Identification of Subunit Binding Positions on a Model Fork and Displacements That Occur during Sequential Assembly of the Escherichia coli Primosome*

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Background: Photo-cross-linking revealed the position of replication restart protein interactions with a fork.

Results: PriA cross-links to the fork until DnaC, DnaB, and ATPγS are added.

Conclusion: PriA remains stably associated with forks until the final stage of helicase assembly, when it is displaced.

Significance: The approach employed should be applicable to complex protein-nucleic acid assembly reactions.

When replication stalls and forks disassemble, the restart primosome is required to reload the replicative helicase so that chromosomal replication can be reinitiated. We have taken a photo-cross-linking approach, using model replication forks containing a phenyl diazirine placed at single locations, to determine the positions of primosomal protein binding and changes in interactions that occur during the assembly reaction. This approach revealed a novel mode for single-stranded DNA-binding protein (SSB)-DNA binding, in which SSB interacts with both the leading and lagging single-strand segments and the parental duplex of the fork. Cross-linking to a novel region within SSB is observed only when it is bound to forked structures. This binding mode is also followed by PriB. PriA binds to the fork, excluding SSB and PriB, interacting with the primer terminus, single-stranded leading and lagging strands and duplex in immediate proximity of the fork. SSB binds to flanking single-stranded segments distal to the fork in the presence of PriA. The addition of PriB or DnaT to a PriA-SSB-fork complex does not lead to cross-linking or displacement, suggesting that their association is through protein-protein interactions at early stages of the reaction. Upon addition of DnaC and the DnaB helicase in the presence of ATPγS, helicase is assembled, leading to contacts within the duplex region on the tracking (lagging) strand and strong contacts with the displaced leading single strand near the fork. PriA is displaced from DNA upon helicase assembly.

Not all replication forks established at the origin complete the two megabase journey to the terminus. Presumably, the helicase occasionally spontaneously disassembles because of less than absolute processivity. In addition, unrepair ed lesions are sometimes encountered that can be bypassed by the action of error-prone Pol2 Y family polymerases. The disposition of the helicase during polymerase switching has not been well studied. Unrepaired lesions can also be processed by error-free recombinational repair, a process that leads to DnaB helicase dissociation prior to re-establishment of a repaired replication fork (1). These processes require reloading of the DnaB helicase in a reaction independent of the DnaA protein.

At least two distinct mechanisms for restart of replication forks exist in E. coli. These pathways are led by association of PriA or PriC with the forked DNA structure and through interaction with intermediate protein factors lead to the reloading of the DnaB helicase (2, 3). Most bacteria do not contain a PriC homolog and, presumably, are solely dependent upon PriA. Even in E. coli, PriA and PriC are not completely redundant. PriA mutants are very sick. They grow slowly and suffer from low viability as indicated by a low replating efficiency (2).

Gel shift assays suggest an ordered assembly of primosomal proteins (4, 5). PriA binds first, followed by binding of PriB and then DnaT. This protein-DNA complex can support ATP-dependent loading of the DnaB helicase onto the fork by the DnaC helicase loader.

PriA binds broadly to D loops or forked structures with a primed leading strand preferentially (6, 7). This specificity appears to be determined, in part, by a 3′-OH binding pocket within the PriA N-terminal domain (8). The C-terminal domain of PriA contains a 3′ → 5′ helicase (9, 10). The presence of a primer on the leading strand template apparently orients PriA such that the helicase activity is directed toward the lagging strand template instead of advancing into the unreplicated duplex of a replication fork (11). Its function is thought to be clearing of a lagging strand duplex if present adjacent to the fork to provide a single-stranded site for helicase loading. The C-terminal tail of SSB binds PriA (12).

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2 The abbreviations used are: Pol, polymerase; SSB, single-stranded DNA-binding protein; HE, holoenzyme; ATPγS, adenosine 5′-O-(thiotriphosphate); nt, nucleotide(s).
On a D loop with a primed leading strand template, PriA yields a large footprint with interactions with the leading strand primer and its complementary strand and regions progressing into the duplex downstream of the primer (5, 7). On templates with duplex lagging strands behind the fork, PriA can progress into that region using its helicase activity and generate a footprint on the lagging strand template (7).

Emphasizing its central role in primosome function, PriA also serves as a checkpoint protein, blocking the intrinsic strand displacement activity of the DNA polymerase III holoenzyme (Pol III HE) until DnaB is assembled (13, 14). This is accomplished by competing with Pol III HE for binding to the leading strand primer terminus (15).

PriB is an SSB homolog. Structures indicate that it is a dimer with two OB folds (16, 17). PriB interacts with the isolated helix-domain of PriA free in solution, but not with full-length PriA. A DNA-PriA complex binds PriB tightly, suggesting a conformational change in PriA, revealing the PriB binding site (18). In the absence of PriA, DnaT and single-stranded DNA appear to compete for binding to PriB. On the basis of this data, a handoff model has been proposed for primosome assembly (18).

DnaB can self-assemble on model replication forks with free 5'-lagging strand template flaps. In a study initiated to define the minimal template required for primosome assembly on model forks to support this work, we showed that the DnaB self-loading reaction can be blocked sterically by attachment of streptavidin to a biotin near the 5'-end of the lagging strand (15). This observation suggests that the DnaB self-loading reaction proceeds by threading of the opening in the DnaB hexameric ring and that anything that gives the DNA fiber a diameter greater than the DnaB6 central pore blocks this reaction. Consistent with this hypothesis, SSB coating the lagging strand flap also blocks the self-assembly reaction (15).

