Cells defective for replication restart undergo replication fork reversal

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We have studied the fate of blocked replication forks with the use of the *Escherichia coli priA* mutant, in which spontaneously arrested replication forks persist owing to the lack of the major replication restart pathway. Such blocked forks undergo a specific reaction named replication fork reversal, in which newly synthesized strands anneal to form a DNA double-strand end adjacent to a four-way junction. Indeed, (i) *priA recB* mutant chromosomes are linearized by a reaction that requires the presence of the Holliday junction resolvase RuvABC, and (ii) RuvABC-dependent linearization is prevented by the presence of RecBC. Replication fork reversal in a *priA* mutant occurs independently of the recombination proteins RecA and RecR. *recBC* inactivation does not affect *priA* mutant viability but prevents *priA* chronic SOS induction. We propose that, in the absence of PriA, RecBC action at reversed forks does not allow replication restart, which leads to the accumulation of SOS-inducing RecA filaments. Our results suggest that types of replication blockage that cause replication fork reversal occur spontaneously.

Keywords: recombination; RecBCD; RuvABC; PriA; *Escherichia coli*; replication arrest

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INTRODUCTION

In all organisms, inactivation of replication forks triggers the induction of a checkpoint, and failure to restart replication properly causes genome instability (reviewed in Rothstein *et al*, 2000; Kolodner *et al*, 2002). The frequency and the origin of naturally occurring replication arrest are not well understood. Encounter of progressing replication forks with DNA damage or with sequences being transcribed or repaired may cause replication arrest and disassembly of the replication machinery. Because in *Escherichia coli*, forks blocked by different types of obstacles are restarted by different pathways (reviewed in Michel *et al*,

2001), this bacterium allows us to address the question of the main types of spontaneous replication arrest.

In *E. coli*, as in most bacteria, restart of inactivated replication forks is catalysed by a set of proteins that allow the reassembly of replication proteins at forked or D-loop structures, regardless of the primary DNA sequence. The key protein of replication restart is PriA (reviewed in Sandler & Marians, 2000; Polard *et al*, 2002). PriA recognizes and binds to fork structures and recombination intermediates, and, via a cascade of protein interactions, triggers the loading of the DnaB helicase by the helicase loader DnaC (Xu & Marians, 2003 and references therein). The helicase–primase complex (DnaB–DnaG primosome) promotes binding of DNA polymerase III holoenzyme at blocked forks and formation of a complete replisome. Cell viability is largely compromised in *priA* null mutants, in which replication restart relies on an inefficient PriA-independent pathway (Sandler & Marians, 2000). Furthermore, mutations that enhance the frequency of replication arrest by impairing replication progression render PriA essential for growth (Nurse *et al*, 1991; Flores *et al*, 2002).

Depending on the cause of replication arrest, replication restarts from blocked forks in different ways. For example, in gyrase mutants in which replication is halted by an excess of positive supercoils, PriA acts directly at blocked forks (Grompone *et al*, 2003). In other replication mutants, forks are processed by recombination proteins before restart (reviewed in Michel *et al*, 2001). Indeed, the properties of several replication mutants are accounted for by a model called replication fork reversal, according to which newly synthesized strands anneal at blocked forks, allowing the re-annealing of the template strands and resulting in the formation of a Holliday junction and a DNA double-strand end (Seigneur *et al*, 1998; Fig 1). Resetting of the reversed fork requires RecBCD, the complex that specifically recognizes DNA double-strand ends in *E. coli* (Fig 1C,D). In the absence of RecBC, the Holliday junction formed by fork reversal is acted upon by RuvABC, the *E. coli* complex that migrates and resolves Holliday junctions, which causes chromosome linearization (Fig 1E). Replication fork reversal was proposed to occur (i) in helicase mutants (in a *rep* mutant that lacks the Rep helicase proposed to remove DNA-binding proteins from the path of replication forks, and in a *dnaBts* mutant affected for DnaB, the main replicative helicase (Seigneur *et al*, 1998, 2000)) and (ii) in three mutants (*hold*, *dnaEts* and *dnaNts*) affected for Pol III

