

Requirements for Replication Restart Proteins During Constitutive Stable DNA Replication in *Escherichia coli* K-12

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Manuscript received September 28, 2004
Accepted for publication December 31, 2004

ABSTRACT

Constitutive stable DNA replication (cSDR) is a mechanism for replisome loading in *Escherichia coli* K-12. This occurs in a *dnaA*-independent fashion in an *mhA* mutant. cSDR is dependent on *recA*, *priA*, and transcription. In this report, it is shown that *dnaA mhA* mutants using cSDR for initiation of their DNA replication additionally require *priB*, but not *priC*, for viability. Two subtle *priA* missense mutations either eliminated the ability to grow using cSDR (*priA301* C479Y) or resulted in very small colonies (*priA300* K230R). *DnaC809*, a *priA* suppressor, failed to allow *priA* or *priB* mutants to grow using cSDR to initiate DNA replication. Furthermore, unlike *dnaC*⁺ strains, *dnaC809* strains require *priC* for cSDR. *DnaC809,820*, a *priC*-independent suppressor of *priA2::kan* phenotypes, allowed *priA* and *priC* (but not *priB*) mutants to grow using cSDR to initiate DNA replication. It is also shown that *rep* and *mhA* mutations are synthetically lethal. *DnaC809* and *dnaC809,820* mutations suppress this lethality. *Rep* is further shown to be required for cSDR in a *dnaC809* strain. A model whereby these different sets of replication restart proteins interact preferentially with substrates associated with either *RecA* or *SSB* during replication restart and cSDR, respectively, is proposed.

IN *Escherichia coli*, a critical step in DNA replication is the loading of the replicative helicase, DnaB, onto the DNA by DnaC. Loading DnaB at *oriC* is a sequence-specific, cell-cycle-regulated event regulated and initiated by the DnaA protein (reviewed in MESSER and WEIGEL 1996; KATAYAMA 2001; MESSER 2002). Loading DnaB away from *oriC* at a repaired replication fork occurs by multiple pathways involving the replication restart proteins (RRPs): PriA, PriB, PriC, DnaT, and Rep (Figure 1 and SANDLER 2000). Two types of *priA* suppressor mutations have been found and both map to the *dnaC* gene. The first type is typified by *dnaC809* and is dependent on the *rep* and *priC* genes (SANDLER 2000). The second type of *priA* suppressor has an additional mutation in *dnaC* relative to *dnaC809* and makes this gene's suppression of *priA* mutant phenotypes independent of *priC* and *rep*. This *dnaC* allele is called *dnaC809,820* (SANDLER 2000; SANDLER *et al.* 1999). The multiplicity of replication restart pathways may be of a more general nature in bacteria since *Bacillus subtilis* also has a similar arrangement of PriA-dependent and PriA-independent pathways (BRUAND *et al.* 2001; MARSIN *et al.* 2001).

Complexes formed during DnaA-dependent initiation of DNA replication at *oriC* and replication restart differ in their protein components and DNA structure. Both, however, provide a mechanism to unwind the DNA helix and make protein-DNA complexes that are

recognized by the DnaC-DnaB complex to load DnaB. Kogoma and colleagues showed that *oriC*/DnaA-independent initiation of DNA replication could take place in an *mhA* mutant strain (KOGOMA and VON MEYENBURG 1983). They called this phenomenon constitutive stable DNA replication (cSDR). Figure 2 presents a model for cSDR. Initiation is thought to begin at a R-loop. An R-loop is a structure of three strands of nucleic acid: two DNA and one RNA. The one strand of RNA is annealed with its complementary strand of DNA, which causes the other strand of DNA to be displaced from the helix. The RNA for the R-loop is transcribed by RNA polymerase and it is thought that *RecA* is necessary to either form or stabilize the R-loop (KASAHARA *et al.* 2000). The 3'-end of the RNA in the R-loop is then extended by DNA polymerase I to form a structure similar to a D-loop (KOGOMA 1997). Kogoma and colleagues showed that cSDR was also dependent on *priA* (MASAI *et al.* 1994). They suggested that the rest of the replication restart machinery, now known to include PriA, PriB, PriC, DnaT, and Rep, would identify the proper substrate made by RNA polymerase, *RecA* and DNA polymerase I, and load DnaB and the rest of the replisome. Here we test whether subtle *priA* missense mutations or mutations in other replication restart genes such as *priB*, *priC*, and *rep*, affect cSDR.

