The PriA Replication Restart Protein Blocks Replicase Access Prior to Helicase Assembly and Directs Template Specificity through its ATPase Activity*

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Running Title: PriA specificity and function

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Background: PriA initiates restart at stalled replication forks.
Result: A FRET assay detects E. coli and B. subtilis PriA-mediated helicase loading.
Conclusion: PriA ATPase activity directs specificity toward larger gaps on leading strands. PriA blocks replicase binding.
Significance: This work reveals a novel role of an ATPase in regulating DNA structural specificity and establishes the mechanism of PriA as a checkpoint protein.

SUMMARY
The PriA protein serves as an initiator for the restart of DNA replication on stalled replication forks and as a checkpoint protein that prevents the replicase from advancing in a strand displacement reaction on forks that do not contain a functional replicative helicase. We have developed a primosomal protein-dependent fluorescence resonance energy transfer (FRET) assay using a minimal fork substrate composed of synthetic oligonucleotides. We demonstrate that a self-loading reaction, which proceeds at high helicase concentrations, occurs by threading of a pre-assembled helicase over free 5' ends—an event that can be blocked by attaching a steric block to the 5'-end or coating DNA with SSB. The specificity of PriA for replication forks is regulated by its intrinsic ATPase. ATPase-defective PriA K230R shows a strong preference for substrates that contain no gap between the leading strand and the duplex portion of the fork, as previously demonstrated. Wild-type PriA prefers substrates with larger gaps, showing maximal activity on substrates on which PriA K230R is inactive. We demonstrate that PriA blocks replicase function on forks by blocking its binding.

In bacteria, DNA replication initiates at a unique chromosomal origin directed by sequence-specific binding of multiple copies of DnaA with ensuing loading of the replicative helicase by a helicase loader (1,2). Once replication forks are established, they often encounter a block before they reach DNA replication termination points, roughly 2 Mbp away on the E. coli chromosome (3). Once the replisome dissociates, DnaA no longer functions to re-establish the replication fork. This reaction is driven by the PriA protein in both Gram-negative and Gram-positive model organisms, E. coli and B. subtilis (4,5).

In E. coli, several proteins are thought to act sequentially in building up the apparatus that can attract the helicase loader and assemble an active replication fork. Gel shift assays indicate that PriA binds initially, followed by PriB and then DnaT (6). The resulting complex recruits the E. coli helicase loader/helicase (DnaC/DnaB), leading to helicase assembly in the presence of ATP (6).

Thus, PriA appears to be the lead protein that directs assembly of the restart primosome. This is consistent with its substrate specificity in binding to D loops, but not bubbles, and three-stranded structures that provide models for
replication forks (7,8). PriA contains an intrinsic 3'-5' helicase that has been suggested to function to clear an annealed lagging strand product from the replication fork, creating a site for primosome assembly and helicase loading (9,10). However, PriA mutants that are defective in helicase activity remain active and are even more effective than their wild-type counterparts on substrates containing single-stranded lagging strands (11). This has been proposed to be due to the mutant form remaining resident at the fork and not migrating away. PriA has also been shown to be a checkpoint protein that blocks the intrinsic strand displacement activity of DNA polymerase III holoenzyme (Pol III HE) on forks before an active replicative helicase has been assembled (12,13).

Early in the primosome assembly reaction, a handoff mechanism has been proposed, sequentially, between PriA, PriB and DnaT. Weak PriA-PriB and PriB-DnaT interactions are strengthened in the presence of single-stranded DNA. The binding sites on single-stranded DNA are partially shared by PriA, PriB and DnaT. It has been suggested that the primosome assembly process is a dynamic one in which these proteins hand off the fork substrate to one another, culminating with DnaT binding to PriB and displacing it from DNA to provide a landing site for the helicase loader and helicase (14).

A replication restart protein-initiated rolling circle replication system has been established for *E. coli* that recapitulates the rate observed for replication forks in vivo (15,16). Using this system, many of the basic principles of fork dynamics have been established, including the reversible association of primase with the replicative helicase, regulating Okazaki fragment length (16,17) and the association of the τ subunit of the Pol III holoenzyme with the replicative helicase. The latter association tethers a dimeric replicase containing both leading and lagging strand polymerases to the helicase, binding all components active in DNA replication together at the fork in one large replisome assembly (18).

