Mechanisms of replication fork restart in *Escherichia coli*

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Replication of the genome is crucial for the accurate transmission of genetic information. It has become clear over the last decade that the orderly progression of replication forks in both prokaryotes and eukaryotes is disrupted with high frequency by encounters with various obstacles either on or in the template strands. Survival of the organism then becomes dependent on both removal of the obstruction and resumption of replication. This latter point is particularly important in bacteria, where the number of replication forks per genome is nominally only two. Replication restart in *Escherichia coli* is accomplished by the action of the restart primosomal proteins, which use both recombination intermediates and stalled replication forks as substrates for loading new replication forks. These reactions have been reconstituted with purified recombination and replication proteins.

Keywords: replication fork; PriA; recombination-directed replication

1. REPLISOME ASSEMBLY

While it was evident to Watson & Crick (1953) that replication of the genome would proceed in a template-directed manner, it was over 30 years before replication of small, circular, double-stranded plasmid genomes was reconstituted *in vitro* with all the purified enzymes required (Kaguni & Kornberg 1984; Minden & Marians 1985). These and subsequent biochemical efforts have led to a thorough understanding of the activities present in the replisome that are necessary for replication fork progression. In *Escherichia coli*, the 10-subunit DNA Pol III HE is responsible for synthesizing the nascent leading and lagging strands of DNA. The Pol III HE has a dimeric core of two active polymerase centres that are linked by the τ -subunit (McHenry 1982). Thus, synthesis of the leading and lagging strands is physically coupled in space (Kim *et al.* 1996b). Primers for initiation of lagging-strand synthesis are manufactured by DnaG, the primase (Bouche *et al.* 1975), which associates transiently with DnaB (Tougu *et al.* 1994), the replication fork DNA helicase (LeBowitz & McMacken 1986), that is driving replication fork progression. The association of the helicase and primase at the replication fork is a common theme in bacteria and their combination is termed a primosome (Marians 1992). Rapid progression of the replication fork requires a protein–protein interaction between the τ -subunit of the Pol III HE and DnaB (Kim *et al.* 1996a; Yuzhakov *et al.* 1996). This interaction literally cements the replisome together. Exposed ssDNA, which is generated only on the lagging-strand template (Mok & Marians 1987), is coated with the SSB.

Formation of a replication fork is limited by the ability to load DnaB to the DNA. While this protein can be

loaded by DnaC, with which it forms a complex, to ssDNA, it cannot be loaded to SSB-coated ssDNA. Instead, other proteins must participate in the loading process. At the origin of replication, *oriC*, DnaA, the replication initiator protein, binds specifically to sequences in the origin (Fuller *et al.* 1984). Protein–protein interactions between DnaA and DnaB (Marszalek *et al.* 1996; Sutton *et al.* 1998) lead to the introduction of the latter to a region of the origin that has been unwound by DnaA. Once DnaB is on the DNA, subsequent replication fork formation proceeds by the ordered execution of some of the protein–protein interactions described above. Binding of DnaG to DnaB results in the synthesis of a primer on the template that serves as an attractor for the Pol III HE. Establishment of the τ –DnaB interaction and additional primer synthesis then completes formation of the replisome.

A second DnaB loading mechanism, involving PriA, PriB, PriC, DnaT, DnaB, DnaC and DnaG, was uncovered as a result of studies on the replication of ϕ X174 bacteriophage DNA *in vitro*. On this ssDNA, PriA recognizes and binds to a hairpin-like region (Shlomai & Kornberg 1980) (termed a primosome assembly site (Marians 1984)). A series of protein–protein interactions then results in the formation of the ϕ X-type primosome (Marians 1992), consisting of PriA, PriB, DnaT, DnaB, DnaG and possibly PriC, on the DNA (Ng & Marians 1996a,b). This primosome can support replication fork progression (Mok & Marians 1987). However, reconstitution of the replication of *oriC*-carrying plasmids *in vitro* made it clear that PriA, PriB, PriC and DnaT were not involved in initiation of chromosomal replication (Kaguni & Kornberg 1984).