A critical deficit in our understanding pertains to identification of the sites of interaction of primosomal proteins with a replication fork in the context of the full complement of primosomal proteins. This would also reveal displacements and arrangements that occur as the complex is built up. Accordingly, we have applied a photo-cross-linking approach in an initial effort to address this issue using a series of model forks with a photo-cross-linker placed at a unique position within each fork. We chose to use a phenylaziridine, whose utility for nucleic acid applications was largely popularized by Bartholomew and co-workers (19). Irradiation at 350 nm generates a highly reactive short-lived carbene. Carbene can insert into the carbon-hydrogen bonds of every amino acid side chain. Thus, they do not show the selectivity exhibited by other photo-cross-linkers, such as 5-bromo- or 5-iodo-deoxyuridine. Thus, negative data can be interpreted with certainty. If a protein is not cross-linked to a phenylaziridine placed at a given position within a model fork, that protein does not bind to that position. When a carbon-hydrogen bond is not adjacent to the reactive carbene, it reacts with water to form an alcohol, rapidly quenching the reactive species and preventing false positive results.

### EXPERIMENTAL PROCEDURES

**Oligonucleotides and Model Forks**—All oligonucleotides were obtained from Biosearch Technologies. Oligonucleotides containing amino-modified C2 dT from Glen Research were derivatized with a phenylaziridine photo-cross-linker and HPLC-purified as described (20). Substrates used in all photo-cross-linking reactions were assembled from HPLC-purified oligonucleotides listed in Fig. 1A. Prior to annealing, the phenylaziridine-containing oligonucleotide was labeled with $^{32}$P (6000 Ci/mmol) on the 5'-end using T4 polynucleotide kinase. Unincorporated [γ-$^{32}$P]ATP was removed by a Microspin-G25 column (GE Healthcare).

Substrates were assembled by combining 1 μM phenylaziridine-containing oligonucleotide with 2 μM of the appropriate unlabeled oligonucleotides in a final volume of 50 μl in a buffer containing 10 mM Tris-HCl (pH 7.75), 50 mM NaCl, and 1 mM EDTA. Samples were heated to 95 °C for 5 min and cooled to 25 °C, decreasing the temperature by 1 °C every 10 min. Forked substrates were prepared from oligonucleotides listed in Fig. 1A. Photo-cross-linking substrate −35 was constructed from T-35, uLeT, and uLeP; substrate −3 from T-3, uLeT, and uLeP; substrate +3 from T+3, uLeT, and uLeP; substrate distal end from T3′end, uLeT, and uLeP; substrate −6 from T-6, uLaT, and uLeP; and substrate −12 from P-12, uLeT, and uLaT. Substrate −6 with no flap on the lagging strand template was constructed from T−6, uLeP, and unlabeled 45-mer, 5′-TATA-TTATCATGAATCTAACATGTTATAGTAATGATCTAT-ACGGC-3′. Annealed products were desalted by Microspin-G25 column.

**Proteins**—E. coli primosomal proteins were purified as described previously: SSB (21), PriA (22), PriB (22), DnaT (22), DnaB (22), and DnaC (22).

**Photo-cross-linking**—Photo-cross-linking reactions were carried out in a final volume of 50 μl containing 20 μM radiolabeled phenylaziridine-containing substrate in a buffer containing 50 mM Heps (pH 7.5), 10 mM magnesium acetate, 10 mM dithiothreitol, 20% (v/v) glycerol, 0.02% (v/v) Nonidet P-40 detergent, 100 mM potassium glutamate, and 100 μM ATPγS (unless noted otherwise). Protein-DNA solutions were incubated for 10 min at room temperature in the dark before irradiating. Reactions were irradiated at room temperature in siliconized 15-ml Pyrex tubes (filters light < 300 nm) measuring 1.25 cm inner diameter (Sigma-Aldrich #CLS982016X) covered on the top in Parafilm. Irradiation took place in a Rayonet Photochemical Reactor-200 equipped with 16 lamps (Southern New England Ultraviolet Company RPR-3500) outputting 4.5 W at 350 nm for 1 h. Reactions were irradiated 10 cm from the light source and rotated at 5 rpm.

For reactions where the 5′-end of the lagging strand template was sterically blocked by binding of streptavidin to incorporated biotin, 200 nM streptavidin was incubated at room temperature with the substrate for 5 min prior to the addition of other protein components. Where proteolysis is indicated, 30 μg of Proteinase K was added to the reaction after irradiation. Reactions were incubated at 37 °C for 1 h in the dark prior to denaturation of macromolecules.
DNA and proteins were denatured by addition of an equal volume of 2X SDS-PAGE sample buffer: 0.25 m Tris-HCl (pH 6.8), 5% (w/v) SDS, 8 m urea, 0.1 m mercaptoethanol, 10% (v/v) glycerol, 0.005% (w/v) bromphenol blue, and 0.005% (v/v) xylene cyanol FF. Reactions were boiled at 95 °C for 10 min prior to gel loading.