In the evolutionarily distant Gram-positive bacterium *B. subtilis*, conserved PriA proteins and the replicative helicase (termed DnaC in *B. subtilis*) participate in the replication restart primosome, but novel proteins, DnaD and DnaB, which are not homologs of *E. coli* primosomal proteins, intervene between PriA and association with the helicase/helicase loader. The *B. subtilis* helicase loader, DnaI, is also poorly conserved, so *B. subtilis* and other low GC Gram-positive bacteria may follow a novel pathway for primosome assembly. An ordered assembly mechanism (PriA-DnaD-DnaB-DnaI-DnaC) has been proposed (19). Another important difference between the *E. coli* and *B. subtilis* replication processes is that the proteins that act at intermediate stages between PriA and the helicase loader also participate in DnaA/origin-dependent initiation (20).

We have established a fully functional *B. subtilis* rolling circle replication system on mini-circular templates that recapitulate the known in vivo replication rate and the genetically defined protein requirements (21). This system allowed the function of two discrete Pol IIIIs (DnaE and PolC) to be established. The PolC HE (PolC, τ-complex and β2) serves as the replicase for both the leading and lagging strands. Unlike in *E. coli*, the major replicase cannot efficiently elongate a primer provided by the DnaG primase. Instead, a protein functionally analogous to DNA polymerase α in eukaryotes, DnaE, extends the RNA primer a short distance and hands off the product to the PolC HE.

Both the *E. coli* and *B. subtilis* rolling circle replication systems require an extensive preincubation in a reaction where the helicase is recruited and assembled before elongation is initiated. This is necessary to isolate the elongation events so they can be studied without the kinetic complications of a rate limiting initiation. Thus, a well-defined system amenable to convenient acquisition of kinetic data is needed to study PriA-dependent assembly of the restart primosome.

FRET assays have been developed using synthetic forks that permit monitoring helicase function (22,23). In this report, we adapt this FRET assay and show that blocking the 5'-end of the lagging strand template sterically precludes self-assembly of the helicase and makes the reaction dependent on PriA and the other components of the replication restart primosome. We use this system to reveal that PriA specificity for replication fork structure is determined by the
PriA ATPase. We also explore the mechanism by which PriA acts as a checkpoint protein, blocking the intrinsic strand displacement activity of the Pol III HE on incompletely assembled replication forks.

EXPERIMENTAL PROCEDURES

Oligonucleotides—All oligonucleotides were obtained from Biosearch Technologies. Substrates used in all FRET experiments and strand displacement reactions carried out in solution were assembled from the HPLC-purified oligonucleotides listed in Fig. 1E.

Substrates for fluorescent helicase assays and strand displacement reactions in solution were assembled by combining 1 μM fluorescent leading strand template, 1 μM quenching lagging strand template, and 1 μM of the appropriate leading strand primer (or no primer) in a final volume of 25 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/min.

Unprimed forked template was constructed from FT90 and QT90; 0 nucleotide (nt) gap forked template from FT90, QT90, and P0g; 2 nt gap forked template from FT90, QT90, and P2g; 5 nt gap forked template from FT90, QT90, and P5g; 10 nt gap forked template from FT90, QT90, and P10g; and 20 nt gap forked template from FT100, QT90, and P20g.

For experiments on streptavidin beads, biotinylated 10 nt gap forked template was constructed from biotinylated primer 35-mer-5’-TT(biotin)GAAGATTCTTACATTAGCCGACA AAATCATATT-3’ (biotin is conjugated to preceding modified thymidine); leading strand template 90-mer-5’-CGCGTATAGATCATTACTATAACATGTTAG ATTCATGATAATACGAGATGACGATA TGATTGGTCGCTAATGTAAGAATCTTCAA A-3’; lagging strand template 90-mer-5’-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
the non-fluorescent quenching lagging strand template. The 20 nM standard was measured in the absence of quenching lagging strand template.

**PriA Blocking Strand Displacement Reactions**—The leading strand primer was labeled with $^{32}$P on the 5'-end using T4 polynucleotide kinase according to the manufacturer's instructions (Invitrogen) prior to annealing. Unincorporated [$^{32}$γ-P]ATP was removed using a Microspin-G25 spin column (GE Healthcare). Experiments were carried out in the same reaction buffer as FRET experiments.

