Unwinding of the Nascent Lagging Strand by Rep and PriA Enables the Direct Restart of Stalled Replication Forks*1

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During origin-independent replisome assembly, the replication restart protein PriC prefers to load the replication fork helicase, DnaB, to stalled replication forks where there is a gap in the nascent leading strand. However, this activity can be obstructed if the 5'-end of the nascent lagging strand is near the template branch point. Here we provide biochemical evidence that the helicase activities of Rep and PriA function to unwind the nascent lagging strand DNA at such stalled replication forks. PriC then loads the replicative helicase, DnaB, onto the newly generated, single-stranded template for the purposes of replisome assembly and duplex unwinding ahead of the replication fork. Direct rescue of replication forks by the Rep-PriC and PriA-PriC pathways in this manner may contribute to genomic stability by avoiding the potential dangers of fork breakage inherent to recombination-dependent restart pathways.

Although the two replisomes that proceed from oriC possess enough innate processivity to complete the replication of the entire Escherichia coli chromosome, the replication forks that begin replication are often not the ones that finish the job at the terminus. Endogenous DNA damage, template nicks, and frozen protein-DNA complexes collapse the fork at a high frequency. Therefore, in addition to DNA repair processes, there must be an accurate system for replication restart in order for the cell to maintain genomic integrity and viability. Loading of DnaB, the replicative helicase, to the chromosome is the key step in replisome assembly. Thus, this process must be highly regulated. In solution, DnaB is maintained in a tight complex with DnaC, melted DNA (5, 6). Besides providing duplex unwinding activity at the origin of replication, DnaB interacts transiently with the primase, DnaG (7), and assembles into a complex with the DNA wrapped around the periphery (3). AT-rich repeats are melted (4) and DnaA promotes the transfer of DnaB to the DnaB-DnaC complex onto the melted DNA (5, 6). Besides providing duplex unwinding activity at the replication fork, DnaB interacts transiently with the primase, DnaG (7), which synthesizes a short primer. The DNA polymerase III helazyme (Pol III HE) gains access to the DNA by recognizing the primer-template, and the replisome becomes fully assembled with a protein-protein interaction between DnaB and the 7 subunit of the holoenzyme (8, 9).

In cases where the replication fork either encounters a nick in the DNA template or a stalled replication fork undergoes breakage, fork collapse will result in a broken chromosome that is repaired by homologous recombination with an intact sister duplex to generate a D-loop structure (10). The restart proteins PriA, PriB, and DnaT fulfill the requirement for a loading system to reassemble the replisome and resume replication by delivering DnaB to the structure. PriA has been shown to bind D-loops with high affinity (11, 12) and form a complex with PriB and DnaT (13). This complex is competent to load DnaB from the DnaB/DnaC complex onto the displaced strand of the D-loop. Although PriA possesses 3' → 5' DNA helicase activity (14, 15), this activity is required neither for replication restart from a D-loop containing molecule in vitro (16, 17), nor to complement many of the phenotypes associated with the loss of PriA (18). Instead, the helicase activity may be important in a direct, non-recombinogenic form of replication restart. To load DnaB to the lagging strand template of a fork for restart, at least 20 nucleotides of single-stranded DNA is required at the branch point (19). On model forks with insufficient single-stranded DNA, PriA was shown to unwind the nascent lagging strand, generating a structure conducive to restart (20). This form of restart was proposed to act in concert with the branch migration protein RecG (21).

If replication restart is absolutely essential for cell survival, then PriA-independent restart mechanisms must exist, because the elimination of PriA, although harmful to the cell, is not lethal (22, 23). Disruption of the genes encoding either PriC or Rep led to inviability in the absence of PriA (24), implicating at least these two proteins in the PriA-independent pathway. In support of the model for multiple restart pathways, strains lacking both PriA and PriB, or PriC and Rep remain viable because the other pathway can compensate in each case. The mechanism for PriA- and PriB-dependent restart has been well characterized, but the restart pathway involving Rep and PriC is less understood. Furthermore, genetic evidence indicates the presence of a third restart pathway, about which little is known, that involves both PriA and PriC. We have recently reported evidence that PriC has the capability to load DnaB and restart replication on specific fork structures in reactions that are distinct from the PriA- and PriB-dependent system (25). However, in those studies, because Rep was not required in the reactions, its contribution toward replication restart could not be elucidated.