**Markers**—Markers that indicate the position of individual proteins photo-cross-linked to DNA were made by performing incubations at high protein concentrations so that the equilibrium was pushed sufficiently that binary complexes formed in the absence of other proteins. They were then subjected to the same photo-cross-linking protocol described above. Forked template with a photolabel at the −6 position (unless designated otherwise) (20 nm) was incubated with either 500 nm SSB₉₋₀, 500 nm PriA, 550 nm DnaB₀₋₉, or 500 nm PriB₂ before irradiation. The DnaC marker was generated using a procedure whereby the McMacken lab first elucidated a previously cryptic interaction of DnaC with DnaB during the DnaB loading reaction (23). 100 nm DnaB₀ and 200 nm DnaC were incubated with forked template in the presence of 100 μM ATPγS.

**Gel Electrophoresis**—Reactions were resolved by 4–20% SDS-PAGE containing 4 m urea. Gels were run at 15 W for 4 h using 25 m Tris base, 190 m glycine, 0.1% (w/v) SDS as the electrophoresis buffer. Gels were exposed to a Storage Phosphor Screen (GE Healthcare) overnight prior to being scanned by a PhosphorImager.

**FRET Helicase Assay**—Assay was performed as we have described previously (15). Substrate used in FRET experiment was assembled from the following HPLC-purified oligonucleotides: leading strand template 90-mer, 5′-tetrachlorofluorescein, CGCGTATAGATCATTACTATAAACATTTGATTTTGTCGG-CTAATGTAAGAATCTTCAA-3′ contained fluorescent 5′-tetrachlorofluorescein at the 5′ terminus; and lagging strand template 90-mer, 5′-TT(biotin)T₄ATTATATCTGAATCTTAATGTTATATGTTATATGTTAAGATGACAAAATCATATT-3′ contained biotin conjugated to preceding thymidine and Black Hole Quencher-1 at the 3′ terminus that quenches fluorescent 5′-tetrachlorofluorescein dye. The primer was 35-mer, 5′-TTGAAGATTTTCAAGATGAGTTGACAAATCATATT-3′. The substrate was assembled from equimolar amounts of each oligonucleotide. Trap oligonucleotide 45-mer 5′-TATA-TTATCATGAATCCTAATGTTATATGTTATATGTTAAGATGACAAAATCATATT-3′ was used to capture the displaced leading strand to prevent renatting to the lagging strand template that contained the fluorescence quencher.

**RESULTS**

We employed photo-cross-linking using a series of model replication forks that contained a phenylaziridine linked to the 5-position of dU (Fig. 1). Each substrate was photolabeled at only one position. Our intention was to use these substrates to map the position of primosomal protein binding and also to detect changes in primosomal subunit arrangements upon handoffs that occur during sequential assembly. Phenylazirines are particularly well suited for this application because they are highly reactive and nonselective in that they can insert into carbon-hydrogen bonds in the side chains of all amino acids (19). Thus, negative data can be interpreted with a high level of confidence. If a protein does not cross-link to a photo-labeled nucleotide, the protein does not bind to that position.

**Detection of a Novel Interaction between SSB and the Fork Junction**—SSB plays a key role in primosome assembly and interacts, minimally, with the PriA subunit (12). Thus, we examined SSB binding first, expecting to see broad binding to all single-stranded positions within model forked templates. Consistent with this expectation, we observed one prominent cross-link (designated SSB-90-mer A in the margin of Fig. 2) at the −35 position within the 90-nt lagging strand template of the fork that corresponded, in electrophoretic mobility, to a cross-link of SSB to a single-stranded oligonucleotide of the same length (Fig. 2, compare lane 1 in A with lanes 1 and 2 in B). The presence of protein in the identified band A was confirmed by treatment with Proteinase K prior to denaturation (Fig. 2A, lane 2). At the distal end of the duplex substrate a cross-linked band of similar mobility was observed, presumably because of SSB binding to the ends of a frayed substrate (Fig. 2A, lane 3). A cross-link with the 35-nt leading strand primer was also observed. Because the resulting band corresponded to the mobility of a control cross-link to the primer in single-stranded form, this species was also assigned as species A and may result from SSB binding to the frayed 3′-terminus within the forked substrate (Fig. 2, compare lane 11 in A with lanes 11 and 12 in B). The same cross-linked species A was observed when the photolabel was placed at position −3, +3, and −6 (Fig. 2B).

At positions close to the fork, additional cross-linked species were observed. When a photo-cross-linker was placed 3 nt internal to the duplex at the fork junction (+3 position), three bands of decreased mobility relative to the radiolabeled substrate were observed. The slowest mobility band did not contain protein, because it was unaffected by protease treatment (Fig. 2A, compare lanes 7 and 8). This band was assigned as a cross-link between the 90-nt leading and lagging strand templates. Prominent DNA-DNA cross-links were not observed at the other positions or in the absence of SSB. The carbene resulting from photoactivation of a phenylaziridine (Fig. 1C) can rapidly insert into an oxygen-hydrogen bond of water to generate the corresponding alcohol. Presumably, this reaction is dominant in the presence of the high (~55 m) concentrations of water when exposed in solution. However, upon protein binding and dehydration, cross-linking to the opposing strand of DNA would be expected to occur.