For reactions carried out in solution, 20 nM substrate was added to protein components from the indicated source organism: 500 nM PriA, 500 nM SSB$_4$, 500 nM β$_2$, 80 nM τ$_3$ complex, and 80 nM Pol III core (exo-) (or indicated B. subtilis polymerase) in a final reaction volume of 30 μL. The samples were incubated for 5 min at room temperature. dNTPs were added to 100 μM (final concentration) and incubated with the reaction for 5 min. The reactions were quenched in a sample of equal volume containing 96 % formamide, 20 mM EDTA, and 1 mg/mL bromophenol blue, then heated to 95 °C for 4 min. The samples were resolved by denaturing electrophoresis at 10 W for 2 h in a 12 % polyacrylamide gel (19:1 acrylamide:bisacrylamide) containing 8 M urea using 100 mM Tris-borate and 2 mM EDTA as electrophoresis running buffer. Gels were dried onto DEAE paper and scanned by a Phosphorimager.

For reactions carried out on streptavidin beads, 600 fmol of radiolabeled, biotinylated primer/10 nt gap forked template were bound to 25 μL of streptavidin beads (Promega Tetralink Tetrameric Avidin) pre-equilibrated in the reaction buffer. The forked template was incubated with the beads for 10 min at room temperature. The beads were then washed three times with 200 μL of reaction buffer to remove any unbound substrate. The bead-bound substrate was then incubated with 500 nM PriA, 500 nM SSB$_4$, 500 nM β$_2$, 80 nM τ$_3$ complex, and 80 nM Pol III core (exo-) at room temperature for 15 min. The beads were then washed five times with 50 μL of the reaction buffer to remove free protein. dNTPs and/or primosomal proteins (PriB, DnaT, DnaB, and DnaC) were added to the reaction and incubated at room temperature for 15 min. The helicase loading conditions were independently optimized for this assay: 50 nM PriB$_2$, 500 nM DnaT$_3$, 12 nM DnaB$_6$, and 25 nM DnaC (supplemental Fig. S4). The reaction was quenched with 100 mM EDTA and 2 % SDS. The product was removed from the bead by incubating with 20 μg of protease K at 37 °C for 1 h. A portion of the supernatant was then added to an equal volume of buffer containing 100 mM Tris-borate, 2 mM EDTA, 20 % (v/v) glycerol, 1 mg/mL bromophenol blue, and 1 mg/mL xylene cyanol FF prior to resolving in a native gel to assay helicase activity. These samples were resolved by electrophoresis at 75 V for 18 h in a 12 % polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) using the same electrophoresis buffer as denaturing gels. The gel was dried and scanned by a Phosphorimager. A separate portion of the supernatant was analyzed by denaturing PAGE.

**RESULTS**

**Development of a FRET assay for primosome function**—We adapted a FRET based assay for helicase function (22) and optimized it for studying the helicase loading apparatus from two representative Gram-negative and Gram-positive organisms, *E. coli* and *B. subtilis*. The system employed model replication forks constructed from synthetic oligonucleotides with a fluorophore opposed by a quencher in the opposing strand (Fig. 1). Splitting the two strands apart results in an increase in fluorescence. We used an excess of a trapping oligonucleotide that was complementary to the duplex region of the leading strand template to sequester strands split apart by helicase so that they do not re-anneal. The leading strand template contained an annealed primer that modeled the leading strand replication product. Primers were synthesized with a variable sized gap between their 3'-ends and the fork. The 5'-end of the lagging strand also contained a biotin to which streptavidin could be bound, providing a sterical block.

Titrating high concentrations of either the *E. coli* DnaB helicase or the *B. subtilis* DnaC replicative helicase onto templates in the absence of other proteins allowed helicase self-loading and function leading to extensive unwinding (Fig. 2A). To determine whether the hexameric helicase self-
assembles on DNA by a mechanism where it threads over a free 5’-end, we blocked the 5’-end of model substrates with streptavidin (Fig. 1). When the 5’-end of the lagging strand template at the fork was blocked, neither E. coli nor B. subtilis helicases were able to self-assemble (Fig. 2B). In the absence of streptavidin, the cognate SSB also prevents helicase self-loading in both systems (Fig. 2C).

To establish an optimal system for helicase loading that is dependent upon E. coli and B. subtilis primosomal proteins, we blocked the single-stranded 5’-end of forked templates with streptavidin to eliminate the helicase self-loading background and titrated each component to determine its optimum concentration (Fig. 3 and 4). The optimal level was selected for subsequent experiments except for SSB. We selected an excess, slightly inhibitory level, that proved effective in blocking helicase self-loading (Fig. 2C).