MATERIALS AND METHODS

Bacterial strains: All bacterial strains used in this work are derivatives of *E. coli* K-12 and are described in Table 1. The protocol for P1 transduction has been described elsewhere

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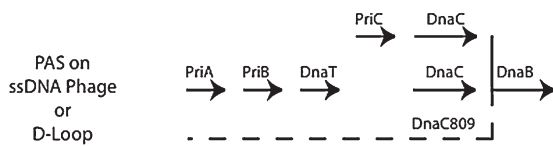
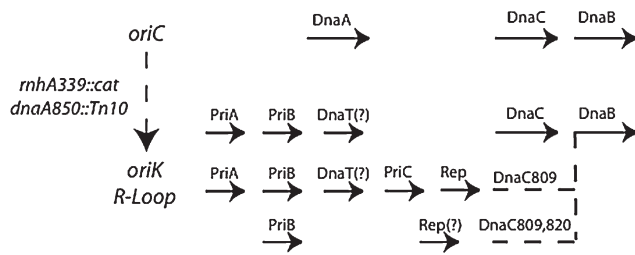
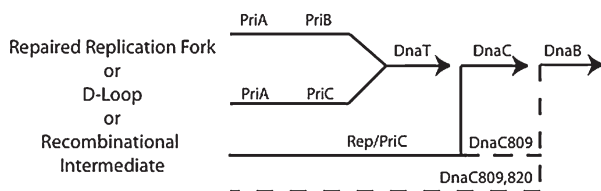
ϕ X174-type primosome***In vitro* initiation of DNA Replication*****Escherichia coli*****Initiation of Chromosomal DNA Replication****Reloading of Replisome during Chromosomal DNA Replication**

FIGURE 1.—Comparison of different methods studied either *in vivo* or *in vitro* for the loading of the DnaB helicase onto DNA and the relationships of the RRP studied under a particular condition. (Top) Pathways formed by the RRP using D-loops or ϕ X174 single-strand DNA as a substrate. Historically, the RRP were considered to form a single biochemical pathway. Recently, Marians and colleagues have shown that this reaction is actually the sum of two separate reactions involving PriA, PriB, and DnaT, on the one hand, and PriC on the other hand (K. MARIANS, personal communication). Both reactions use DnaC to load DnaB. DnaC809 forms a new suppressor pathway denoted by the dashed line. (Middle) The loading of DnaB during initiation of DNA replication as it occurs either at *oriC* in a DnaA-dependent fashion or using cSDR at *oriK* in a DnaA-independent fashion. The pathways using the RRP summarize the new data presented in this report. Each line or reaction shows the gene products required when the strain is *dnaC*⁺, *dnaC809*, or *dnaC809,820*. DnaT is listed with a question mark since its role has not been explicitly tested. It is hypothesized that since *dnaT* mutants have the same phenotype as *priA* mutants (McCool *et al.* 2004a), the requirement of *dnaT* will mirror that of *priA* in these strains. The role of Rep in the *dnaC809,820* strain was not tested because the strain grew too poorly and so is listed with a question mark. The dashed lines represent the pathways used by the mutant *dnaC* proteins. (Bottom) Model showing how the RRP form multiple pathways to restart replication forks during log-phase growth (Sandler 2000). The dashed lines show pathways available in the presence of particular *dnaC* alleles.

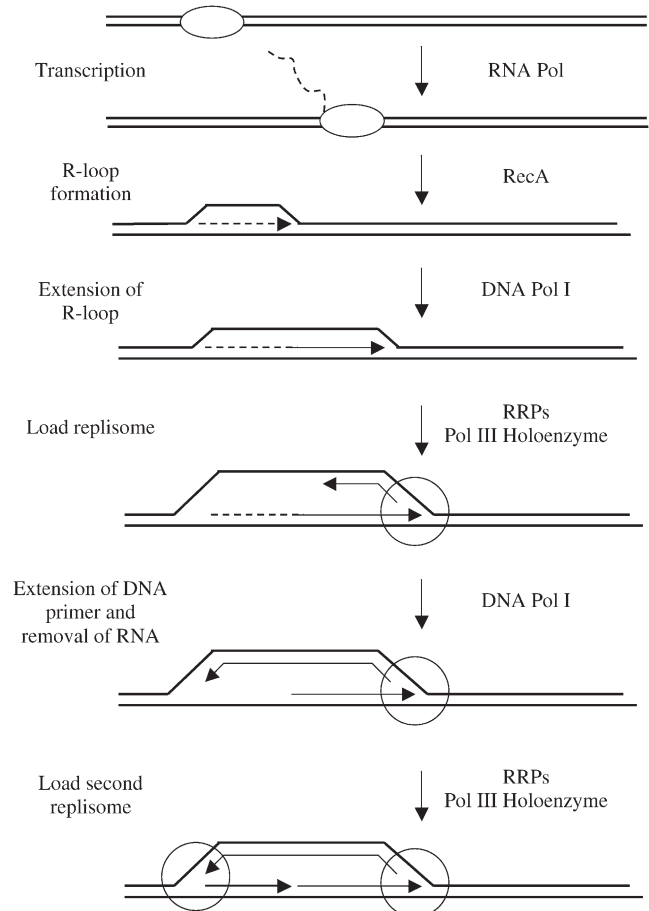


FIGURE 2.—A model for the bidirectional initiation of DNA replication at an *oriK* using cSDR. This diagram is derived and modified from KOGOMA's (1997) model. The oval and the circle represent RNA polymerase and the replisome, respectively. Dashed lines indicate RNA. The wording on the left denotes the process occurring at that step and the wording on the right denotes the enzymes involved.