Surprisingly, two distinct SSB-DNA cross-links arose from cross-linking to the +3 position (Fig. 2A, lane 7). One was the previously identified cross-link A, and the second, of slower mobility (designated B), is novel. We consider it unlikely that the slower migrating cross-link B is the result of incomplete denaturation because the samples were subjected to a 10-min boiling step in the presence of high concentrations of SDS and 4 m urea. As an additional control, we autoclaved the sample for 60 min in the presence of the same denaturants, and the band corresponding to cross-link B persisted (Fig. 3). Thus, the new protein-DNA cross-link likely arises via a novel interaction of SSB with the fork.

Substrates in which the photolabel was placed in single-stranded regions near the fork (−3 in the lagging strand tem-
plate and −6 within the leading strand template) also generated the slower migrating SSB-DNA cross-link B, but not DNA-DNA cross-links (Fig. 2A, lanes 5 and 9). Remarkably, only cross-link B is generated when SSB cross-links to the −6 position within a 10-nt gap in the substrate. This species is dependent upon an opposing single strand at the fork. If the 45-nt single-stranded half of the lagging strand template is omitted, species A is generated (Fig. 3). Thus, the unique region of SSB that interacts with the first few nucleotides of the fork duplex is also involved in binding adjacent single-strands. We eliminated the possibility that band B resulted from heterogeneity or something peculiar to the photolabeled oligonucleotide used to construct the corresponding forked substrates by demonstrating that generation of species B is dependent upon a forked structure. Only species A is generated when a cross-link occurs between SSB and a single-stranded oligonucleotide (Fig. 2B).

**PriA Binds Exclusively to the Fork Junction**—We next examined interactions of PriA with the fork, initially in the absence of SSB. PriA cross-linked to the −3 and the +3 positions, but not the −35 position within the lagging strand template (Fig. 4). On the leading strand, cross-links to both the −6 position and the penultimate nucleotide (−12) within the primer occurred. No interaction with the distal end of the duplex was observed. Thus, its interactions are limited to the vicinity of the fork in contrast to the broader binding observed in the absence of SSB and other proteins (5–7).

As with SSB, template-template cross-links were observed within the duplex +3 region of the fork junction, as judged by their insensitivity to protease digestion (Fig. 4). Unlike SSB, PriA binding also led to DNA-DNA cross-links within the single-stranded region of the fork junction, at the −3 and −6 positions of the lagging and leading strand templates, respectively. The mobility of markers generated by incubation of high con-

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**FIGURE 1. Substrates used in photo-cross-linking reactions.** A, oligonucleotide sequences used in photo-cross-linking experiments. The position of the phenyldiazirine photo-cross-linker is indicated by an encircled red lowercase t. B, diagram of forked substrate used in photo-cross-linking studies. The positions marked by stars indicate placements of the phenyldiazirine photo-cross-linker. The position number is given as the number of nucleotides away from the fork junction with the exception of the distal end. C, structure of thymine-linked phenyldiazirine photo-cross-linker inserted at the indicated positions and formation of carbene upon irradiation.
centrations of PriA with single-stranded oligonucleotides corresponded to the mobility of the PriA cross-links observed on forked substrate (data not shown).

When PriA and SSB were added sequentially to the forks, PriA was observed to largely exclude SSB from the regions to which PriA binds (−3 and −6) and to the primer terminus (−12) (Fig. 5). No binding or greatly reduced binding of SSB was observed at these positions. In Fig. 5, no cross-link of PriA to the duplex region of the fork in the presence of SSB was observed. This result was variable: PriA cross-linked to the duplex in a similar experiment shown in Fig. 6 (lane 3). In either case, PriA binding is excluding SSB from the fork. At positions to which PriA does not bind (−35 position and distal end of duplex template) prominent SSB cross-links are observed (Fig. 5). PriA can rapidly access a fork in the presence of high concentrations of SSB during the strand displacement reaction catalyzed by Pol III HE in the absence of helicase (14). However, in our static fork model, exclusion of SSB by PriA is dependent upon addition of PriA first. If SSB is added first, it bound to all photolabeled positions and excluded PriA (Fig. 6).

DnaB helicase self-assembles in the absence of SSB, binding to the fork junction and making a prominent contact with the displaced strand. In the absence of steric blocks or SSB, bacterial replicative helicases can self-assemble by threading over the free 5′-end of the lagging strand template (15). When self-assembly was allowed to occur in the presence of ATP, no cross-links were observed (Fig. 7). This was expected because the active helicase should split the two template strands and disso-

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**FIGURE 2.** A novel interaction occurs between SSB and the replication fork junction. A, two different SSB-DNA cross-linked species are formed, with the slower migrating cross-link only resulting from interaction with the fork junction. 500 nM SSB4 was combined with 20 nM forked substrate. Lanes that indicate protease were subjected to treatment with Proteinase K postirradiation to identify protein-containing bands. B, the more slowly migrating cross-linked species B does not occur when reactions are conducted using ssDNA. In reactions containing only single-stranded DNA (designated by (·) in the fork-labeled row above the gel), 500 nM SSB4 was reacted with 20 nM of the radiolabeled phenyldiazirine-containing oligonucleotide. Refer to Fig. 1 for nomenclature indicated in the position row above each panel of this and the following figures.

**FIGURE 3.** Slower migrating bands are not caused by incomplete denaturation and are caused by fork structure. 500 nM SSB4 was combined with 20 nM substrate. Lane 6 contains substrate where the 45-nt single-stranded portion of the lagging strand template is omitted. Lanes 1–6 were treated prior to gel loading as described under “Experimental Procedures.” Lanes 9–12 were autoclaved for 60 min in gel loading buffer prior to gel loading. The “no flap” designation in lane 6 refers to an experiment performed on a substrate containing the photolabel at position −6 on the leading strand template but lacking the single-stranded 45-nucleotide 5′-flap of the lagging strand template (refer to Fig. 1).