2. PriA IS INVOLVED IN REPLICATION RESTART

The role played by PriA in replication fork restart and the importance of restart as a housekeeping function were

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revealed in large part by the phenotypes of mutations null for PriA function. Initial studies demonstrated that *priA::kan* strains were constitutively induced for the SOS response (Nurse *et al.* 1991) and were UV-sensitive (Lee & Kornberg 1991). To account for the SOS induction, we proposed that PriA was involved in origin-independent rescue of inactivated replication forks (Nurse *et al.* 1991; Zavitz & Mariani 1991). A similar suggestion had been made earlier to account for DnaT-dependent initiation of lagging-strand synthesis at regions outside of *oriC* during minichromosome replication in crude extracts (Seufert & Messer 1986). To account for the almost complete penetrance of the phenotype, we also suggested that even under normal growth conditions, the replication forks formed at *oriC* were unlikely to complete replication of the chromosome.

Subsequent studies demonstrated that *priA::kan* mutants were deficient in homologous recombination

Figure 1. Restart primosome-directed assembly of a replication fork at a D loop. (a) Template construction. The form II D loop template was constructed by first annealing a 100 nt long primer containing a central 42 nt long non-complementary region to f1 phage DNA. This primer was extended in the presence of SSB by the Pol III HE. During the last 2 min of this incubation, 2',3'-dideoxy-TTP and ATP were added so that the complementary strand would be terminated with a dideoxynucleotide and thus could not serve as an active 3'-OH in subsequent reactions. A 5'-³²P-minus strand 42 nt long oligonucleotide was then annealed to complete the D loop. (b) Primosomal protein requirements for D loop-dependent replication. The complete reaction contained 50 mM Hepes-KOH (pH 8.0), 10 mM MgOAc, 10 mM dithiothreitol, 80 mM KCl, 200 µg ml⁻¹ bovine serum albumin, 2 mM ATP, 40 µM dNTPs, 0.42 nM [³²P]form II D loop DNA template, 0.5 µM SSB, 225 nM DnaC and 30 nM PriA, PriB, PriC, DnaT, DnaB and DNA polymerase III holoenzyme and was incubated at 37 °C for 10 min. The indicated proteins were omitted from the reaction mixtures. Analysis was by electrophoresis through a denaturing 1% alkaline agarose gel.

(Kogoma *et al.* 1996; Sandler *et al.* 1996), double-strand break-repair (Kogoma *et al.* 1996), and both forms of stable DNA replication (Masai *et al.* 1994). This latter finding led Kogoma to propose that PriA acted to assemble replication forks at D loops. To account for all the phenotypes, both Kogoma (Kogoma 1996; Kogoma *et al.* 1996) and Sandler *et al.* (1996) proposed that PriA could assemble replication forks at recombination intermediates.

This suggestion received strong biochemical support from the finding that PriA bound D loops (McGlynn *et al.* 1997). We showed that PriA was recognizing the intersection between the two template strands and the 3'-OH end of the invading strand (Nurse *et al.* 1999). This structure is equivalent to a replication fork where the nascent leading strand, but not the nascent lagging strand, is present.

3. PriA-DIRECTED REPLICATION FORK ASSEMBLY AT A D LOOP

We began our efforts to model replication fork restart *in vitro* by first asking whether we could assemble a replication fork at a D loop. To do so we used the template shown in figure 1a, where a nicked, double-stranded DNA circle was engineered to carry a D loop of 42 nt. If a replication fork is to form at a D loop, the 3'-OH of the invading strand should serve as the primer for leading-strand synthesis. To ensure that we could observe such a reaction, the only ³²P label present in the reaction was at the 5'-end of the invading strand. The products of synthesis were observed by electrophoresis through a denaturing alkaline agarose gel. If a replication fork formed at the D loop and extended the invading strand completely around the circular template, the label should shift from the position of the 42-mer invading strand to that of full-length DNA (6 kb).

Complete replication of the template was observed in the presence of the ϕ X-type primosomal proteins, PriA, PriB, PriC, DnaT, DnaC, and DnaG, SSB, and the Pol III HE. Full-length synthesis was dependent on all of the

Figure 2. Pathways of repair of stalled or collapsed replication forks. Replication forks stall when they encounter template damage and collapse when they encounter nicks in the template (i). Stalled forks undergo replication fork regression (iv), where the nascent strands pair, forming a Holliday junction. This event regenerates the template opposite the lesion, allowing repair. Reversal of regression restores the fork that is then a substrate for restart. Or, the Holliday junction can be cleaved by RuvC, yielding a detached sister chromosome arm (ii), which is the same situation that occurs when a fork encounters a nick in the template strand. Under these circumstances, the only path to replication fork reactivation is a strand invasion event between the broken and intact sister arms of the chromosome, generating a D loop (iii), which is a substrate for replication fork restart as described in the legend to figure 3*a*.

primosomal proteins except PriC, which appeared to stimulate the reaction by about a factor of three (figure 1*b*). The lack of a requirement for PriC is explained in the final section of this report.