(WILLETT *et al.* 1969). All P1 transductions were selected on 2% agar plates made with either Luria broth or 56/2 minimal media (WILLETT *et al.* 1969) supplemented with 0.2% glucose, 0.001% thiamine, specified amino acids, or 0.2% cass-amino acids. Selection used the antibiotics kanamycin (25 μ g/ml), tetracycline (10 μ g/ml), or chloramphenicol (50 μ g/ml). All transductants were grown at 37° and purified on the same type of media on which they were selected.

Transductional assay and controls: cSDR refers to the type of initiation of DNA replication that occurs in *rnhA* mutant cells in the absence of DnaA (KOGOMA 1997). Experimentally, this can be achieved in a *dnaA850::Tn10 rnhA339::cat* double-mutant strain. The test for whether a mutation would affect cSDR was performed by first combining the mutations of interests: *priA300*, *priA301*, *del(priB)302*, *priC303::kan*, *dnaC809*, and/or *dnaC809,820* with *rnhA339::cat* and then attempting to introduce *dnaA850::Tn10* using transduction with a donor P1 lysate grown on AQ12257. If Tet^R transductants were found at high frequency, then the mutation is judged not to have any major effect on cSDR. If not, then the mutation has an effect. To be sure that the mutations truly caused the effect and that the lack of Tet^R transductants was not due to other mechanisms, two types of control experiments were performed. The first is that all recipients were tested for their

TABLE 1

Strain list

Strain	<i>priA</i>	<i>priB</i>	<i>priC</i>	<i>dnaA</i>	<i>mhA</i>	<i>dnaC</i>	Other relevant genotype	Source or derivation
AQ12551	+	+	+	+	339	+		T. Kogoma
AQ12257	+	+	+	850	339	+		T. Kogoma
CAG18431	+	+	+	+	+	+	<i>ilv-500::Tn10</i>	<i>E. coli</i> Stock Center
CAG18442	+	+	+	+	+	+	<i>thr-34::Tn10</i>	<i>E. coli</i> Stock Center
JC13509 ^a	+	+	+	+	+	+		Lab stock
JC19008	+	+	+	+	+	809		SANDLER <i>et al.</i> (1996)
JC19165	+	+	303	+	+	+		SANDLER <i>et al.</i> (1999)
JC19257	+	+	+	+	+	809,820		SANDLER <i>et al.</i> (1999)
JJC213	+	+	+	+	+	+	<i>del(rep)::kan</i>	B. Michel
SS135	+	302	+	+	+	+		SANDLER <i>et al.</i> (2001)
SS138	+	302	+	+	+	+	<i>thr-34::Tn10</i>	CAG18442 → SS135 ^b
SS140	+	302	+	+	+	809		JC19008 → SS138 ^c
SS141	+	302	+	+	+	809,820		JC19257 → SS138 ^c
SS145	+	+	303	+	+	+		SANDLER <i>et al.</i> (2001)
SS156	+	+	+	+	+	+	<i>thr-34::Tn10</i>	CAG18442 → JC13509 ^b
SS170	2	+	+	+	+	+	<i>thr-34::Tn10</i>	JC19008 → SS156 ^c
SS171	2	+	+	+	+	809		JC19008 → SS170 ^c
SS172	2	+	+	+	+	809,820		JC19257 → SS170 ^c
SS494	300	+	+	+	+	+		SANDLER <i>et al.</i> (2001)
SS496	301	+	+	+	+	+		SANDLER <i>et al.</i> (2001)
SS1091	+	+	+	+	+	809		JC19008 → SS1213 ^c
SS1092	+	+	+	+	+	809,820		JC19257 → SS1213 ^c
SS1099	+	+	303	+	+	809		JC19165 → SS1091 ^d
SS1100	+	+	303	+	+	809,820		JC19165 → SS1092 ^d
SS1213	+	+	+	+	+	+	<i>thr-34::Tn10</i>	CAG18442 → JC13509 ^b
SS1531	+	+	303	+	339	+		AQ12551 → SS145 ^e
SS1537	+	302	+	+	339	809		AQ12551 → SS140 ^e
SS1538	2	+	+	+	339	809		AQ12551 → SS171 ^e
SS1539	+	+	303	+	339	809		AQ12551 → SS1099 ^e
SS1580	+	302	+	+	339	809,820		AQ12551 → SS141 ^e
SS1581	2	+	+	+	339	809,820		AQ12551 → SS172 ^e
SS1582	+	+	303	+	339	809,820		AQ12551 → SS1100 ^e
SS1627	301	+	+	+	339	+		AQ12551 → SS496 ^e
SS1628	300	+	+	+	339	+		AQ12551 → SS494 ^e
SS1731	+	+	+	+	+	+	<i>del(rep)::kan</i>	JJC213 → JC13509 ^d
SS1732	+	+	+	+	339	809		AQ12551 → SS1091 ^e
SS1733	+	+	+	+	339	809,820		AQ12551 → SS1092 ^e
SS1734	+	302	+	+	339	+		AQ12551 → SS135 ^e
SS2288	+	+	+	+	339	809	<i>del(rep)::kan</i>	JJC213 → SS1732 ^d
SS2299	+	+	+	+	339	809,820	<i>del(rep)::kan</i>	JJC213 → SS1733 ^d
SS3005	+	+	+	+	+	+	<i>ilv-500::Tn10 del(rep)::kan</i>	CAG18431 → SS1731 ^f