**FIGURE 4.** PriA selectively binds near the replication fork junction. 500 nM PriA was combined with 20 nM forked substrate. Lanes that indicate protease were subjected to treatment with Proteinase K postirradiation to identify protein-containing bands.
ciate. If nucleotide was omitted, only weak cross-linking was observed. However, in the presence of 100 μM ATPγS, prominent cross-links were observed, indicating that this nonhydrolyzable ATP analog stabilizes DnaB interaction with a fork junction but does not support progression of the helicase to separate the two template strands.

**FIGURE 5.** PriA remains stably bound to the replication fork junction in the presence of SSB. 20 nm forked substrate was combined with 500 nm PriA followed by 500 nm SSB4 (~30 s later). Lanes that indicate + protease were subjected to treatment with Proteinase K postirradiation to identify protein-containing bands.

**FIGURE 6.** The order of addition between SSB and PriA determines which protein is excluded at the fork juncture. 20 nm forked substrate was combined with 500 nm PriA and/or 500 nm SSB4. subunit 1 indicates the subunit added to the reaction first, and subunit 2 indicates the subunit added to the reaction second. Where no protein is listed for subunit 2, only subunit 1 was present in the reaction. Lanes that indicate + protease were subjected to treatment with Proteinase K postirradiation.

**FIGURE 7.** DnaB self-loading in the presence of ATP versus ATPγS. A, 20 nm forked substrate photo-labeled at the −6 position was combined with 550 nm DnaB6 with either 2 mM ATP (lane 1), no nucleotide (lane 2), or 100 μM ATPγS (lane 3). B, 20 nm forked substrate photo-labeled at the −6 position was combined with 550 nm DnaB6 with either 10 μM ATPγS (lane 1) and 100 μM ATPγS (lane 2).
Photo-cross-linking of Primosome to Forks

We exploited the DnaB self-loading reaction that occurs in the presence of ATPγS to determine contacts made by DnaB in the absence of other primosomal proteins. Cross-links were observed on the lagging strand template that goes through the center of the hexameric helicase ring (−3 and +3 positions) (Fig. 8, lanes 5 and 7). Interestingly, a strong cross-link was observed between the displaced leading strand template and the helicase at the −6 position (Fig. 8, lane 9). Template-template cross-links are also observed in the presence of bound protein.

Self-assembly of DnaB is blocked by attachment of streptavidin to the 5′-end of model replication forks (15). We conducted a parallel set of reactions in the presence of streptavidin attached to a biotin incorporated into the penultimate nucleotide at the 5′-end of the lagging strand template flap (Fig. 8, lanes 13–17). The presence of streptavidin eliminated DnaB cross-linking. Interestingly, streptavidin tethered only nine nucleotides away from the −35 label fails to yield detectable cross-links (Fig. 8, lane 17). Given its monomeric molecular mass (13.2 kDa), we would have expected a streptavidin−90-mer cross-link to migrate with a mobility between the free 90-mer and SSB−90-mer (A). Thus, proximity is insufficient to yield cross-links. Cross-linking apparently requires direct association to compete effectively with the rapid insertion of a carbene into an oxygen-hydrogen bond of water.

Loading of DnaB by the Primosomal Pathway Can Be Detected by Photo-cross-linking—Using streptavidin linkage to the 5′-end of the lagging strand template to block self-assembly renders the DnaB loading reaction dependent upon primosomal proteins (15). Using the −6 photolabel in the displaced leading strand as a probe, we conducted a DnaB assembly reaction in the presence of the full complement of primosomal proteins in the presence of ATPγS to stabilize DnaB interactions with the fork. We observe an efficient cross-link of DnaB (Fig. 9A, lane 3). Omission of any of the primosomal proteins results in loss of the cross-link, consistent with the biochemical dependences established by functional assays (Fig. 9A, lanes 4–9) (15, 24, 25). The faint band with approximately the same mobility as a DnaB-DNA cross-link observed in the reaction in which PriA was omitted (Fig. 9A, lane 4) corresponds to a third minor cross-link observed between SSB and DNA at positions near the fork (compare lane 2). The presence of PriA (lanes 5–9) excludes SSB from the fork and eliminates this minor band.

Surveying the full complement of photo-cross-linking substrates employed in this study, we observe that DnaB cross-links are restricted to +3 on lagging strand template and −6 on the displaced strand. The −3 cross-link observed in the DnaB self-assembly reaction does not occur (Fig. 9B, lanes 7, 9, and 11). We note that some PriA and SSB cross-links are still detected, even in the presence of DnaB. These bands are light and presumably due to incomplete DnaB loading.