Like PriA, Rep exhibits 3' → 5' DNA helicase activity (26) and also plays a role in the replication of the phX174 chromosome (27). Although the processivity of Rep helicase activity is rather low on its own, it has the ability to displace bound proteins from the DNA template (28). The rep mutant was reported to have a slower rate of replication fork movement, about 50–60% of that of wild-type cells (29). Consequently, cells are larger, contain more DNA, and have more replication forks per cell (30). In combination with recB or recC disruptions, the rep mutant is inviable (31), suggesting an increased frequency of double-strand breaks.
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Indeed, the rep mutant does accumulate linear DNA when temperature-sensitive RecB and RecC are inactivated by high temperature (32). One explanation is that frequent replication pauses occur in the absence of Rep, perhaps because of an inability to remove blocking proteins, which leads to chromosome breakage.

In this report, we describe how Rep participates in the direct, PriA-independent rescue of stalled replication forks and the role of the helicase activities of Rep and PriA in the restart process. In the Rep-PriC pathway, the latter protein plays a crucial, multifunctional role by loading both Rep and DnaB to branched structures. Both Rep and PriA have the ability to unwind nascent lagging strand DNA at the fork branch point, activities that are focused to the proper template strand by the presence of SSB. The newly single-stranded template is then utilized by PriC to load DnaB, which nucleates replisome reassembly and provides the replicative helicase activity. Thus the Rep-PriC and PriA-PriC pathways of replication restart exhibit similar properties and are likely to act at stalled forks where a blockage of leading strand synthesis has allowed the 5′-end of the nascent lagging strand to progress further along the template than the 3′-end of the nascent leading strand.

**EXPERIMENTAL PROCEDURES**

**DNA and Protein**—The restart primosomal proteins PriA, PriC, DnaB, DnaC, and DnaG were all expressed in *E. coli* and purified as described previously (33). Oligonucleotides (oligos) were obtained from GeneLink. The forked, linear DNA template was prepared as described previously (25). The Rep open reading frame was amplified by PCR from *E. coli* W3110 genomic DNA using a forward primer with the sequence 5′-CGGCCGCCGATGCTCAAACCGGCCAAC-3′, and a reverse primer with the sequence 5′-GCCGCCGGATCCTTATTTCCTGTTTTCGCGCC-3′, and ligated into Ndel- and BamHI-digested pET-21a (Novagen) to create pRH72. Rep was purified from soluble extracts of BL21(DE3)(pLysS)(pRH72) by sequential chromatography on Q-Sepharose, Bio-Rex 70, single-stranded DNA cellulose, and hydroxylapatite, a combination and modification of previously published procedures (34, 35). SSB and the DNA Pol III HE (reconstituted from Pol III′ and β) was prepared as described previously (36). The fork substrates used in unwinding assays were prepared with the following oligos: (i) 1b-98, 5′-GACAGCCTTCTCAAGTGCCGACGGC-3′; (ii) 3L-98, 5′-GACTATCACTCCAGCGCCTCAGGCGGCGCATATGCGTCTAAACCCCGGCCAAC-3′; (iii) 11b-38, 5′-TACGTGCTGGCCTGTTGTAAGGCTGTAGAAGGCTTGC-3′; (iv) B-33, 5′-AGTCTGGCGGCCGAGCCTCCGACGATAGTC-3′. The basic fork is composed of oligos 1b-98 and 3L-98; to this, the leading strand oligo 11b-38 or the lagging strand oligo B-33 was annealed to the fork or omitted, as noted. All fork substrates were 5′-end-labeled on 3′-end-labeled on 3L-98

**Fork Unwinding Assays**—Reaction mixtures (15 μl) containing 50 mM HEPES-KOH (pH 8.0), 40 μg/ml bovine serum albumin, 2 mM dithiothreitol, 2 mM ATP, 4 mM MgOAc₂, 1 μM DNA substrate, and 125 nM SSB where indicated, were preincubated at 25 °C for 3 min. Reactions were started by addition of the indicated concentrations of Rep, PriC, PriA, DnaB, and DnaC, and incubated at 37 °C for 10 min, unless otherwise noted. Unwinding was terminated by addition of EDTA, SDS, and proteinase K to 20 mM, 0.5%, and 0.2 mg/ml, respectively, followed by incubation at 37 °C for 30 min. The samples were analyzed by electrophoresis at 17 V/cm for 1 h through a 10% polyacrylamide gel (30:1, acrylamide to bisacrylamide) using 100 μM Tris borate (pH 8.3), 2 mM...
EDTA as the electrophoresis buffer. The gel was fixed by soaking in 10% methanol, 7% HOAc, 5% glycerol, dried, exposed to a phosphorimager screen, and then autoradiographed. The fraction of intact substrate and unwound product bands were quantitated using a phosphorimager.