^aJC13509 has the following genotype: *sulB103⁻ lacMS286 Φ80dIIIacBK1 argE3 his-4 thi-1 xyl-5 mtl-1 Sm^R T6^R*. The *lacMS286 Φ80dIIIacBK1* code for two nonoverlapping deletions in the *lac* operon. See KONRAD (1977) and ZIEG and KUSHNER (1977).

^bSelection for tetracycline resistance.

^cSelection for prototrophic growth and screen for the presence of the *dnaC* allele by using PCR followed by restriction analysis for the *HinFI* restriction site polymorphism.

^dSelection for kanamycin resistance on minimal media.

^eSelection for chloramphenicol resistance on minimal media.

^fSelection for tetracycline resistance on Luria media and then screen for kanamycin resistance.

competence for transduction to verify that they are not P1 resistant and that they are recombination proficient by being crossed with a P1 donor (CAG18431) containing the *ilv-500::Tn10* mutation. It was found that, for some recipients, it was possible to introduce *ilv-500::Tn10* but not *dnaA850::Tn10*. In these cases, a second control experiment was done. This was to test for the possibility that *dnaA850::Tn10* could not be introduced because this insertion mutation may be polar on downstream *dnaN* and *recF* expression. To do this, a plasmid

expressing *dnaA* [pAB3 (GINES-CANDELARIA *et al.* 1995)] was introduced into each recipient where *dnaA850::Tn10* could not be introduced. The transduction introducing *dnaA850::Tn10* was then repeated with the modified recipient. If the introduction of *dnaA850::Tn10* was now successful, it was concluded that the failure to inherit *dnaA850::Tn10* (in the recipient without pAB3) was not due to polar effects. These tests are summarized in Table 2 for all crosses.

The failure to introduce *dnaA850::Tn10* to the *mhA* mutant

does not necessarily mean that the additional mutations interfere with cSDR. It is possible that the *dnaA850::Tn10* mutation is synthetically lethal with any one or a combination of the mutations in the recipient strain [*priC303::kan* was found to be synthetically lethal with certain temperature-sensitive alleles of *dnaA* at the permissive temperature of 30° (HINDS and SANDLER 2004)]. Individual combinations of mutations and *dnaA850::Tn10* cannot be tested because *dnaA* is an essential gene and this *dnaA* mutant is viable only in the absence of *rmhA*.

RESULTS

***priA300* and *priA301* affect cSDR:** PriA has at least four types of activities: ATPase, helicase, the ability to load the replisome, and the ability to interact with other proteins. While it has been shown that the *priA1::kan* null mutation removes the ability to do cSDR (MASAI *et al.* 1994), it is unclear which activities of PriA are needed for cSDR. Although this issue was partially addressed by the analysis of different mutants on multi-copy plasmid (TANAKA *et al.* 2003), it has not been addressed by studying subtle *priA* missense mutants, single copy on the chromosome. By subtle, it is meant that the mutation by itself causes very little if any measurable effect on the cell in terms of growth, DNA repair capacity, and basal levels of SOS expression. Two mutations that have been placed on the chromosome that meet this criteria are *priA300* (K230R) and *priA301* (C479Y) (SANDLER *et al.* 2001). The former is a mutation in its Walker P-loop motif and is deficient in both ATPase and helicase activities but is still able to load the replisome (ZAVITZ and MARIANS 1992). The latter affects the cysteine-rich region of PriA thought to be important for Zn⁺² binding, helicase activity, and or protein-protein interactions (ZAVITZ and MARIANS 1993; LIU *et al.* 1996). As a single-copy allele on the chromosome in an otherwise wild-type strain, the only discernible phenotype reported for these mutants thus far is a four- to fivefold lower plating efficiency of *Mu* phage (SANDLER *et al.* 2001). These mutations, however, are not without consequence. For instance, both mutations when combined with a *del(priB)302* mutation yield a cell that is phenotypically like a *priA* null mutant (SANDLER *et al.* 2001). In combination with other mutations, other phenotypes for *priA300* have also been reported (FLORES *et al.* 2002; JAKTAJI and LLOYD 2003).