DnaB Is Not Present at the Fork in the Presence of High Inhibitory Concentrations of DnaC—DnaC is the helicase loader (26). In multiple functional studies, DnaC titrations have yielded sharp optima with activity decreasing sharply at DnaC concentrations beyond the optimum (26). Formally, it is possible that this inhibition is due to DnaC association with DnaB on the fork, stalling its activity. Alternatively, inhibition could be due to unloading of DnaB from the fork or failure of complexes to form. We employed cross-linking to determine the status of DnaB at the fork in the presence of high inhibitory concentra-
Photo-cross-linking of Primosome to Forks

**FIGURE 10.** DnaB is not present at the fork in the presence of high concentrations of DnaC. A, markers were prepared as described under “Experimental Procedures.” The PriA marker (lane 10) was also treated with Proteinase K prior to gel loading to provide a marker for DNA-DNA cross-links (lane 11). Lanes 2–9 and 15–18 contain streptavidin-blocked –6 photolabeled substrate and 150 nM PriA, 50 nM PriB, 50 nM DnaT, 500 nM SSB, 100 μM ATPγS, and the indicated concentrations of DnaB, and DnaC. The reaction products were resolved in two separate gels run under identical conditions. B, FRET assay (15) monitoring helicase loading by helicase loader DnaC on 20 nM substrate blocked with streptavidin. DnaC was titrated in the presence of 150 nM PriA, 50 nM PriB, 50 nM DnaT, 60 nM DnaB, and 500 nM SSB. The reaction was performed in triplicate. The average values were plotted, and standard deviation is indicated by error bars.

Photocrosslinking of Primosome to Forks

We observe an optimal concentration of DnaC for DnaB cross-linking that parallels its optimum in functional assays based on the helicase activity of DnaC (Fig. 10, A, lanes 3 and 16, and B). At high DnaC concentrations, DnaB is not present at the fork, consistent with either removal or inhibition of the assembly reaction (Fig. 10A, lanes 5 and 18).

The Binding of PriB to Model Forks Mimics the Characteristics of SSB Binding—PriB is a homolog of SSB and will bind DNA directly by itself (17). We observe a pattern of binding that mimics that observed with SSB with the generation of a novel cross-linked species with slower mobility when PriB is photo-cross-linked to templates containing photolabel near the replication fork (Fig. 11A). Cross-linking of PriB to a single-stranded region 35 nt in front of the fork yields a single cross-link (designated species A to be consistent with the convention we adapted for SSB) that is identical whether cross-linked to a model fork or corresponding photolabeled single strand (Fig. 11A, lanes 1 and 3). Near the fork (–3 position in lagging strand, +6 position in leading strand, or in +3 position in fork duplex), a slower migrating species B is dominant (Fig. 11A, lanes 6, 11, and 8, respectively). Cross-linking to the single-stranded form of the photolabeled oligonucleotides used to construct the model forks yields only cross-linked species A (Fig. 11A, lanes 7, 13, and 10, respectively). DNA-DNA cross-links also occur when PriB is bound as demonstrated by protase resistance (Fig. 11A, lanes 9 and 12). PriB also cross-links weakly to the leading strand primer terminus (Fig. 11A, lane 14).

**PriA Is Not Displaced by PriB**—Based on protein requirements to achieve strong binding by gel shift assays, it has been proposed that PriA, PriB, and DnaT act sequentially in the assembly of a primosome (4, 5). A handoff model has been proposed for primosome assembly (18). We used our newly developed cross-linking tool to determine the presence of PriA at the fork at intermediate stages of primosomal assembly.

If we add PriA to model forks and then titrate in PriB, we observe strong binding and cross-linking of PriB to the –35 position far from the fork where PriA does not bind (Fig. 11B, lanes 2 and 3). When positions near the fork are probed (–3 on the lagging strand, +3 in the fork duplex, –6 on the leading strand, and –12 at the leading strand primer terminus), PriA is not displaced (Fig. 11B, lanes 9, 12, 15, and 18, respectively). Furthermore, the intensity of PriB cross-links near the fork is very weak compared with PriB binding to the same forked substrate alone (Fig. 11, A and B).

If we serially add PriA and SSB (like in Fig. 5) and then add PriB, we do not observe PriB effectively competing with SSB at the –35 single-stranded position upstream from the fork (Fig. 11C, lane 5). PriA interaction with positions near the fork are not diminished either (Fig. 11D, lanes 7, 9, 11, and 13). If the experiment conducted in Fig. 11D is repeated with adding DnaT and PriB simultaneously, the same result is obtained (Fig. 11E), except PriA interactions also occurred more broadly at the –35 position and the distal end of the duplex.

**DISCUSSION**

Previous studies have established an ordered assembly of replication restart primosomal proteins and have suggested a handoff mechanism in the loading of the DnaB helicase and restarting stalled *E. coli* replication forks (4, 5, 18). Footprinting and binding experiments have provided information regarding the regions in which single restart primosomal proteins can bind (5–8, 11). However, little information is available regarding the positions to which restart primosomal proteins bind in the context of their functional partners and how binding positions change during the competitions imposed during complex assembly and helicase loading. We chose to address this issue to gain a deeper understanding of the mechanism of restart primosome function using highly reactive photo-cross-linking moieties attached to specific positions within a model replication fork.