Because DNA helicases were being introduced to the DNA during primosome assembly, extension of the invading strand could result from one of two processes: either assembly of a replication fork at the D loop followed by elongation of the leading strand coupled with unwinding of the duplex DNA template or uncoupled unwinding of the template DNA leaving an oligonucleotide annealed to the viral ssDNA that could be elongated in a primer-extension reaction by the polymerase. To determine that replication fork assembly was occurring, we assessed the requirement for the τ -DnaB interaction. As discussed in a previous section, the signature manifestation of this interaction is a replication fork that moves at 1000 nt s^{-1} , whereas in its absence, elongation of nascent DNA proceeds at *ca.* 35 nt s^{-1} . By measuring the speed of elongation in the replication system described in figure 1, we found that a bona fide replication fork had indeed formed at the D loop (Liu *et al.* 1999). These studies provided direct evidence that PriA could direct the assembly of a replication fork on a recombination intermediate such as a D loop. The combination of genetic and biochemical data indicated clearly that the ϕ X-type primosomal proteins were involved in replication restart in the cell. Thus, we renamed these proteins the replication restart primosomal proteins (Sandler & Marians 2000).

4. DOUBLE-STRAND BREAK-REPAIR BY RECOMBINATION-DEPENDENT REPLICATION

Studies by a number of laboratories have demonstrated that replication restart can proceed in a number of ways

(Kuzminov 1999; Cox *et al.* 2000; Kowalczykowski 2000; Rothstein *et al.* 2000; Lusetti & Cox 2002) (figure 2). Forks that have stalled completely undergo nascent strand regression, where the nascent leading- and lagging-strands pair and the parental duplex rewinds, generating a Holliday junction. This regression reaction can, by template-strand switching, also provide an accurate template for repair of the lesion. Regression can then presumably be reversed and restart effected at the restored fork. Alternatively, it has also been suggested that stalled forks might break, leading to replication fork collapse (Horiuchi & Fujimura 1995). Fork breakage has, under certain conditions, been shown to require the Holliday junction resolvase, RuvC (Seigneur *et al.* 1998), or fork breakage may be spontaneous. Replication forks will also collapse if they encounter a nick in one of the template strands. In either example of replication fork collapse, the only route to restoration of chromosomal replication is via the establishment of a joint molecule between the intact and broken sister arms by the combined action of the RecBCD helicase/nuclease and the RecA homologous strand-pairing protein. The D loop formed by this event can then serve as a nucleus for restoration of replication with restart primosome assembly introducing the lagging-strand machinery and the 3'-end of the invading strand acting as the primer for leading-strand synthesis (figure 3*a*). The Holliday junction formed is then presumably resolved subsequently to disjoin the sister chromosomes.

We have reconstituted this reaction using purified proteins. A supercoiled plasmid DNA serves as the intact sister arm of the chromosome (χ -1 DNA; figure 3*a*(ii)) and an 800 bp-long PCR product complementary to χ -1 DNA that contains a χ site roughly in the middle (figure 3*a*(i)) serves as the broken chromosomal sister arm. RecA and RecBCD, in the presence of SSB, form a joint molecule

Figure 3. (Caption opposite.)

between the two DNAs (figure 3*a*(iii)) that serves as the substrate for the assembly of a replication fork, mediated by the restart primosomal proteins. The product of this reaction takes the form of a late replication intermediate (figure 3*a*(v)) where there is some region of unreplicated parental DNA remaining. The reason for this is that DNA

gyrase cannot support the final stages of χ -type DNA replication (Minden & Mariani 1986) because of its inability to remove precatenanes (Hiasa & Mariani 1996; Peter *et al.* 1998). Completely replicated molecules (figure 3*a*(vi)) can be formed if topoisomerase IV is included in the reaction (Xu & Mariani 2003). In the absence of PriA, a non-