**Replication Restart Reactions**—Replication restart reaction mixtures (15 μl) containing 50 mM HEPES-KOH (pH 8.0), MgOAc$_2$ (5.5 mM for Rep-PriC reactions and 8 mM for PriA-PriC reactions), 10 mM dithiothreitol, 100 μg/ml bovine serum albumin, ATP (0.5 mM for Rep-PriC reactions and 1 mM for PriA-PriC reactions), 200 μM NTPs, 40 μM [α-32P]dATP (4000–5000 cpm/pmol), 40 μM dCTP, dTTP, and dGTP, 1 μM linear, forked template DNA, 20 nM Pol III*, 30 nM β subunit of the HE, 250 nM SSB, Rep, and the primosomal proteins as indicated were incubated at 37 °C for 30 min. When present, PriA was at 32 nM (except when varied as indicated), Rep was at 200 nM (except when varied as indicated), PriC was at 320 nM, DnaB was at 60 nM, DnaC was at 400 nM, and DnaG was at 300 nM. DNA synthesis was terminated by the addition of EDTA to 33 mM. Total DNA synthesis was determined by assaying an aliquot of the reaction mixture for acid-insoluble radioactivity. Samples were prepared for electrophoresis by adding one-fifth volume of a loading dye mixture containing 150 mM NaOH, 10 mM EDTA, 6% sucrose, and 0.1% bromophenol blue. DNA products were analyzed by alkaline gel electrophoresis through a 0.8% agarose gel at 2 V/cm for 15.5 h using 30 mM NaOH and 2 mM EDTA as the electrophoresis buffer. For DNA size reference, a 5'-[32P]-1 kb ladder (NEB) was analyzed alongside the samples. Gels were neutralized with 5% trichloroacetic acid, dried, exposed to a phosphorimager screen, and then autoradiographed.

**RESULTS**

PriC Stimulates Rep, but Not PriA, Helicase Activity—In considering the relationship between Rep and PriC, we asked whether PriC affected Rep helicase activity. When these analyses were conducted using partial duplex DNA substrates, no effect of PriC was detected (data not shown), however, when forked duplex substrates were used, PriC had a significant stimulatory effect (Fig. 1). The framework of the forked helicase substrates used in these experiments has been described previously (25). The substrates are composed of two 98 nucleotide (nt)-long oligos.

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**FIGURE 2.** Either the presence of a leading strand or of SSB suppresses Rep unwinding of the template DNA. Fork unwinding reaction mixtures containing either a model fork substrate carrying only the lagging strand (A and B) or both the leading and lagging strands (C and D) and either only the indicated concentrations of Rep (A and C) or both the indicated concentrations of Rep and 125 nM SSB (B and D) were incubated and analyzed as described under “Experimental Procedures.” Rep concentrations increase by a factor of two from left to right. Positions of the fork substrate and single- and double-stranded products are indicated to the right of the gels. The location on the substrate of the 32P label is marked by an asterisk. Autoradiograms of the dried gels are shown. Quantitation of the extent of unwinding was as described under “Experimental Procedures” and is plotted in each panel. Fork Unw., unwinding of the template strands. Lag Unw., unwinding of the duplex formed by the lagging strand template and the nascent lagging strand.
Annealing the two oligos generates a 60 bp duplex DNA with one blunt end and the other end consisting of two non-complementary, 38-nt long single-stranded tails. Various complementary oligos can be annealed to the single-stranded tails to represent the nascent leading and lagging strands at stalled replication forks.

Alone, Rep exhibited weak activity on a forked substrate that had no oligos representing nascent DNA annealed to it; however, in the presence of PriC, unwinding activity was stimulated 6-fold (Fig. 1A). The DNA substrate is relatively small, so the stimulation of activity most likely represents an increase in Rep loading efficiency, although whether PriC also increases the processivity of Rep cannot be determined by this assay. During rolling circle DNA synthesis of the bacteriophage φX174, an interaction between Rep and the phage-encoded gene A protein at the replication fork enables Rep to accomplish processive unwinding of the duplex DNA of the phage chromosome (27, 37). Based on our finding that PriC facilitates duplex unwinding by Rep, it is possible that PriC represents a cellular analog of the gene A protein.

At a fixed concentration of Rep, introducing increased concentrations of PriC caused unwinding to increase, but stimulation exhibited a threshold effect (Fig. 1B). At low concentrations of PriC, activity was barely increased, but at higher PriC concentrations, activity increased rapidly, resulting in a sigmoidal titration curve. Whether this indicates cooperativity in binding between multiple PriC monomers and DNA or Rep is unclear at this time. In a previous report, a Rep helicase stimulatory protein was purified from E. coli cell extract (38). Like PriC, this protein was small and basic, stimulated unwinding activity with a pronounced sigmoidicity, and maximal stimulation was observed at a protein:DNA ratio of over 350. Because this stimulatory factor shares so many characteristics in common with PriC, they are likely to be the same protein. This stimulatory activity of PriC was specific for Rep. No stimulation of PriA helicase activity could be observed (Fig. 1C).