To test whether a chromosomal allele of *priA300* or *priA301* allows cSDR, a *rmhA* mutation was first combined with each *priA* mutation. These double mutants were then the recipients in a cross with a donor lysate grown on a strain containing *dnaA850::Tn10*. Table 2 shows that for *priA300*, this triple mutant was viable. The resulting triple mutant, however, produced very small colonies after 2–3 days of growth at 37° (data not shown). When the *priA301 rmhA* mutant was used as the recipient, no triple-mutant transductants were seen. It is suggested that PriA's ability to load the replisome is not sufficient for cSDR. It is clear from the small colony

phenotype of the *priA300* mutants that the ATPase and helicase activities of PriA are important for cSDR. There remains, however, at least one other activity of PriA (*e.g.*, protein-protein interactions) that is affected by *priA301*, and not by *priA300*, which is essential for cSDR.

PriB, but not PriC, is required for viability in the *dnaA850::Tn10 rmhA339::cat* strain: Other work has shown that neither *priB* nor *priC* is essential for growth or has any obvious growth or replication phenotype in an otherwise wild-type strain (SANDLER *et al.* 1999). Yet, the *priB priC* double mutant was inviable (SANDLER *et al.* 1999). Further studies suggested that *priB* and *priC* each functioned with *priA* in redundant pathways for replication restart during normal growth (SANDLER 2000). Thus, even though cSDR absolutely required *priA*, it was possible by the multiple pathways model that cSDR could tolerate either a *priB* or a *priC* mutation.

This hypothesis was tested by attempting to introduce the *dnaA850::Tn10* mutation into the *priB rmhA* and *priC rmhA* double mutants. Table 2 shows that the *priC rmhA* mutant was able to tolerate the introduction of the *dnaA850::Tn10* mutation, but the *priB rmhA* mutant could not. I conclude that, unlike replication restart in log-phase wild-type cells, cSDR is able to tolerate the absence of *priC*, but not *priB*. I hypothesize that there are no redundant mechanisms for loading DnaB at a structure initiated by the formation of an R-loop, as there are for cells during replication restart, and that cSDR proceeds by a mechanism that requires both *priA* and *priB*.

***DnaC809* does not suppress the absence of *priA* and *priB* and creates the additional requirement of *priC* in cSDR:** Mutations in *dnaC* (*e.g.*, *dnaC809*) can indirectly suppress *priA2::kan* mutant phenotypes. It has been shown that the inviability of *priB priC* double mutants can be partially rescued by a *dnaC809* mutation. These triple-mutant strains, however, still have some defects that can be rescued by a *dnaC809,820* mutation (SANDLER *et al.* 1999; SANDLER 2000). The molecular basis for this additional suppression is not entirely understood. It has been hypothesized that the *dnaC820* mutation makes the *dnaC809* mutant *priC* and *rep* independent.

To begin to test whether *dnaC809* and *dnaC809,820* could suppress the absence of *priA*, *priB*, or *priC* in cSDR, I first tested whether *dnaC809* and *dnaC809,820* mutants were proficient for cSDR. Since *dnaC809* and *dnaC809,820* have no other obvious phenotypes except the ability to suppress the absence of certain replication restart mutants, it was predicted that these alleles should be proficient for cSDR. This was tested by first combining the *dnaC* and *rmhA* mutations into a single strain and then adding *dnaA850::Tn10*. Table 2 shows that both *dnaC809* and *dnaC809,820* were viable in the *rmhA dnaA* strain. The time to form colonies of the *dnaC809,820* strain was longer than that of the *dnaC809* derivative, which suggests that while both strains are viable,

TABLE 2
Ability to transduce *rnhA339::cat* strains to Tet^R using P1 grown on strains containing Tn10 inserted into *dnaA* or *ilv*

Strain	Genotype of recipient strain					Ability to transduce to Tet ^R ^a			
	<i>priA</i>	<i>priB</i>	<i>priC</i>	<i>dnaC</i>	<i>rep</i>	Donor plasmid:	None	pAB3 ^b	None
SS1651	+	+	+	+	+		+	+	+
SS1628	300	+	+	+	+		+ (sm)	+	+
SS1627	301	+	+	+	+		-	+	+
SS1734	+	302	+	+	+		-	+	+
SS1531	+	+	303	+	+		+	ND	+
SS1538	2	+	+	809	+		-	+	+
SS1537	+	302	+	809	+		-	+	+
SS1539	+	+	303	809	+		-	+	+
SS1581	2	+	+	809,820	+		+	ND	+
SS1580	+	302	+	809,820	+		-	+	+
SS1582	+	+	303	809,820	+		+	ND	+
SS1732	+	+	+	809	+		+	ND	ND
SS1733	+	+	+	809,820	+		+	ND	ND
SS2288	+	+	+	809	<i>kan</i>		-	+	+ ^c

dnaA refers to *dnaA850::Tn10* and *ilv* refers to *ilv-500::Tn10*. All transductions reported in this table have been repeated at least twice. sm, a small colony phenotype; ND, not determined.