The preceding experiment was conducted under conditions using a forked substrate containing a 10 nt gap between the leading strand 3’-primer terminus and the fork. Similar experiments were conducted with a substrate that contained no gap (supplemental Fig. S1 and S2). Similar results were obtained, except higher concentrations of PriA, PriB, DnaT and DnaC were required in the E. coli system and higher levels of PriA, DnaB, DnaD and DnaI were required in the B. subtilis system. Thus, both systems required higher levels of all proteins that act prior to helicase loading, suggesting a lower functional affinity for templates that do not contain a gap in the leading strand.

Our initial forked substrate contained 90 nt leading and lagging strand templates with a 45 nt duplex ahead of the fork. This represented close to the longest practical length considering economic factors and current commercial capabilities for producing substituted oligonucleotides. Systematic efforts to decrease the size resulted in decreased activities (supplemental Table S1). Thus, we retained the use of forks composed of annealed 90-mers for most experiments.

*The intrinsic PriA ATPase is required for primosome assembly on forks containing leading strand gaps*—The preference for forked templates with variable gap length was determined. We observed significantly higher function with templates with the largest (10 nt) gap size in both the E. coli and B. subtilis primosomal systems (Fig. 5A,D). In an earlier study, the opposite result was observed using a PriA derivative in which the intrinsic ATPase required to drive a 3’→5’ helicase activity was inactivated (11,34). To permit a direct comparison, Ken Marians kindly provided some of the PriA K230R used in that study. Using our FRET assay, we reproduced their results (Fig. 5B). Thus, the reversal in gap size preference and the ability to efficiently use templates with gaps requires the activity of the PriA ATP-dependent helicase (Fig. 5C). The results reported in Fig. 5 were obtained under conditions optimized for forked templates containing a 10 nt gap. To ensure that the result was not condition-specific, we repeated the experiment using conditions that had been optimized for templates lacking a gap between the fork and the leading strand and obtained the same result (supplemental Fig. S3).

**PriA functions as a checkpoint protein by binding to forks and blocking Pol III HE binding to the 3’-terminus of the leading strand**—In addition to PriA serving as the lead protein in directing primosomal assembly and replicative helicase loading, it functions as a checkpoint protein, at least in E. coli, blocking the intrinsic strand displacement activity of the Pol III HE on forks lacking the replicative helicase (12,13). We confirmed this result, using our FRET assay template with radiolabeled primers, permitting assay for primer extension, on templates containing 2 and 20 nt gaps (Fig. 6A, lanes 3 and 6). We observed, however, that we needed to add PriA before we added SSB. If we added SSB first, the checkpoint activity of PriA was not observed (compare Fig. 6A lane 2 with 3 and 5 with 6). We also observed that the action of PriA in blocking the progression of Pol III is dependent upon a forked structure. Omitting the lagging strand template from the reaction does not support PriA functioning as a checkpoint protein (Fig. 6A, lanes 9 and 11). A control used to validate the assay (lane 8) shows that SSB is required to support strand displacement (13).

Next, we sought to determine whether B. subtilis PriA blocked the B. subtilis polymerases
PolC HE and DnaE HE. As in the E. coli system, B. subtilis PriA served as a checkpoint protein that blocked the forward progression of both polymerases, but only if added before SSB (Fig. 6B and C).

We next sought to determine if the reaction was specific for cognate polymerases. We observed that both the PriAs could block E. coli Pol III HE and B. subtilis Pol C and DnaE HEs with equal efficiency. Furthermore, B. subtilis PriA blocked a polymerase that is not homologous to Pol IIIIs, T7 DNA polymerase, completely. The E. coli PriA inhibited T7 significantly with only a small portion of the product reaching full length (Fig. 6D). The experiment shown was conducted with a template containing a 2 nt leading strand gap. Essentially the same result was obtained on templates containing a 20 nt gap (data not shown).