The Presence of a Nascent Leading Strand and of SSB Modulates Rep and PriA Helicase Activities at Forked DNA Structures—Whereas the functional biochemical interaction between Rep and PriC indicated by the experiments described in Fig. 1 was consistent with the genetic observations, the helicase activity manifest was inconsistent with replication fork restart. It seemed unlikely that the unwinding of the template strands described would be productive. Rep cannot couple with
the Pol III HE in the absence of the φX174 gene A protein and, unlike DnaB, exhibits no interaction with the primase. Such promiscuous unwinding of the template strands in the absence of replisome formation is sure to be deleterious.

Under normal circumstances, DnaB is prevented from loading onto single-stranded DNA that is coated with SSB (2). Based on our previous observations that PriC was involved in a DnaB loading system that could overcome the SSB inhibition at stalled replication forks containing gaps in the leading strand (25), and that Rep helicase activity appeared to be important for restart, we hypothesized that a more productive role at a stalled replication fork for a non-replicative helicase such as Rep would be to modulate the structure of the fork by unwinding an obstructing DNA strand on the lagging strand template. Such an activity had already been ascribed to PriA (39).

When incubated with a forked substrate carrying a lagging strand oligo that was shortened from the 5'-end, leaving a 5-nt gap between it and the template branch point (this gap was used in order to be able to compare Rep and PriA action, it is required for PriA unwinding of the nascent lagging strand (data not shown and Ref. 39)), Rep could unwind both the template strands (fork unwinding) and the duplex formed by the lagging strand template and the nascent lagging strand (Fig. 2A). The former activity is similar to that described in Fig. 1; however, note that in the absence of PriC a 5-fold higher concentration of Rep was required to sustain the same extent of unwinding (compare the Rep titration in the presence of PriC shown in Fig. 1A to the Rep titration in the absence of PriC shown in Fig. 2A). However, the persistence of the fork unwinding activity was still troubling. We therefore searched for elements that would act to modulate this unwinding activity by examining the influence of both the presence of a nascent leading strand and of SSB, elements likely to be present at a significant fraction of stalled fork structures.

The presence of either a nascent leading strand or SSB suppressed the fork unwinding activity of Rep (Fig. 2, B–D). Both elements presumably act by the same mechanism, i.e. preventing access of Rep to the leading strand template. The ability of Rep to bind the forked substrates in the presence of SSB suggests that this protein, like PriA, possesses structure-specific DNA recognition. This is consistent with our observation that Rep can bind D-loop DNA specifically, even in the presence of SSB.3 These data indicate that the Rep helicase activity will be directed DnaB-loading activity, which is inhibited by the presence of a nascent lagging strand DNA, DnaB loading to this fork required an additional factor, either Rep or PriA, although targeting of these latter two proteins to the forked structures occurred by different mechanisms: PriA could recognize the branched structure independently, whereas Rep loading was mediated by PriC.

Unlike Rep, PriA did not exhibit any fork unwinding activity (Fig. 3). However, also unlike Rep, when a nascent leading strand was present at the fork, unwinding of the nascent lagging strand was suppressed (Fig. 3, A–C). This suppression was reversed by the presence of SSB (Fig. 3, D–F). In this case, we surmise that SSB acts to partially denature the branch point region, allowing PriA to gain access to the lagging strand template after recognition of the branched structure. Thus, SSB plays a critical role in ensuring that at stalled replication forks the helicase activities of Rep and PriA are applied in a fashion that will assist restart: to obviate a potentially inhibitory situation where access of DnaB to the lagging strand template is prevented. As described in the following sections, we proceeded to examine the complete mechanism of replication restart under such conditions.

The observed PriA-catalyzed unwinding of the nascent lagging strand is consistent with a previous demonstration using similar struc-
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FIGURE 5. Reconstitution of the Rep-PriC pathway of replication restart. Replication restart reaction mixtures were incubated and analyzed as described under “Experimental Procedures.” In the reaction shown in A, lane 1, the template carried only a leading strand oligo whose 3’-end was 16 nt from the fork branch point. In the reactions shown in all other lanes in A and all lanes in B, the template carried the leading strand oligo and a lagging strand oligo whose 5’-end was 5 nt from the fork branch point. Reaction mixtures contained 20 nM DNA Pol III HE, 60 nM DnaB, 400 nM DnaC, 300 nM DnaG, 320 nM PriC, and 250 nM SSB. Rep was at 200 nM in the reactions shown in A and as indicated for A (increasing in steps of two from left to right in lanes 3–6). For A and B, a relative DNA synthesis value of 1 represents 5.3 and 3.1 pmol of [32P]dAMP incorporated into acid-insoluble product, respectively.