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Fig 1 | Replication fork reversal model (adapted from Seigneur *et al*, 1998; Flores *et al*, 2001). In step A, the replication fork is blocked, and the two newly synthesized strands anneal forming a Holliday junction, which is stabilized by RuvAB binding (step B). Pathway C: RecBCD binds to the double-strand tail (C1) and initiates a genetic exchange mediated by RecA (C2), and RuvC resolves the first Holliday junction bound by RuvAB (C3). Pathway D: RecBCD-mediated degradation of the tail progresses up to the RuvAB-bound Holliday junction. In *PriA*⁺ cells, replication can restart efficiently from recombined or degraded forks (C and D), whereas in a *priA* mutant replication restart is inefficient because it relies on a weak *PriA*-independent pathway. Pathway E: In cells that lack RecBC, resolution by RuvC of the RuvAB-bound Holliday junction results in linearization of the blocked fork, forming a sigma molecule; as linear chromosomes are observed by PFGE, we assume that the sigma molecule is fully linearized, either because the second fork is then easily broken, or because replication fork reversal also occurs at the other fork. Continuous and discontinuous lines represent the template and the newly synthesized strands of the chromosome, respectively; the arrow indicates the 3' end of the growing strand.

holoenzyme, the main replicative polymerase (Flores *et al*, 2001; Grompone *et al*, 2002). In these mutants, in addition to RecBC, *PriA* is essential for replication restart, regardless of whether the DNA double-strand end formed by fork reversal has been recombined or degraded (Seigneur *et al*, 1998; Flores *et al*, 2002; Fig 1).

Alternatively to replication fork reversal, other types of blocked fork processing are sometimes needed before *PriA* action. For example, replication restart after UV irradiation requires Pol II, RecFOR and RecA, and occurs independently of RecBCD (Khidhir *et al*, 1985; Rangarajan *et al*, 2002 and references therein). Forks blocked by an ectopic replication terminator site (*Ter*/Tus complex) require RecBCD, RecA and RuvABC recombination proteins to recombine DNA double-strand ends generated by the arrival of a second round of replication (Bidnenko *et al*, 2002). This in turn allows restart.

To date, the role of homologous recombination in replication restart has been studied in cells in which a specific kind of fork arrest is induced, whereas the fate of spontaneously arrested

replication forks remains an open question. In the present work, we address this question with the use of the *priA* mutant. As *PriA* is required for efficient replication restart, the main effect of *priA* inactivation is presumably to extend the lifetime of forks blocked by naturally occurring obstacles. We report here that RuvABC-dependent chromosome linearization occurs in a *priA* null mutant in the absence of RecBC and we propose that spontaneously arrested replication forks are reversed in the absence of an efficient restart pathway.

RESULTS

priA recB strains contain a high level of linear DNA

To prevent the accumulation of cells carrying suppressor mutations, *priA* mutants were constructed in the presence of the plasmid pAM-*priA*, which carries the wild-type *priA* gene and a conditional replication origin. This plasmid allows cell propagation in a *PriA*⁺ context and curing of the *priA*⁺ gene at the onset of experiments (see Methods; Grompone *et al*, 2003, 2004).

Table 1|Double-strand break formation in *priA recB* mutants

Strain	Relevant genotype	Percentage of linear DNA			N
		30 °C	37 °C	42 °C	
JJC315	<i>recB</i>	26.8 ± 6.9	32.7 ± 5.3	43.1 ± 3.8	3/10/4
JJC1401	<i>priA2::kan recB</i>	39.6 ± 4.3	55.7 ± 5	62.5 ± 3.8	7/7/13

N is the number of determinations at 30 °C/37 °C/42 °C. The *recB* and *priA recB* strains are highly significantly different at $P=0.0003$ at 30 °C and $P<0.0001$ at 37 and 42 °C. Owing to the Exo V activity of RecBCD, the level of linear DNA is low in wild-type cells (3–8%) and *priA* single mutants (10–15%).

To determine whether the replication restart defect of *priA* mutants causes chromosome breakage, the amounts of linear DNA in *recB* and *priA recB* cells were compared by pulsed-field gel electrophoresis (PFGE) at 30, 37 and 42 °C (Table 1). The *priA* mutation caused a significant increase in the level of linear DNA in *recB* cells at all temperatures, indicating the occurrence of increased chromosome breakage in the absence of PriA.