^a (+) refers to the fact that the number of transductants were similar to the *rnhA339::cat* strain (SS1651) and a 100- to 1000-fold over that seen with a *rnhA*⁺ strain (JC13509). P1 transduction of *dnaA850::Tn10* into JC13509 typically yielded a few Tet^R transductants. These strains are presumably *dnaA*⁺ and reflect background levels. (-) refers to the observation that the number of transductants was equal or less of that of an *rnhA*⁺ strain (JC13509).

^b pAB3 is a plasmid derived from pACYC177 that has a *HindIII-XhoI* fragment containing *dnaA*⁺ (this plasmid was given to me by Mark Sutton). Its original derivation is in GINES-CANDELARIA *et al.* (1995). This plasmid was used to transform the indicated recipient. This new strain was then used as a recipient in a cross with P1 containing *dnaA850::Tn10*. (+) in this case means that the frequency of transduction was 100- to 500-fold greater than that of the strain without pAB3.

^c CAG5052 containing *btuB3191::Tn10* was used as a donor since it is not tightly linked with the *del(rep)::kan* mutation.

the two *dnaC* alleles may differentially impart some effects on the double-mutant cell.

The experiments reported above revealed that cSDR was PriC independent. Thus it was first tested whether *dnaC809* would suppress the absence of *priA* in cSDR. Table 2 shows that the *dnaA850::Tn10* could not be added to a *priA2::kan dnaC809 rnhA* strain. It was then hypothesized that *dnaC809* also should not suppress the absence of *priB* since PriB requires PriA for function (NG and MARIANS 1996). This was tested directly by constructing the *priB dnaC809 rnhA* triple mutant and then attempting to introduce *dnaA850::Tn10*. Table 2 shows that this multiple mutant was not viable. As a control, the *priC dnaC809 rnhA* triple mutant was also tested for its ability to inherit *dnaA850::Tn10*. Since *priC* was not required for cSDR, it was predicted that the triple mutant should allow the introduction of *dnaA850::Tn10*. Surprisingly, Table 2 shows this is not the case. The *dnaC809* strain now required the presence of *priC* for cSDR, whereas a *dnaC* does not. From this it is concluded that in the presence of *dnaC809*, cSDR requires *priA*, *priB*, and *priC*.

***DnaC809,820* is able to allow *priA* and *priC* mutants to grow using cSDR to initiate DNA replication:** The *dnaC809,820* mutation is able to simultaneously suppress the *priA-priC* and *priB-priC* synthetic lethality whereas the *dnaC809* mutation cannot (SANDLER 2000). It was then tested whether *dnaC809,820* could allow growth of *priA*, *priB*, and *priC* mutants using cSDR as their sole method to initiate DNA replication. Once again this was tested by creating the *priA*, *priB*, or *priC dnaC809,820 rnhA* triple-mutant strain and then by attempting to introduce *dnaA850::Tn10*. Table 1 shows that all triple mutants were viable and Table 2 shows that *dnaA850::Tn10* could be added to the strains mutated in *priA* and *priC*, but not *priB*.

***Del(rep)::kan* and *rnhA339::cat* are synthetically lethal:** The *rep* protein has at least two roles in the cell: it stabilizes the replication fork during DNA synthesis (UZEST *et al.* 1995; SEIGNEUR *et al.* 1998) and it functions with PriC in the PriA-independent pathway of replication restart (SANDLER 2000). It was therefore of interest to test if the absence of *rep* would inhibit cSDR. To test this idea, I first attempted to construct the *rep rnhA*

double mutant. Introduction of either the *rep* mutation into the *rnhA* strain or the *rnhA* mutation into the *rep* strain was unsuccessful, suggesting that the two mutations are synthetically lethal. This was further tested by construction of a *ilv-500::Tn10 del(rep)::kan* double mutant (SS3005). These two mutations are tightly linked. This strain (SS3005) was then used as donor in a cross with a *rnhA*⁺ and *rnhA339::cat* recipient. Combining the results of two independent sets of transductions, it was seen that when the recipient was *rnhA*⁺, 32/48 of Tet^R transductants were also Kan^R (thus inheriting *del(rep)::kan*) and when the recipient was *rnhA339::cat*, 0/48 were both Tet^R and Kan^R. I concluded that *del(rep)::kan* and *rnhA339::cat* are synthetically lethal.