...only one unwinding product was observed from this SSB-coated model fork containing a lagging strand oligo. The nascent lagging strand was unwound exclusively, whereas the parental duplex was left intact. On the other hand, PriA bound the substrate and unwound the lagging strand equally well in either the presence or absence of PriC (lanes 10 and 11). Unwinding of the nascent lagging strand oligo provided a single stranded template for the loading of DnaB to the fork by PriC when all components were present: PriC, DnaB, and DnaC, in the presence of either PriA or Rep. Only under these conditions was DnaB loaded to the lagging strand template and able to use its 5’ → 3’ helicase activity to unwind the parental duplex (Fig. 4, lane 9 for Rep and lane 13 for PriA). The loading of DnaB to such a stalled replication fork should be sufficient to reassemble the replisome and allow replication to proceed. To test this supposition, we reconstituted the Rep-PriC and PriA-PriC replication restart reactions.

Reconstitution of Replication Restart Mediated by the Rep-PriC and PriA-PriC Pathways—We have described previously a long linear forked template that we used to model replication restart at a stalled replication fork (25). The template is blunt-ended on one side, contains 6.9 kb of duplex DNA, and has two 38-nucleotide noncomplementary arms on the other end. The advantage of this template is that oligos of different lengths can be annealed to its arms, thereby modeling the structures of different types of stalled replication forks. The restart reactions are performed in the presence of [α-32P]dATP to label the DNA products, which are analyzed by alkaline agarose gel electrophoresis. The nascent leading strand migrates as a nicked DNA 7 kb in length, whereas the nascent lagging strand Okazaki fragments distribute as a population centered around 1 kb in length (note that neither DNA ligase nor DNA polymerase I, required to process and join Okazaki fragments together, are present in the reaction mixture). To maximize the DnaB loading activity of PriC, the template carried a nascent leading strand that left a gap of 16 nt between the template branch point and the 3’-OH. (As noted previously above, this gap is used in order to maximize PriC-directed DnaB loading activity.)

As we reported previously (25) this template supported DNA replication in the presence of the DNA Pol III HE, SSB, PriC, DnaB, DnaC, and DnaG (Figs. 5A and 6A, lane 1). However, when a template was used that also carried a nascent lagging strand oligo (with a 5-nt gap between the branch point and the 5’-end of the oligo), replication was completely inhibited because loading of DnaB to the lagging strand template was prevented (Figs. 5A and 6A, lane 2). Introduction of either Rep (Fig. 5A, lanes 3–6) or PriA (Fig. 6A, lanes 3–6) led to a recovery of replication activity, consistent with our proposal that these DNA helicases act to unwind the nascent lagging strand at stalled replication forks to provide a loading site for DnaB. That the PriC replisome loading system was still operative under these conditions is demonstrated by the single component omission experiments shown in Figs. 5B and 6B, where it is evident that replication was completely dependent on all of the components of either the Rep-PriC or PriA-PriC system except the primase, DnaG. Omission of DnaG should result in only the loss of Okazaki fragment synthesis. Studies in several reconstituted replication systems have demonstrated that its presence is not required for replisome assembly (16, 40), consistent with its transient association with the replisome (41, 42) and the presence of an available 3’-OH end on the leading strand oligo. Nevertheless, the strong dependence exhibited on the presence of DnaG in the experiments shown in Figs. 5 and 6 suggests that a significant fraction of leading strand synthesis may be initiating de novo. This aspect of the replication restart systems is currently under investigation.

DISCUSSION

Various types of lesions on the DNA template such as pyrimidine dimers, abasic sites, interstrand cross-links, and frozen protein-DNA complexes cause the interruption and arrest of replication fork progression. Strand breakage can occur at such a stalled fork by directed enzy-
mastic means, but replication forks can also collapse upon encounter with a DNA strand discontinuity. With such a myriad of types and possible locations of damage, it is not surprising that the cell has evolved multiple pathways to deal with the task of restarting DNA replication in order to achieve complete duplication of the chromosome and the accurate transmission of genetic material. Replication restart can be loosely divided into two types of pathways: those that involve fork breakage and recombination between the broken end and the intact sister duplex to generate a D-loop molecule, and forms of direct replication restart in which the substrate is an intact fork structure. The D-loop takes only one structural form, but the stalled fork has the potential to contain nascent leading and lagging strand DNA at the branch point, or to contain nascent DNA with large gaps. How the different restart pathways deal with these DNA structures has been the focus of intense research.

Both Rep and PriC act together genetically in a replication restart pathway that is distinct from the well studied PriA-PriB pathway; nevertheless, PriA is also located genetically in a pathway that includes PriC (24). Although each of the three restart pathways contains its own distinct set of proteins, one common feature is thus the presence of a helicase with 3′→5′ polarity, either PriA or Rep. However, both biochemical and genetic data suggest that the PriA helicase activity is dispensable for recombination-dependent replication restart, the presumed primary restart mechanism that utilizes the PriA-PriB pathway. Reconstitution of the replisome loading activity of the PriC pathway did not require Rep (25). Of what significance then are the Rep and PriA helicase activities?