Two models exist for PriA function. When the checkpoint action of the PriA protein was initially discovered, it was suggested that PriA may just act as a steric block, denying access of the Pol III HE to the primer terminus (12). Later, a multi-function protein from bacteriophage T4, gp59, that is not homologous to PriA but exhibits similar checkpoint activity, was shown to function by binding to the blocked T4 polymerase at forks, forming an inactive ternary complex (35) until helicase is loaded which releases the inhibition. We chose to distinguish these two models, using E. coli PriA and Pol III HE. A system was developed where the forked template was immobilized on streptavidin beads, allowing rapid washing and determination of the proteins bound functionally (Fig. 7A). The Pol III HE once bound to DNA in an initiation complex requires bumpers to prevent it from sliding off. Thus, we moved the biotin from the lagging strand template to the 5' end of the leading strand primer. The fork functioned as a bumper on the other end of the primer. The presence of SSB served to prevent self-assembly of helicase.

In the absence of primosomal proteins, the Pol III HE could efficiently form initiation complexes on primed bead-bound replication forks that survived washing and extensively elongated the leading strand primer in a strand displacement reaction upon addition of dNTPs (Fig. 7B, lane 3). The observed reaction was dependent upon the presence of $\beta_2$, indicating it proceeds from authentic initiation complexes rather than residual polymerase not removed by the washing step (Fig. 7B, lane 8). Elongation did not occur in the absence of SSB, a required cofactor for the strand displacement activity of Pol III HE (13) (Fig. 7B, lane 9).

Addition of PriA to the reaction in the absence of other primosomal proteins prevented extension of primers, either by blocking Pol III HE binding or arresting the polymerase in an inactive state (Fig. 7B, lane 4). The remaining primosomal proteins were added to permit PriA-directed helicase assembly, yielding an active helicase. Control reactions run on native gels detect a labeled primed leading strand (35/90) split from the lagging strand template permitting an independent assessment of helicase function (Fig. 7C). These control experiments indicated that 40% of the bead-bound forks contained an active helicase (Fig. 7C, lane 7). Yet, no elongation product was observed in the corresponding lane 7 in Fig 7B. If Pol III HE was present, sequestered in an inactive complex with PriA, elongation would have been expected at a 40% of the level we observed in lane 3 once helicase was loaded, relieving inhibition by PriA. We could have detected elongation at a level of 1%, judging by the control shown in Fig. 7B lane 13. Another control reaction where the washing step was omitted confirmed that PriA inhibition of the Pol III HE is relieved on the bead-bound substrate upon helicase assembly (Fig. 7D).

To check whether the result observed was caused by the 10 nt gap being too small to accommodate both Pol III HE and PriA at a fork, we repeated the experiment reported in Fig. 7 using a substrate containing a 20 nt gap and observe the same result (supplemental Fig. S5). We conclude that PriA acts as a checkpoint protein by blocking Pol III HE binding.

**DISCUSSION**

In this work, we have used synthetic model replication forks to study aspects of the replication restart reaction in divergent Gram-negative and Gram-positive model organisms. It has been estimated that E. coli and B. subtilis diverged approximately two billion years ago, a greater evolutionary distance than yeast and humans ((36) and references therein). Important differences
have been observed in the replication systems of *E. coli* and *B. subtilis* that suggest that the extensive replication studies conducted with *E. coli* do not always present an accurate model for replication in all bacteria (37). For example, *B. subtilis* requires two DNA polymerase IIIs for replication while *E. coli* requires only one (38). It has been demonstrated that the second Pol III (DnaE) has a specialized role in lagging strand primer processing analogous to the role of DNA polymerase α in eukaryotes (21). Additional proteins participate in initiation reactions, both at the origin and at the restart replication fork, that have no homologs in *E. coli* (39). Thus, having a Gram-positive system to compare with *E. coli* will permit further understanding of the mechanistic basis for their functional divergence.

We adapted a simple FRET assay that has been used in the study of other helicases (22). By blocking the 5'-end of the lagging strand, we were able to make both the *E. coli* and *B. subtilis* systems dependent on PriA and the remaining primosomal proteins. This indicates that the replicative helicase self-assembly reactions proceed by threading of pre-assembled hexamers over free 5'-ends of model forks. An example of the alternative model, where a hexameric helicase could transiently open and close, sequestering a single-stranded template, has been provided by the double-stranded RNA virus Φ12 helicase (40). This latter type of assembly should not be inhibited by a steric block on the 5'-end of the lagging strand template. The helicase self-assembly reaction only takes place at very high, non-physiological concentrations of helicase. We can conclude that this does not represent a common pathway in vitro consistent with genetic evidence that it does not take place in vivo (41,42).

