raise some interesting questions. First, why is Gp59 essential for RDR but nonessential for ODR? We postulate that features of the DNA unwinding element present at T4 origins and of its incorporation into R-loops favor helicase recruitment to the initial fork even in the absence of Gp59. Lack of Gp59 leads to aberrant retrograde replication, however, as leading strand synthesis proceeds without coupling to lagging strand synthesis (13). The more stringent requirement for Gp59 in RDR is probably due to the strong inhibition of Gp41 helicase and prisma-some functions by recombination proteins UvsX and UvsY (30), which necessitates the targeting of helicase assembly onto clusters of Gp32-ssDNA (Fig. 9B). UvsX and UvsY are not expressed during early infection and so are unlikely to interfere with ODR. The fact that Gp59 is excluded from DNA that is coated with UvsX and UvsY (30) may also explain why RDR is not bidirectional; any retrograde fork would have to initiate at a 5′ D-loop margin or Holliday junction that is complexed with UvsX and UvsY. Second, how can a PBC persist at the 5′ R-loop margin in the presence of Gp32? We have shown that Gp32 binding to lagging strand ssDNA, as would occur at the 3′ R-loop margin (Fig. 9C), converts a PBC into an HLC and unlocks polymerase. But the 5′ R-loop margin is structurally distinct from the 3′ margin. At the 5′ margin, lagging strand ssDNA is annealed to RNA. Gp32 clusters occupy the opposite strand in the opposite polarity with respect to the margin (“tails” toward the 5′ margin, “heads” toward the 3′ margin). The ability of Gp59 to form a PBC at the 5′ R-loop margin indicates that the orientation of Gp32 clusters matters in the remodeling of Gp59-fork DNA interactions. HLC formation may require the proximity of Gp59 to the head of a Gp32 cluster. Gp59 could also use such a sensing mechanism to determine the correct strand on which to load Gp41 helicase. These and other questions, such as the role(s) of Gp59 in bubble migration synthesis (SDSA (synthesis-dependent strand annealing)) and replication fork restart, provide abundant opportunities for further study.

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