Figure 3. Reconstitution of recombination-dependent DNA replication. (a) Possible replication pathways from recombinant joint molecules. This figure is described in the text. (b,c) Requirements for recombination-directed DNA replication. The complete reaction contained 50 mM HEPES-KOH (pH 8.0), 16 mM MgOAc, 10 mM dithiothreitol, 100 $\mu\text{g ml}^{-1}$ bovine serum albumin, 2 mM ATP, 40 μM [α - ^{32}P]dATP (1000–2000 cpm pmol $^{-1}$), 40 μM dCTP, dGTP and TTP, 7.5 mM creatine phosphate, 62 ng ml $^{-1}$ creatine kinase, 12.5 nM 800-mer, 1.1 nM pBR χ -1 DNA, 3.5 μM RecA, 1 nM RecBCD, 160 nM RecO, 160 nM RecR, 0.8 μM SSB, 16 nM DNA gyrase, 5 nM PriA, 5 nM PriB, 5 nM PriC, 60 nM DnaB, 0.96 μM DnaC, 300 nM DnaG, 160 nM DnaT, 20 nM Pol III* and 20 nM β -subunit of the HE and was incubated at 37 °C for 12 min. The indicated proteins were omitted from the reaction mixtures. Analysis was by electrophoresis through 1% agarose gels. Dried gels were exposed to phosphorimager screens and the amount of radioactivity in the 'ds product' band was quantitated. In (b) and (c), 100% incorporation is 16 680 and 17 140 PSL (photostimulated luminescence), respectively. ds prod, double-stranded product, item (v) in (a); ss prod, single-stranded product, item (iv) in (a); F II, form II, item (vi) in (a); FX, form X, an unwound form generated by the action of the RecA filament.

specific DNA synthesis reaction occurs where the 3'-end of the invading strand can be elongated by any DNA polymerase and replication is driven around the circular template DNA by the combined action of gyrase and SSB (Xu & Marians 2002). The product is a form II DNA with the displaced template strand linked to it (figure 3a(iv)).

The recombination-dependent replication reaction required RecA, RecBCD, SSB, gyrase, the Pol III HE, PriA, PriB, DnaB, DnaC, DnaG and DnaT (figure 3b,c). As with PriA-directed assembly of a replication fork on a form II DNA carrying a D loop, PriC was not required, but did stimulate the reaction by a factor of 2.5. Interestingly, this reaction is stimulated by the RecO and RecR proteins (figure 3b). These two proteins form an epistasis group with RecF (Lloyd *et al.* 1987, 1988; Mahdi & Lloyd 1989) and it has been thought that they were involved in post-replication, daughter-strand, gap repair (Clark & Sandler 1994). However, observations indicating that they were involved in replication fork restart after the encounter of a fork with UV damage (Courcelle *et al.* 1997) prompted us to examine their effect on the recombination-directed replication reaction described here. RecF appears to modulate the action of RecOR in this reaction. When it is included, the observed stimulation by RecOR is suppressed (Xu & Marians 2003).

Joint molecules formed by the action of RecA and RecBCD can be the substrate for a number of reactions that all have somewhat different outcomes. The heteroduplex formed can be branch-migrated either by the growing RecA filament itself or by the branch migration enzymes RuvAB and RecG. Alternatively, replication fork formation can be initiated. Little is known currently about how a recombinant joint formed between the two sister arms of a replicating chromosome is apportioned between these competing reactions. The development of the recombination-directed replication reaction described here will allow us to probe the nature and outcomes of these competing reactions.

5. MULTIPLE PATHWAYS OF PRIMOSOMAL PROTEIN-DIRECTED REPLICATION FORK RESTART

The comparison of phenotypes of mutations in *priB* and *priC* with those resulting from mutations in *priA*, an analysis of the synthetic lethality of different combinations of these and other mutations, and the ability of different suppressor mutations to rescue these phenotypes have led to the proposal that there are multiple pathways of replication fork restart that are, to some extent, redundant (Sandler 2000). The first suggestion that there were multiple pathways arose from the fact that *priA* $^{-}$ cells were viable, although quite debilitated. The biochemical events required for assembly of a primosome on ϕX174 DNA predicted that PriB and PriC would be in the same pathway as PriA. However, cells that are either *priB* $^{-}$ or *priC* $^{-}$ are only marginally affected, if at all. But cells that are both *priB* $^{-}$ and *priC* $^{-}$ are even more debilitated than cells that are *priA* $^{-}$. Furthermore, the *priA* $^{-}$ *priC* $^{-}$ and *priA* $^{-}$ *rep* $^{-}$ combinations proved to be synthetically lethal, whereas the *priA* $^{-}$ *priB* $^{-}$ and *priC* $^{-}$ *rep* $^{-}$ combinations were viable.