Our initial forked substrate contained 90 nt single stranded leading and lagging strand templates with a 45 nt duplex ahead of the fork. Systematic efforts to decrease their size resulted in significantly decreased activity. This result contrasts markedly from the requirements of model forks for reactions that only contain replicative helicase in self-assembly reactions (43). There, a 15 nt lagging strand arm is sufficient to support an efficient reaction and a 10 nt arm is adequate for the reaction, although a 5 nt arm is not. In our system, a 15 nt lagging strand arm is inert (supplemental Table S1). Since it is thought that only the first 6-10 nts of the lagging strand tail productively interact with the helicase, the remaining length requirement must be for additional protein factors to bind. At least part of the binding site for PriA, PriB and DnaT is near the fork (14), but these factors productively interact with SSB (44,45) and additional lagging strand length is likely required to accommodate that interaction. The requirement for a long duplex ahead of the fork and within the leading strand arm is less explicable. Future studies, perhaps led by DNA site-specific crosslinking will likely provide insight into which factors interact within these regions.

We also examined the effect of the size of the gap between the 3'-end of the leading strand and the fork. We found differences depending on whether the ATPase within the PriA protein was active. PriA K230R with an inactive ATPase exhibits a strong preference for substrates with small or no gaps. Templates with gaps of 10 nt are inactive, consistent with published observations (34). However, with ATPase-proficient wild-type PriA, the result is just the opposite, with discrimination against substrates with short gaps and the highest activity on substrates with 10 nt gaps. To our knowledge, this observation is without precedent. While a large number of nucleic acid transactions are driven by ATPases and a large number of reactions are activated or dissociated by ATPase activity, we know of none where specificity in two robust reactions is reversed by the presence of an ancillary ATPase.

In addition to its key role in initiating primosome assembly, PriA serves as a checkpoint protein that prevents Pol III HE from catalyzing a strand displacement reaction in the absence of a replicative helicase at the fork (12). One model initially proposed was that PriA merely blocked access of the Pol III HE to replication forks. Another model was provided by the established mechanism of phage T4 gp59. Gp59 serves as a helicase loader and also as a checkpoint protein. Although not homologous, its function is analogous to PriA. Gp59 binds to forks and the T4 DNA polymerase, locking it into an inactive conformation in a stable ternary complex (35). Using forked templates bound to beads allowed rapid washing after complex formation so that
bound components could be determined. We showed that PriA excluded Pol III HE, as initially hypothesized. Control experiments showed that in the absence of PriA, Pol III HE could form initiation complexes on primed forked templates.

Further supporting a non-specific steric role for PriA in blocking strand displacement is our observation that PriA can block non-cognate polymerases. *E. coli* PriA can block strand displacement catalyzed by the *B. subtilis* Pol C and DnaE HEs, and *B. subtilis* PriA blocks strand displacement by the *E. coli* Pol III HE. It is conceivable that conserved protein interaction sites could enable the inhibition observed. But, we also demonstrated that both the *B. subtilis* and *E. coli* PriA proteins could block T7 DNA polymerase, which is not homologous to DNA Pol IIIIs. These experiments also establish a checkpoint role for *B. subtilis* PriA, showing the original *E. coli* observation (12) is general.

In all assays conducted for this study, we observed that PriA had to be added before SSB for it to display function. Presumably, if added first, SSB covers the PriA binding site and prevents its interaction with the fork. However, in dynamic reactions where Pol III HE is catalyzing a strand displacement reaction that is dependent upon its interaction with SSB on the lagging strand, addition of PriA immediately stops the reaction (13). This could be explained if PriA can bind to an initial segment of single-stranded DNA exposed on the lagging strand of the fork that is too small to stably or rapidly bind SSB. In this reaction, there could be transient interactions with Pol III HE, facilitating its displacement, but our results show a stable complex is not formed.

The substrates, assays and basic principles established by this study provide a foundation for further pursuit of the mechanistic basis of differences between the replication restart reaction in Gram-negative and low GC Gram-positive bacteria. The simple oligonucleotide substrates should be amenable to crosslinking studies, using nucleotide position-specific modifications, kinetic studies, screens for small molecule inhibitors and structural studies.
REFERENCE LIST


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FOOTNOTES
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2 The abbreviations used are: Pol III HE, DNA polymerase III holoenzyme; Pol III, DNA polymerase III; PolC HE, PolC holoenzyme (a combination of B. subtilis PolC, β2, and τ-complex); FRET, fluorescence resonance energy transfer; nt, nucleotide; DnaE HE (a combination of B. subtilis DnaE, β2 and τ-complex); SSB, single-stranded DNA binding protein; TET, tetrachlorofluorescein; BHQ-1 or BHQ, blackhole quencher 1.