Clues to the answer of this question came from our demonstration that the PriA-PriB and PriC restart pathways exhibited preferential recognition of stalled replication forks as a function of the position of the 3′-end of the nascent leading strand: the PriA-dependent system was inhibited by gaps between the leading strand and the fork branch point, whereas the PriC-dependent system was stimulated by the presence of such gaps (25). A DNA structure with gaps in the nascent leading strand could be generated if the replication fork encounters a blocking lesion located on the leading strand template (43). Leading strand DNA synthesis should halt, but the two DNA polymerases may become uncoupled, allowing helicase unwinding and lagging-strand DNA synthesis to continue for a limited distance. Based on our previous studies, it was clear that PriC would recognize this type of stalled fork, but would also be unable to load DnaB to the lagging strand template for restart because the nascent lagging strand DNA blocked access. To overcome the obstruction, the nascent lagging strand DNA must be unwound in order to provide a landing pad for DnaB. We therefore investigated whether the 3′→5′ helicase activities of Rep and PriA could catalyze this enabling event. This possibility proved to be the case for both proteins.

Both Rep and PriA could remove the nascent lagging strand from model fork substrates that did not carry a nascent leading strand. Rep, but not PriA, also manifested significant template strand unwinding under these conditions. The presence of either the nascent leading strand or of SSB inhibited this latter Rep-catalyzed reaction, leaving nascent strand unwinding unaffected. On the other hand, in the absence of SSB the presence of a leading strand inhibited PriA-catalyzed unwinding of the lagging strand, whereas in the presence of SSB, PriA unwinding of the lagging strand was stimulated. Thus, under the conditions most likely to be extant when forks stall, i.e. any exposed single-stranded DNA is bound by SSB, the helicase activities of the two proteins are directed toward unwinding the nascent lagging strand.

Therefore, a significant degree of similarity exists between how the helicase activities of the two proteins would elaborate at stalled forks, particularly because a presumed promiscuous and nonproductive template unwinding reaction catalyzed by Rep is suppressed in the cellular environment. PriA does not possess such an activity. An important aspect of these analyses is that it is clear that both proteins can unwind the nascent lagging strand irrespective of the presence of the nascent leading strand, thus ensuring that such an activity can be manifested at all types of stalled forks. However, although both proteins appear capable of recognizing the stalled forks (Rep has similar structure-specific DNA binding characteristics as PriA)3 PriA recognition occurs at high
affinity, with binding constants less than 5 nM even in the presence of SSB, whereas Rep binding under these conditions is significantly less avid, with binding constants greater than 50 nM. It is here, then, that the demonstrated PriC stimulation of Rep loading comes into play, effectively lowering the concentration of Rep required by nine-tenths, bringing it into the same functional range as that of PriA. PriC, therefore, is an unusual protein in that it must recognize and coordinate the loading of two helicases (Rep and DnaB) for DNA replication and restart.

Reconstitution of the complete Rep-PriC and PriA-PriC replication reactions demonstrated that the presence of a nascent lagging strand does, indeed, prevent PriC-directed DnaB loading and subsequent replisome assembly and that both Rep and PriA could overcome this inhibition by removing the blockage, generating an SSB-coated region on the lagging strand template to which DnaB could be transferred. Thus, the Rep-PriC and PriA-PriC restart reactions are biochemically equivalent, with the Rep-PriC combination being functionally equivalent to PriA in structure-specific DNA recognition and lagging strand unwinding (Fig. 7). Both of these pathways fall into the direct, non-recombinogenic category of replication restart. The cell may favor such pathways over recombination dependent pathways because potential dangers involving fork breakage are avoided.