***DnaC809* and *DnaC809,820* suppress the *rep-rnhA* synthetic lethality:** Several scenarios can be thought of to explain the *rep-rnhA* synthetic lethality. One idea is that Rep is needed to restart replication forks that have specifically stopped or stalled at R-loops (KOGOMA *et al.* 1993; McCOOL *et al.* 2004b). Since *dnaC809,820* (and not *dnaC809*) suppresses *priA2::kan* in a *rep*- and *priC*-independent fashion (SANDLER 2000), it is predicted that *dnaC809,820* (and not *dnaC809*) should suppress the *rnhA-rep* synthetic lethality. Table 1 shows, however, that both *dnaC809* and *dnaC809,820* suppress the *rnhA-rep* synthetic lethality. It was also seen that the growth of colonies of the *dnaC809* strain is greater than that of the *dnaC809,820* strain (data not shown). Large colony variants, probably containing suppressor mutations, were often seen in the *dnaC809,820 rnhA rep* multiple-mutant strain (data not shown). Since some *dnaC* mutations can suppress the *rnhA-rep* synthetic lethality, it is tentatively concluded that the likely cause of the lethality is the failure to restart replication forks that have stopped at R-loops.

***Rep* is required for cSDR in *rnhA dnaC809* strain:** While *rep*'s role in *dnaC*⁺ strains could not be tested, it was possible to test if *rep* was needed during cSDR in a *dnaC809* strain. Thus I tested if *dnaA850::Tn10* could be introduced into the *dnaC809 rnhA rep* triple mutant. Table 2 shows that this cross did not yield any viable quadruple-mutant transductants. A control cross was done using P1 grown on a strain carrying *btuB3191::Tn10* instead of *ilv-500::Tn10*, because it is not tightly linked with the *del(rep)::kan* mutation. This marker was inherited without any problem, so I conclude that *rep* is required for cSDR in the presence of *dnaC809* (like *priA*, *priB*, and *priC*).

DISCUSSION

This report tests whether several mutations known to affect the reloading of the replisome during replication restart, a process that occurs during log-phase growth of a wild-type strain, influence the loading of the replisome during cSDR. This study is important because it shows that the process of loading the replisome at different

structures can have different mechanisms even though the same repertoire of proteins are used.

It is interesting that *priA* and *priB* are essential for cSDR but not for replication restart in log-phase cells. There are several reasons why this might be true. The first is that there may be only a single pathway for loading a replisome at an R-loop. Evidence of multiple pathways would require the demonstration of genetic redundancy. While it is tempting to speculate that the one proposed pathway for loading during cSDR is very similar to or identical to the PriA-PriB-DnaT Φ X174 *in vitro* system and the PriA-PriB (DnaT) pathway for replication restart, the behavior of the *dnaC809* mutant using cSDR suggests that there may be some differences. This will be further addressed below. A second reason why cells using cSDR as their method for initiation of DNA replication require both *priA* and *priB* for growth (and cells using DnaA/oriC-dependent initiation of DNA replication do not) is because these cells use *priA* and *priB* to both initiate their chromosomal DNA replication and restart repaired replication forks. This may be further compounded if the lack of *rnhA* creates more situations where replication may stop and then need to be restarted (KOGOMA *et al.* 1993; McCOOL *et al.* 2004b). Finally, changes in gene expression created by *dnaA850::Tn10* may increase the need for *priA* and *priB* in *dnaA rnhA* mutants. DnaA-binding sites are found at *oriC* and other sites scattered throughout the chromosome (ROTH and MESSER 1998; OGAWA *et al.* 2002). DnaA binding can bend the DNA (SCHAPER and MESSER 1995) and if these sites are within promoter regions, its presence or absence can affect the activity of promoters (QUINONES *et al.* 1997; GLINKOWSKA *et al.* 2003; ORTENBERG *et al.* 2004). Thus the expression of many genes may be altered in *dnaA rnhA* mutants and this in turn may create a greater need for *priA* and *priB*.

Several differences are seen in the behavior of some of the replication restart mutants during cSDR in *dnaA rnhA* mutants as compared to normal log-phase growth. The first is between *priA300* and *priA301*. In previous studies, these two mutant alleles *in vivo* behave similarly in terms of having no phenotype by themselves and creating a *priA2::kan*-like phenotype in the presence of *del(priB)302* (SANDLER *et al.* 2001). These two mutations affect different regions and functions of PriA. PriA300 has been extensively studied biochemically. While this mutant has no ATPase and helicase activities, it maintains the ability to load replisomes (ZAVITZ and MARIANS 1992; KOGOMA *et al.* 1996; SANDLER *et al.* 1996). PriA301 has not been characterized biochemically. However, other PriA proteins mutant in the zinc-binding/protein interactions/helicase region have been (ZAVITZ and MARIANS 1993; LIU *et al.* 1996; TANAKA *et al.* 2003). Many of these are proficient in ATP hydrolysis and deficient in helicase activity. Thus one can speculate that the difference between these two mutants during cSDR is due to the ability to interact with other proteins.