Like PriA, Rep is a 3' $\rightarrow$ 5' DNA helicase. In studies of mechanisms that lead to the generation of double-strand breaks *in vivo*, Seigneur *et al.* (1998) demonstrated that *rep* $^{-}$ *recB* $^{-}$ combinations were lethal and suggested that the absence of Rep led to more frequent replication fork arrest. This group had shown previously that fork arrest leads to the production of double-strand breaks (Michel *et al.* 1997). Replication forks in *rep* mutants have been reported to travel at one-half the speed of those in wild-type cells (Lane & Denhardt 1975). These observations are consistent with a role for Rep in replication fork restart.

Taken together, the analysis of mutations in *priA*, *priB*, *priC* and *rep* and the probable role of Rep in replication fork restart suggest that there are both PriA-dependent and PriA-independent pathways of replication fork restart. The two PriA-dependent pathways require at least PriA and either PriB or PriC, whereas the PriA-independent pathway requires at least both PriC and Rep. It has also been suggested that replication fork repair via the PriA-dependent pathway gives rise to a crossover between the two sister chromosomes, whereas repair via the PriA-independent pathway does not generate a crossover (McCool & Sandler 2001).

We have begun to investigate these alternative pathways biochemically. We find that, like PriA, Rep binds D loop DNA even in the presence of SSB. Again, similar to PriA, Rep prefers to bind three-way junctions with a 5'-single-stranded tail as opposed to a 3'-tail (data not shown). This preferred substrate is none other than a replication fork with a nascent leading strand but lacking a nascent lagging strand. We have also identified a D loop unwinding reaction that requires Rep, PriC, DnaB and DnaC. This latter reaction suggested that PriC might be able to load DnaB to the lagging-strand template at a replication fork. To investigate this issue, we purified joint molecules, formed as described in the previous section, and asked if they would support replication in the presence of PriC, DnaB, DnaC, DnaG, the Pol III HE, SSB and DNA gyrase. This proved to be the case (figure 4). The LRI band that indicates replication fork formation and progression of the

Figure 4. Reconstitution of PriA-independent replication restart. Joint molecules were formed by the combined action of RecA, RecBCD and SSB, treated with proteinase K, and then isolated by gel filtration chromatography. This DNA was used as a substrate in a replication reaction that included the Pol III HE, DNA gyrase, SSB, DnaB and DnaG at the concentrations indicated in the legend to figure 3, 360 nM DnaC and the indicated concentrations of PriC. Incubation and analysis were as in the legend to figure 3.

fork around the circular template was formed only when PriC and DnaB and DnaC were present together. Thus, this replication reaction appears to be the biochemical equivalent of the PriA-independent pathway of replication fork restart that has been described genetically. At the moment, though, despite the Rep-dependent unwinding reaction described above, inclusion of Rep in the replication reaction leads only to an inhibition of DNA synthesis. We are currently exploring the substrate specificity of this reaction and anticipate that a requirement for Rep will emerge.

6. CONCLUSIONS

It has become clear that there are many different substrates for replication fork restart. These substrates are dictated by the nature of the event that caused replication fork arrest and by the action of the recombination proteins RecA, RecBCD, RecFOR, RuvABC and RecG. All replication restart appears to proceed via different combinations of the replication restart primosomal proteins. In trying to ensure capture of the cellular replication fork helicase DnaB, ϕ X174 phage obviously combined both the PriA-dependent and PriA-independent pathways of restart.

The crucial nature of replication fork restart is reflected in the fact that while one or two of the genetically defined pathways can be inactivated, elimination of all of them is lethal. It is worth noting that these cells are unstressed with respect to exogenous DNA damaging agents. Thus, replication restart is a housekeeping function that is necessary for survival of the organism.

Replication restart appears to hold similar importance in eukaryotic cells, where it has been suggested that the gene products of such cancer predisposition genes as *BLM* and *BRCA2* are involved in this process (Pierce *et al.* 2001).

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GLOSSARY

- LRI: late replication intermediate
 Pol III HE: polymerase III holoenzyme
 SSB: single-stranded DNA-binding protein
 ssDNA: single-stranded DNA
 UV: ultraviolet