Previously characterized replication mutations that cause an increase in the level of linear DNA also render RecBC proteins essential for viability, whereas *priA recB* cultures are still viable. The plating efficiency of *priA* and *priA recB* mutants grown to exponential phase is twofold to threefold lower than that of wild-type cells, a plating defect that has been previously reported for a *priA* mutant (Sandler et al, 1996) and which suggests the presence of 50–70% nondividing cells in *priA recB* cultures. Accordingly, comparison of the generation times of wild-type, *priA* and *priA recB* cells showed that by the time the wild-type strain has undergone one generation (about 39 min) the *priA* and *priA recB* mutants have undergone 0.77 and 0.70 generations, respectively (Table 2). Knowing that nondividing cells accumulate in a cell culture, the production of 30% of nondividing cells per generation correlates with the twofold to threefold difference in plating efficiency and with the presence of more than 50% of linear DNA in *priA recB* cultures.

Linear DNA formation requires RuvABC

Linearization of chromosomes upon replication fork blockage could result from (i) direct breakage of replication forks, (ii) over-replication of blocked forks, or (iii) replication fork reversal. The last mechanism is characterized by a requirement of the Holliday junction resolution complex RuvABC for linear DNA formation (Fig 1E). We could not test directly whether RuvABC is required

for linear DNA formation in *priA recB* mutants, as strains that lack simultaneously PriA and RuvABC have a very low viability (Grompone et al, 2004). This low viability is due to their failure to resolve recombination intermediates formed by the RecA/RecFOR pathway, and viability can be improved by inactivation of *recA* or *recR* (Grompone et al, 2004). We thus used *recA* or *recR* mutations in combination with *priA recB* to test the effects of RuvABC on the formation of linear DNA. Inactivation of *recA* or *recR* did not significantly modify the level of linear DNA in the *priA recB* mutant, indicating that chromosome linearization occurs independently of these two proteins (Table 3). Inactivation of RuvABC decreased the amount of linear DNA to the level observed in PriA⁺ cells, indicating that chromosome breakage due to *priA* inactivation results from the action of RuvABC proteins (Table 3). In contrast, chromosome breakage in PriA⁺ cells was independent of RuvABC, indicating that replication fork reversal is not detectable when replication restart is functional. We conclude from these experiments that a RuvABC substrate is formed specifically in *priA recB* mutants, independently of the RecA and RecR proteins, the resolution of which leads to chromosome linearization. This observation argues in favour of replication fork reversal occurring at blocked forks in a *priA* mutant.

Linear DNA does not form in the presence of RecBC

RuvABC action is prevented at reversed forks by the presence of RecBC, presumably because RecBC acts on the DNA double-strand end before RuvABC-catalysed resolution of the Holliday junction (Seigneur et al, 1998; Flores et al, 2001). To test RuvABC-dependent linearization in the presence of RecBCD, we measured DNA degradation in *priA recA* mutants. If RuvABC acts before RecBCD (Fig 1E), this should lead to extensive RuvABC-dependent degradation of chromosomes, due to the exonuclease V (Exo V) activity of RecBCD. Conversely, if RecBC acts first (Fig 1C,D), only limited DNA degradation should take place, as Exo V would be stopped by the Holliday junction regardless of the presence of RuvABC, as previously observed in the *hld* mutant (Flores et al, 2001). To measure the effects of a *ruvABC* mutation on DNA degradation in a *priA* mutant, *priA recA* cells were used because the *priA ruvABC* mutant is not viable enough to be tested, owing to the occurrence of RecA-, RecFOR-dependent homologous recombination in *priA* mutants (Grompone et al, 2004). A higher level of DNA degradation was observed in the *priA recA* mutant than in the *recA* single mutant, which importantly was entirely independent of RuvABC (Fig 2). Increased DNA degradation when *priA* is inactivated in *recA* cells is presumably due to a defect in RecFOR–RecA recombinational repair, as *priA recFOR* and *priA recA* mutants require exo V for viability and DNA degradation is also observed in *priA recFOR* mutants (Grompone et al, 2004;

Table 2|*Escherichia coli* growth is affected in *priA*, *priA recA* and *priA recB* mutants to similar levels

Strain	Relevant genotype	Generation time (min)	N _g ^a
JJC40	Wild type	38.8 ± 3.1	1
JJC1398	<i>PriA</i>	61.8 ± 2.1	0.77
JJC1401	<i>PriA recB</i>	78.6 ± 10	0.70

Generation times are averages calculated from three independent growth curves. Exponential cultures were grown in minimal medium at 37 °C. Names of pAM-*priA* plasmid-carrying strains are indicated. The plasmid was segregated at the onset of the experiments (see Methods). ^aN_g is the number of generations undergone by a mutant strain, during one generation time of the wild-type strain. N_g is $10^{G_{ref} \log 2 / G_{mutant} / 2}$, where G_{ref} is the generation time of the reference strain (wild type) and G_{mutant} is the generation time of the mutant strain.