A second difference between the behavior of muta-

tions during replication restart and cSDR lies with the *priB* and *priC* mutations. PriB and PriC form redundant, PriA-dependent pathways for reloading replication forks. PriC also participates in a PriA-independent pathway. Initially, few differences were documented between the two PriA-dependent pathways. More recently, it has been shown that the helicase activity of PriA may be important for the PriA-PriC pathway (SANDLER *et al.* 2001) and that *dnaC1331* cannot function with the PriA-PriB pathway (HARINARAYANAN and GOWRISHANKAR 2004). An interpretation of these new results is that the substrate (R-loop) used during cSDR is very specific in nature and requires PriA and PriB (and not PriA and PriC). Although this pathway may be similar to the PriA-PriB pathway used during normal growth and the PriA-PriB-DnaT biochemical pathway for $\Phi X174$ DNA replication in that both do not require PriC, a greater specificity for *dnaC*⁺ is also apparent in that *dnaC809* will not substitute.

RnhA mutations are synthetically lethal with mutations in a number of other genes: *polA* (KOGOMA and MALDONADO 1997), *recB* (ITAYA and CROUCH 1991), and *recG* (HONG *et al.* 1995). In this report, *rep* is added to the list. It is important to note that these synthetic lethality are in cells that initiate DNA replication using DnaA and *oriC*. In some cases (*recG*), the synthetic lethality is thought to occur because the gene products have the ability to remove R-loops on the chromosome and that their combined absence leads to too many R-loops (HONG *et al.* 1995). In other cases (*polA* and *recB*), it is thought that the ability to process DNA damage created by the encounter of replication forks with R-loops is essential (KOGOMA *et al.* 1993). Rep has at least two roles in the cell: stabilization of replication forks and PriA-independent replication restart (UZEST *et al.* 1995; SEIGNEUR *et al.* 1998; SANDLER 2000). While other models are also possible, the observation that *dnaC809* can rescue the *rep-rnhA* synthetic lethality suggests that Rep's essential role in *rnhA* mutants may be to help restart forks that were inhibited at R-loops (KOGOMA *et al.* 1993; MCCOOL *et al.* 2004b).

The behavior of the *dnaC* mutations was surprising on several levels. First, *dnaC809* did not suppress the absence of *priA* in cSDR. All previous experiments show that this allele will suppress all of the phenotypes of *priA2::kan* that have been tested. It was also quite surprising that the presence of *dnaC809* increased the requirement for *priC* during cSDR. It was less surprising that *dnaC809,820* should have a different effect because it is known that the two *dnaC* mutations can also behave quite differently with respect to *priA-rep*, *priA-priC*, and *priB-priC* synthetic lethality (SANDLER 2000). Although the pattern of suppression of *dnaC809,820* was similar in replication restart and cSDR (it suppressed the absence of *priA* and *priC*), the pattern of suppression of *dnaC809* was different. In normal cells during replication restart, *dnaC809* suppresses the absence of *priA* in a way that requires *priC* and *rep*. In cSDR, *dnaC809* did

not suppress the absence of *priA* and it did suppress the *rnhA-rep* synthetic lethality.

It is proposed that the differences in behavior of replication restart mutations during normal log-phase growth and during cSDR are due to the differences in the substrates to which these proteins load. While the models of replication restart during log-phase growth and cSDR that have been postulated so far (KOGOMA 1997; COX *et al.* 2000) have some similarities, there are some differences. The way in which the substrates are formed and the proteins that may be associated with them may explain some of the different requirements. For instance, during replication restart, D-loops are formed by processes involving RecBCD and RecFOR helping RecA onto the DNA (COX *et al.* 2000; KOWALCZYKOWSKI 2000). These proteins can make structures *in vitro* that are substrates for the RRP (XU and MARIANS 2003). The way in which the R-loop is formed is different. It has been proposed that transcription by RNA polymerase creates the RNA. The R-loop is formed and stabilized by RecA. This then creates a primer that can be extended by DNA polymerase I (Figure 2) (KOGOMA 1997; KOGOMA and MALDONADO 1997). The proteins that are left behind after the helix unwinding or repair event that may interact with the RRP are also different. Since recombination is thought to be a highly used pathway for fixing replication forks, it is possible that the repaired fork may still have RecA associated with it at the time of reloading. In cSDR, DNA polymerase I is thought to extend an RNA primer at the R-loop as a prelude to loading the replisome. It is reasonable to hypothesize that the displaced strand that occurs during this DNA synthesis is coated with single-strand DNA binding protein (SSB) to protect it from nucleases. SSB has been shown to bind this type of "bubble" substrate *in vitro* (XU and MARIANS 2000). Thus it is possible that the ability of these different sets of proteins to load the replisome at these two types of substrates is their ability to interact with DNA structures associated with either RecA or SSB. It has been shown recently that PriA can interact specifically with SSB bound to certain substrates *in vitro* (CADMAN and MCGLYNN 2004; CHEN *et al.* 2004). A second idea is that RecA bound to a D-loop is different enough from RecA bound to an R-loop to be seen by the RRP. A drawback to this model is that it has no role for DNA polymerase I, which is known to be required for cSDR.

I thank Ken Marians, Martin Marinus, and Benedicte Michel for reading the manuscript before publication and helpful suggestions. This work was supported by grant RPG-99-194-04-GMC from the American Cancer Society.

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