If Rep is indeed involved in a specific replication restart pathway, then cells lacking Rep function should, as those lacking PriA do, display relevant phenotypes. The overall replication rate is reduced in the absence of Rep (29), manifested by an increase in the total amount of DNA per cell (30, 44) and an increase in the average number of growing forks in exponentially growing cells (45). One potential explanation of these observations is that the actual speed of the replication fork is reduced, but a more likely reason is that the replication fork frequently pauses or stalls. The finding that Rep has the capacity to dislodge DNA-bound proteins during unwinding led to the hypothesis that Rep suppresses fork pausing by removing proteins from the path of the oncoming replication fork (46). In the absence of Rep, RecB and RecC are essential, but RecA is not required (31). To explain the finding, the authors proposed that increased fork stalling led to an increased frequency of double-strand breaks, which must be repaired by the RecBCD recombination pathway. RecA was not required because under those conditions, the linear part of the chromosome resulting from the double-strand break would be degraded, leaving the bulk of the chromosome intact. Upon further analysis, an increase in the amount of linear DNA was detected, corresponding to an increase in the frequency of chromosome breakage, in the absence of functional Rep, RecB, and RecC, but not in the absence of Rep and RecA (32). Although these results can be interpreted to mean that Rep suppresses replication fork stalling that would lead to chromosome breakage, an equally plausible interpretation is that Rep actively participates in the recovery of stalled forks by a means that avoids chromosome breakage and replication restart by recombination. In support of the latter hypothesis, it was found that inactivation of RuvABC restores viability and suppresses the accumulation of double-strand breaks in strains lacking functional Rep, RecB, and RecC (47). In this situation, the loss of viability may be because fork restart is redirected away from Rep-dependent direct restart and toward a pathway involving fork regression, branch migration, and Holliday junction cleavage by RuvABC. The broken chromosome would be lethal to the cell without homologous recombination involving RecB and RecC, but in the absence of RuvABC, the regressed fork would instead be reset and replication restarted directly, presumably in a PriA-dependent fashion. Our finding that Rep unwinding of the parental duplex of a model fork is suppressed by the presence of SSB provides additional evidence that Rep does not act as an accessory replicative helicase. Instead, Rep unwound the nascent lagging strand preferentially, translocating in a direction away from replication fork progression.

Because PriB and PriC have redundant and essential functions with respect to growth rate and viability, the PriA-dependent pathways can be subdivided into the PriA-PriB, and PriA-PriC restart pathways (24, 48). Our reconstitution of the PriA-PriC restart pathway provides a biochemical underpinning to this division but does not explain completely the genetic observations. Both the genetics and biochemistry are congruent in arguing that the PriA helicase activity is important for the PriA-PriC pathway only: Mutations in PriA that specifically abolish
ATPase and helicase activity but preserve the replisome-loading activity have little effect on phenotype, but they have a negative effect on cells lacking PriB (49). However, it was found that cells lacking DnaT behave just like cells lacking PriA, suggesting that DnaT is also involved in the PriA-PriC pathway (50). The replisome-loading reaction described here does not require DnaT to load DnaB to the fork, thus how DnaT might fit into this pathway remains uncertain. Also, because the DNA structure is the same as that used for the Rep-PriC pathway, it is unclear under what circumstances it would be targeted by PriA for unwinding.

Conflicting genetic evidence makes it difficult to determine the relative contribution of the different restart pathways during normal cell growth. The extreme inviability of cells lacking PriA (22) and the lethality associated with loss of PriA and PriC or PriA and Rep support the concept that replication restart is required during every cell generation and suggests that Rep and PriC play only a minor role in this process (24). However, the finding that the dnaC133I mutation is specifically defective in PriA-PriB-dependent restart, but displays a wild-type growth rate suggests that the Rep-PriC pathway is potentially a major contributor to replication restart (51). Eliminating PriB should also inactivate the PriA-PriB pathway, but cells null for priB also display wild-type growth rates and viability, suggesting that the alternate pathways either compensate or are potentially major contributors. In the absence of DnaC function, it was found that 10–12% of cells failed to complete replication initiated at oriC (52). However, there are several examples of DnaC mutants that are differentially affected for different restart pathways (48, 51), so it may be that stalled forks are processed in different ways, changing the frequency of different restart substrates and altering the relative contributions of the restart pathways. An increased focus on the frequency of replication fork stalling in response to the action of different damaging agents during normal growth and how the cell responds to specific damage types should help clarify how often each restart pathway is used in wild-type cells.