We initiated our study by monitoring cross-linking of SSB to forks. We anticipated that this would provide a convenient control and baseline using a well understood protein. As anticipated, binding of SSB to single-stranded DNA yielded a well defined single band presumably between the OB fold and the photolabel placed at a unique position within the single-stranded DNA probe. The cross-linked species exhibited approximately the same mobility whether the photolabel was
Photo-cross-linking of Primosome to Forks

located at either end or central to the oligonucleotide. To our surprise, when experiments were extended to model forks, three cross-links were observed within the double-stranded region of the fork, close to the fork junction, but not at the distal end of the duplex (Fig. 2). One species corresponded to that observed on single-stranded DNA. Another was assigned as a DNA-DNA cross-link because the band remained after protease digestion. DNA-DNA cross-links are not observed on forks in the absence of protein. This is likely because the reactive carbene generated rapidly reacts with water. When bound in a protein binding pocket, the DNA in the vicinity of the photolabel would be expected to be relatively dehydrated, enabling

![Figure 11](image-url)

**FIGURE 11.** PriB mimics SSB binding and does not displace either SSB or PriA from the fork. A, 500 nM PriB was combined with 20 nM forked substrate for lanes where + fork is indicated. Lanes that indicate + protease were subjected to treatment with Proteinase K postirradiation to identify protein-containing bands. For lanes where − fork is indicated, 500 nM PriB was reacted with 20 nM of the single-stranded radiolabeled phenyldiazirine-containing oligonucleotide. B, 500 nM PriA was bound to 20 nM of forked substrate, and PriB was titrated. C, reactions analyzed in lanes 4 and 5 contained 500 nM PriA and 500 nM SSB. For the lane indicating + PriB, 500 nM PriB was added after PriA and SSB to the corresponding reaction. D, reactions analyzed in lanes 2–13 contained 500 nM PriA and 500 nM SSB. For the lane indicating + PriB, 500 nM PriB was added after PriA and SSB to the corresponding reaction. E, reactions analyzed in lanes 2–13 contained 500 nM PriA and 500 nM SSB. For lanes indicating + PriB/DnaT, 500 nM PriB and 150 nM DnaT were added after PriA and SSB to the corresponding reaction. We attempted to generate a DnaT-DNA cross-linked marker but were unsuccessful using DnaT concentrations up to 500 nM. We note that a recent report (35) shows a DnaT-DNA interaction, but only at very high concentrations (>2.8 μM).
reaction of the carbene with nearby protein or nucleic acid residues.

The third band contains protein and is novel, not occurring with the single-stranded DNA controls. Because the mobility is significantly slower on SDS gels, it must result from cross-linking to a position within SSB that is closer to the ends than the species observed with single-stranded DNA. This would result in a more asymmetric, slower migrating band. The same novel band is observed in the single-stranded regions adjacent to the fork, but not at the distal ends of the single-stranded flaps. This novel cross-link in the single-stranded regions requires a forked structure. If the opposing flap is deleted, only the species characteristic of binding single-stranded DNA is observed. Thus, SSB binds both single- and double-stranded regions at the fork differently than single-stranded DNA.

Our findings suggest that a single SSB tetramer can interact with a replication fork, including both single-strands and the flanking duplex. The spacing between the photolabels (6 nt on the lagging strand and 9 nt on the leading strand template) are well within the binding site size for an SSB tetramer (27). The requirement for single strands on both the leading and lagging strand side of a fork for the novel cross-link to occur suggests binding of both by the same SSB tetramer. If true, this opens the possibility that SSB could interact with not only the lagging strand template but the leading strand, perhaps nested between Pol III HE and the DnaB helicase. This would allow the leading strand half of the Pol III HE to enjoy the same stabilization and face the same challenges as the lagging strand half. It has been commonly presumed, without evidence, that SSB binding occurs only on the lagging strand. Additionally, or alternatively, this interaction could support an important intermediate in repair or recombinational reactions in which SSB participates (28). If the interaction of SSB with a fork is sufficiently tight, it could help direct localization of limiting SSB to the fork through cooperative interactions.

PriA is a paralog of SSB, containing an OB fold and binding single-stranded DNA (17, 29). Like SSB, it yields a single cross-linked species on denaturing gels when linked to a single-stranded probe and three distinct species with cross-linked at or near a fork. One species is the same as observed on single-stranded DNA, another is a DNA-DNA cross-link, and the third represents a slower migrating species resulting from a novel interaction with forks, both in the flanking duplex and single-stranded regions (Fig. 11).

Structures are available for PriB bound to single-stranded DNA. Binding takes place through an OB fold in a slightly different mode that uses different interactions (16, 30). Unlike SSB, PriB binds both single- and double-stranded DNA (31). However, our studies indicate the interaction with a fork is different from general double-stranded DNA because the novel cross-linked species are only observed near the fork. It is possible that the binding observed near the distal end of the duplex arises from fraying, because no DNA-DNA cross-link is observed. No structure is available for PriB binding double-stranded DNA, but two models have been proposed (30). In one, the duplex has been modeled to interact with the OB-fold, through some contacts used to bind single strands plus others. In another model, a different binding site mimics binding of double-stranded DNA by the E. coli histone-like HU protein but still shares some interactions with the OB fold (30). Our findings could be explained by either model, provided novel contacts occur that are closer to the ends of the protein than the OB-fold single-stranded DNA contacts.

PriA, in the absence of other proteins, cross-links only to regions close to the fork, including the primer terminus and both flaps and the duplex. Interactions are not observed at the distal single- and double-stranded regions. These observations are consistent with the known binding properties of PriA (5–8). DNA-DNA contacts are also observed at the fork, not only in the double-stranded region, but in the flanking single-strands. This indicates that the PriA complex brings the two opposing single-strands into close proximity in a dehydrated environment.