FIGURE LEGENDS
FIGURE 1. Model replication fork and oligonucleotides used in FRET helicase assays. A, DNA substrate used in FRET unwinding reactions. Fluorescence of tetrachlorofluorescein (TET) on the 5’ terminus of the leading strand increases when separated from blackhole quencher 1 (BHQ-1) on the lagging strand. B, Internal biotin conjugated to modified thymidine. C, BHQ-1 conjugated to 3’-end of the lagging strand. D, TET conjugated to 5’-end of the leading strand. E, Sequences of oligonucleotides used to build substrates depicted in A for FRET assays. T(biotin) indicates the internal biotin modification in B.

FIGURE 2. E. coli and B. subtilis helicases self-load onto replication forks by threading onto a free 5’-end on the lagging strand. Titrations performed against 20 nM unprimed forked template in the absence of streptavidin unless stated otherwise. A, 550 nM E. coli DnaB6 (red) and B. subtilis DnaC6 (black). B, Streptavidin inhibits helicases from self-loading onto replication forks by binding to a biotin near the 5’-end of the lagging strand. Streptavidin titrated using E. coli DnaB6 (red) or B. subtilis DnaC6 (black). C, SSB can block the 5’-end of the lagging strand and prevent helicases from self-loading. E. coli or B. subtilis SSB titrated in the presence of E. coli DnaB6 (red) or B. subtilis DnaC6 (black), respectively.

FIGURE 3. Optimizing E. coli protein concentrations using 10 nt gap forked template. Helicase loading proteins and helicase were titrated sequentially (in the order they appear here) in the presence of all other primosomal proteins; the arrow indicates the chosen optimum for each. In subsequent experiments, that optimal level was used. The starting conditions used were: 75 nM PriB2, 250 nM
DnAT₃, 12 nM DnB₆, 100 nM DnC, and 500 nM SSB₄. The final optimized conditions from this series of experiments: 150 nM PriA, 50 nM PriB₂, 50 nM DnAT₃, 12 nM DnB₆, 50 nM DnC, and 500 nM SSB₄. A, PriA titration. B, PriB₂ titration. C, DnAT₃ titration. D, DnC helicase loader titrated at three different DnB₆ concentrations (6 nM (green), 12 nM (red), and 24 nM (blue)). E, SSB₄ titration. To prevent the helicase self-loading reaction, 500 nM SSB₄ was used for further experiments.

FIGURE 4. Optimizing B. subtilis protein concentrations on 10 nt gap forked template. The experiment was carried out as in Fig. 3. The starting conditions used were: 300 nM DnB, 300 nM DnD, 12 nM DnC₆, 200 nM DnA, and 500 nM SSB₄. The final optimized conditions from this series of experiments: 150 nM PriA, 75 nM DnB, 75 nM DnD, 12 nM DnC₆, 50 nM DnA, and 500 nM SSB₄. A, PriA titration. B, DnB titration. C, DnA titration. D, DnD helicase loader titrated at three different DnC₆ helicase concentrations (6 nM (green), 12 nM (red), and 24 nM (blue)). E, SSB₄ titration. To prevent the helicase self-loading reaction, 500 nM SSB₄ was chosen for further experiments.

FIGURE 5. Wild-type PriA containing a functional ATPase prefers forked substrates with large leading strand gaps. For A, B, and D five substrates were used: unprimed forked template (blue), 0 nt gap forked template (red), 2 nt gap forked template (green), 5 nt gap forked template (purple), or 10 nt gap forked template (cyan). Optimal E. coli protein concentrations listed in the legend of Fig. 3 were used. A, Wild-type PriA (E. coli) titration. B, PriA K230R (E. coli) titration. C, Amount of DNA unwound plotted against gap size at 50 nM wild-type PriA (red) and 50 nM PriA K230R (black) for E. coli. D, B. subtilis PriA titration. Optimal B. subtilis protein concentrations listed in the legend of Fig. 4.