REFERENCES
1. Wickner, S., and Hurwitz, J. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 921–925
2. Lellowitz, J. H., and McMacken, R. (1986) J. Biol. Chem. 261, 4738–4748
3. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell 38, 889–900
4. Brambil, D., and Kornberg, A. (1988) Cell 52, 743–755
5. Baker, T. A., Funnell, B. E., and Kornberg, A. (1987) J. Biol. Chem. 262, 6877–6885
6. Baker, T. A., Sekimizu, K., Funnell, B. E., and Kornberg, A. (1986) Cell 45, 53–64
7. Tougu, K., Peng, H., and Marians, K. J. (1994) J. Biol. Chem. 269, 4675–4682
8. Kim, S., Dallmann, H. G., McHenry, C. S., and Marians, K. J. (1996) Cell 84, 643–650
9. Yuzhakov, A., Turner, J., and O’Donnell, M. (1996) Cell 86, 877–886
10. Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J., and Marians, K. J. (2000) Nature 404, 37–41
11. McGlynn, P., Al-Delb, A. A., Liu, J., Marians, K. J., and Lloyd, R. G. (1997) J. Mol. Biol. 270, 212–221
12. Nurse, P., Liu, J., and Marians, K. J. (1999) J. Biol. Chem. 274, 25026–25032
13. Liu, J., and Marians, K. J. (1999) J. Biol. Chem. 274, 25033–25041
14. Lee, M. S., and Marians, K. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 84, 8345–8349
15. Lasken, R. S., and Kornberg, A. (1988) J. Biol. Chem. 263, 5512–5518
16. Liu, J., Xu, L., Sandler, S. J., and Marians, K. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3552–3555
17. Xu, L., and Marians, K. J. (2003) Mol. Cell 11, 817–826
18. Zavitz, K. H., and Marians, K. J. (1992) J. Biol. Chem. 267, 6933–6940
19. Jezewska, M. J., Kim, U. S., and Bujalowski, W. (1996) Biochemistry 35, 2129–2145
20. Jones, J. M., and Nakai, H. (1999) J. Mol. Biol. 289, 503–516
21. McGlynn, P., and Lloyd, R. G. (2000) Cell 101, 35–45
22. Nurse, P., Zavitz, K. H., and Marians, K. J. (1991) J. Bacteriol. 173, 6686–6693
23. Lee, E. H., and Kornberg, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3029–3032
24. Sandler, S. J. (2000) Genetics 155, 487–497
25. Heller, R. C., and Marians, K. J. (2005) Mol. Cell 17, 733–743
26. Yarranton, G. T., and Gefter, M. L. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1658–1662
27. Scott, J. F., Eisenberg, S., Bertsch, L. L., and Kornberg, A. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 193–197
28. Yancey-Wrona, J. E., and Matson, S. W. (1992) Nucleic Acids Res. 20, 6713–6721
29. Lane, H. E., and Denhardt, D. T. (1975) J. Mol. Biol. 97, 99–112
30. Lane, H. E., and Denhardt, D. T. (1974) J. Bacteriol. 120, 805–814
31. Uzest, M., Ehrlich, S. D., and Michel, B. (1993) Mol. Microbiol. 11, 1177–1188
32. Michel, B., Ehrlich, S. D., and Uzest, M. (1997) EMBO J. 16, 430–438
33. Mariani, K. J. (1995) Methods Enzymol. 262, 507–521
34. Scott, J. F., and Kornberg, A. (1978) J. Biol. Chem. 253, 3292–3297
35. Lohman, T. M., Chao, R., Green, J. M., Sage, S., and Runyon, G. T. (1989) J. Biol. Chem. 264, 10139–10147
36. Xiawa, H., and Marians, K. J. (1996) J. Biol. Chem. 271, 21529–21535
37. Arai, N., and Kornberg, A. (1981) J. Biol. Chem. 256, 5294–5298
38. Smith, J. R., Yang, T., and Matson, S. W. (1989) J. Biol. Chem. 264, 6119–6126
39. Jones, J. M., and Nakai, H. (2001) J. Mol. Biol. 312, 935–947
40. Mok, M., and Mariani, K. J. (1987) J. Biol. Chem. 262, 16644–16654
41. Wu, C. A., Zechner, E. L., and Marians, K. J. (1992) J. Biol. Chem. 267, 4030–4044
42. Wu, C. A., Zechner, E. L., Reems, J. A., McHenry, C. S., and Mariani, K. J. (1992) J. Biol. Chem. 267, 4074–4083
43. Higuchi, K., Katayama, T., Iwai, S., Hida, M., Horiuichi, T., and Maki, H. (2003) Genes Cells 8, 437–449
44. Trun, N. (2003) FEMS Microbiol. Lett. 236, 187–193
45. Colasanti, J., and Denhardt, D. T. (1987) Mol. Gen. Genet. 209, 382–390
46. Matson, S. W., Bean, D. W., and George, J. W. (1994) Bioessays 16, 13–22
47. Seigneur, M., Bidvenko, V., Ehrlich, S. D., and Michel, B. (1998) Cell 95, 419–430
48. Sandler, S. J., Mariani, K. J., Zavitz, K. H., Couto, J., Parent, M. A., and Clark, A. J. (1999) Mol. Microbiol. 34, 91–101
49. Sandler, S. J., McCool, J. D., Do, T. T., and Johansen, R. U. (2001) Mol. Microbiol. 41, 697–704
50. McCool, J. D., Ford, C. C., and Sandler, S. J. (2004) Genetics 167, 569–578
51. Harinarayanan, R., and Gowrishankar, J. (2004) Genetics 166, 1165–1176
52. Maisnier-Patin, S., Nordstrom, K., and Dasgupta, S. (2001) Mol. Microbiol. 42, 1371–1382