When SSB is added to established PriA-fork complexes, SSB is excluded from the fork but cross-links to the distal single-stranded regions of the flap and duplex, presumably by fraying it. A contact between PriA and the primer terminus remains, consistent with there being a specific binding pocket within PriA for the primer terminus. PriA binds the C terminus of SSB (12).

For SSB to be excluded from the fork, a specific order of addition in which PriA is added first is required. If SSB is added first, PriA is excluded from the fork (Fig. 6). However, PriA rapidly arrests strand displacement by Pol III HE in the absence of DnaB helicase in the presence of SSB (14). Perhaps PriA can bind to a smaller site than SSB, giving it earlier entry, or it interacts with Pol III HE, facilitating its entry.

The DnaB helicase can self-assemble on forks by threading itself onto the free end of a 5′-flap in the absence of steric blocks provided by SSB or attached proteins (15). This provides an avenue to study the positional binding specificity of this helicase in the absence of other proteins. To obtain a stable interaction, we must provide ATPγS (Fig. 7). In the absence of nucleotide or in the presence of ATP, no DnaB-fork cross-links are observed. In the presence of ATP, DnaB certainly loads but proceeds with its helicase activity splitting the strands and dissociating. ATPγS stabilizes binding but will not support helicase action. Cross-linking of DnaB is observed in the duplex region and the single-stranded flaps, all limited to the region of the fork junction (Fig. 8). This technique has proven useful in studying protein-protein interactions at the fork. For example, the affinity of the τ subunit of the Pol III HE for DnaB is enhanced when the latter is assembled onto a DNA fork in the presence of ATPγS.3

Addition of streptavidin to a biotin attached to the 5′ flap blocks the DnaB self-loading reaction and makes it dependent on the full complement of primosomal proteins (15). We observe a full dependence upon primosomal proteins for DnaB loading in the photo-cross-linking reaction as well. Omission of any single primosomal protein results in the loss of DnaB assembly in the presence of ATPγS. Like PriA, we see more precise positioning of DnaB in the presence of functional protein partners. When loaded by the primosomal pathway, the

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3 L. Wilson, B. P. Glover, and C. S. McHenry, unpublished observation.
results show that hydrolysis is not necessary, because ATP that ATP is required for the helicase to engage the arm. Our essential for processive action of the helicase. They observed observed that a leading strand arm of at least 14 nucleotides is dehydration to be required to enable cross-link formation. our knowledge of the chemistry of the competing reactions, we suggest their presence in the complex. Our results do not exclude tran- have been observed when these proteins are added to PriA-DNA complexes (4), indicating orients the helicase to unwind the lagging strand side of arrested repli- complexes we form in the presence of ATPγS. SSB remains associated to peripheral single-stranded regions on the fork. contact with the single-stranded portion of the lagging strand is lost because the helicase presumably progresses into the duplex for one helicase step, without hydrolysis. We do not observe significant DnaC cross-links with forked substrates in complete reactions in the presence of ATPγS and DnaB. On single-stranded DNA, DnaC binds DNA in the presence of ATPγS and excludes DnaB from binding (23). We have also confirmed a DnaC-ssDNA interaction in our experiments (Fig. 10A, lane 13). Thus, the DnaC-ATPγS-DNA complex observed (23) likely occurs transiently, but the reaction proceeds further on a forked structure without ATP hydrolysis. We observe a strong DnaB cross-link with a photolabel attached to the displaced strand, 6 nucleotides in front of the fork (Fig. 8). Because we observe no cross-link between streptavidin and the photolabel attached to the −35 position, we know that proximity is not sufficient to support a cross-link. From our knowledge of the chemistry of the competing reactions, we would expect sequestration in a binding pocket that leads to dehydration to be required to enable cross-link formation. Reports of contacts with the displaced strand by helicases have been inferred from functional data. Bujalowski et al. (32) have observed that a leading strand arm of at least 14 nucleotides is essential for processive action of the helicase. They observed that ATP is required for the helicase to engage the arm. Our results show that hydrolysis is not necessary, because ATPγS will suffice. Raney and co-workers (33) have observed that at least a 6-nucleotide leading strand tail is required for efficient action of the Dda helicase. Trakselis and co-workers (34) detected a contact with the displaced strand from an archaeal MCM helicases and showed that interaction made important contributions to stability and function. We do not observe competition by PriB for the fork. PriB is excluded from the fork but binds to distal single-stranded regions. In the presence of both SSB and PriA, no PriB (or DnaT or DnaC) binding is observed to any of the photolabeled positions. We know both proteins are part of the complex because supershifts in labeled forks occur on native gels in their presence (4, 5). Thus, the proposed handoff mechanism (18), although it likely occurs, probably awaits the presence of all protein partners with all interactions occurring rapidly and transiently before dissociation upon loading DnaB. Thus, our observations are consistent with a model in which PriA binds to forks, probably as a multimer binding both single-stranded flaps at the replication fork and the primer terminus (Fig. 12). SSB binds to flanking single-stranded regions and forms a specific contact with PriA, leading to precision in its positioning. The addition of PriB and DnaT does not change any of the existing contacts nor create new ones, so their association is peripheral. Upon addition of DnaC and ATP, DnaB loading occurs, probably with undetected additional rapid transient contacts before the remaining primosomal proteins dissociate. Upon reassociation of dimeric Pol III HE on the leading strand, replication can resume.

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