FIGURE 6. PriA blocks the strand displacement reaction by Pol III HE in E. coli and by both B. subtilis Pol IIIIs. ³²P-labeled products of strand displacement were resolved in 12 % polyacrylamide with 8 M urea. Substrates are either 2 nt gap forked template (giving a 90-mer product) or 20 nt gap forked template (giving at 100-mer product). Substrates without a lagging strand consist of just primer bound to leading strand template. In reactions where PriA was not added first (lanes 2 and 5 in A, B, and C), but is present in the reaction, PriA was added immediately after SSB, but before HE. A, E. coli Pol III (exo-) HE and E. coli PriA. Lanes 9-12 contain a substrate composed of only primed template. Substrates in lanes 9 and 10 are constructed from FT90 and P2g and lanes 11 and 12 from FT100 and P20g. B, B. subtilis PolC HE and B. subtilis PriA. C, B. subtilis DnA HE and B. subtilis PriA. D, PriA blocks the strand displacement reaction across species. All reactions were carried out on 20 nM 2 nt gap forked template. Reactions with Eco Pol III HE and with T7 polymerase were performed with exonuclease-deficient polymerase. Lanes 1 and 2 contain markers to indicate the migration of the primer (43-mer) and the expected product (90-mer).

FIGURE 7. PriA and holoenzyme do not coexist on PriA-inhibited replication forks. A, Diagram depicting two possible models of PriA inhibiting the strand displacement reaction and the expected result of each for reactions on streptavidin beads. The primer is labeled on the 5′-end with ³²P so that primer extension and helicase activity can be monitored. The primer contains a biotin near the 5′-end so that substrates can be conjugated to streptavidin-linked beads. Scheme 1 depicts PriA blocking the 3′-OH of the primer, physically preventing Pol III HE from binding. Scheme 2 portrays an inhibition model where both PriA and Pol III HE bind to the substrate. Primosomal proteins (PriB, DnAT, DnB, and DnA) were added as described under Experimental Procedures. B, Denaturing gel analysis to monitor primer extension by E. coli Pol III (exo-). Lanes 11-13 are dilutions of the positive control lane 3 to establish detection limits. For both B and C, lanes 8 and 9 contain the full Pol III HE but in lane 8 the β subunit was omitted and in lane 9 SSB was omitted. C, Native gel analysis to monitor substrate unwinding by E. coli DnA helicase. The upper band is the replication fork and the 90/90 duplex product in those cases where replication occurs. The lower band is the displaced leading strand primer-template. In lane 5 ~45 % of the substrate was unwound by the helicase. In lane 7 ~40 % of the substrate was unwound by the helicase. In all other lanes, the amount of substrate unwound is not significantly above background. D,
Denaturing gel analysis to monitor primer extension by *E. coli* Pol III (exo-) on immobilized substrate without a washing step. Experiment carried out as described in *Experimental Procedures*, except after incubation with Pol III HE components, the washing steps were omitted. In lane 5 ca. 45% of the primer is elongated.
Fluorescent Leading Strand Template 90-mer (FT90)
5'-TET-CGCCGTTAAGATACATCTTTAATACATATACATATACTCAGCTATATAGATGATTTGCTGCTAATGTAACGAAATCTTCAA-3'

Quenching Lagging Strand Template 90-mer (QT90)
5'-TT(biotin)-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
Figure 2

A

[B] or [C] (nM)

[unwound DNA] (nM)

0 100 200 300 400 500 600 700

20 15 10 5 0

Figure 3

A

[ PriA] (nM)

[unwound DNA] (nM)

0 100 200 300 400 500 600 700

12 10 8 6 4 2 0

B

[ PriB] (nM)

[unwound DNA] (nM)

0 20 40 60 80 100 120 140 160

C

[DnaT] (nM)

[unwound DNA] (nM)

0 200 400 600 800 1000

7 6 5 4 3 2 1 0

D

[DnaC] (nM)

[unwound DNA] (nM)

0 200 400 300 400

E

[SSB] (nM)

[unwound DNA] (nM)

0 100 200 300 400 500 600 700 800 900 1000

6 5 4 3 2 1 0
Figure 4

A

B

C

D

E

[unwound DNA] (nM)

[PriA] (nM)

[DnaB] (nM)

[DnaD] (nM)

[DnaI] (nM)

[SSB] (nM)
The PriA Replication Restart Protein Blocks Replicase Access Prior to Helicase Assembly and Directs Template Specificity through its ATPase Activity
Carol Manhart and Charles S. McHenry

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