Table 3 | Double-strand break formation in *priA recB* mutant is RuvABC dependent and RecA, RecR independent

Strain	Relevant genotype	Percentage of linear DNA (37 °C)	N
JJC1105	<i>recB recA</i>	28 ± 6.5	6
JJC1681	<i>priA2::kan recB recA</i>	50.3 ± 5.7	5
JJC2072	<i>priA2::kan recB recR</i>	54.2 ± 6.1	5
JJC1152	<i>recA recB ruvABC</i>	23.8 ± 2.9	4
JJC2123	<i>priA recB recA ruvABC</i>	27.1 ± 1.8	4
JJC2100	<i>priA recB recR ruvABC</i>	26.2 ± 2.9	4

N is the number of determinations. Names of pAM-*priA* plasmid-carrying strains are indicated. The plasmid was segregated at the onset of the experiments (see Methods).

G.G. and B.M., unpublished results). As no RuvABC-dependent DNA degradation is observed in *priA recA* cells, RuvABC-dependent linear molecules observed in *priA recA recB* cells by PFGE are not formed in the presence of RecBC.

SOS induction is RecB dependent in *priA* mutants

The SOS regulon consists of more than 40 genes, many of which are involved in the repair of DNA damage (Courcelle *et al*, 2001). The SOS response is constitutively expressed at a high level in a *priA* mutant (Sandler *et al*, 1996). SOS is induced when RecA filaments promote autocleavage of the LexA repressor. Similarly to RecA filaments that participate in homologous recombination, SOS-inducing RecA filaments require either the RecBCD or the RecFOR presynaptic proteins for their formation (McPartland *et al*, 1980). To determine which presynaptic proteins are required for chronic SOS expression in a *priA* mutant, the level of SOS induction was compared in *priA*, *priA recB* and *priA recF* mutants with the use of an *sfIA::lacZ* fusion, in which the level of expression of the *lacZ* gene from the *sfIA* promoter is a measure of SOS induction (Table 4). As expected, SOS was induced more than tenfold by *priA* inactivation. *recB* inactivation completely abolished SOS induction in the *priA* mutant, whereas *recF* inactivation had a much weaker effect (Table 4). The twofold effect of *recF* inactivation correlates with the occurrence of RecFOR-mediated recombination in a *priA* mutant (Grompone *et al*, 2004). The much larger effect of *recB* inactivation indicates that, in the *priA* mutant, most of the SOS-inducing RecA filaments are formed via RecBC.

DISCUSSION

We have explored here the role of recombination proteins at arrested replication forks that fail to restart owing to the absence of PriA. Studies of *priA recFOR* mutants suggested that RecFOR does not act directly at blocked forks in *priA* cells (Grompone *et al*, 2004). Here, we have studied the role of RecBC.

The model of replication fork reversal is supported by three lines of evidence (reviewed in Michel *et al*, 2001): (i) RuvABC-dependent breakage occurs in *recBC* mutants (Fig 1E); (ii) RuvABC-dependent breakage is prevented by the presence of RecBC (Fig 1C,D); and (iii) RecBC proteins are required for viability whereas RecA is not (Fig 1D). A significant level of RuvABC-

Fig 2 | DNA degradation is RuvABC independent in *priA recA* mutants. Chromosomes were labelled by growing cells in the presence of tritiated thymidine. Labelled cells were then shifted to unlabelled medium and loss of incorporated tritiated thymidine was measured to determine the extent of DNA degradation. The ratio of tritiated thymidine that remains incorporated is shown. Closed squares, *PriA*⁺ *recA*; open squares, *priA recA*; stars, *priA recA ruvABC*.

dependent DNA linearization is observed in the *priA recB* mutant, which is prevented in the presence of RecBC and is not observed in *PriA*⁺ cells. This points to the occurrence of replication fork reversal when spontaneously arrested replication forks fail to restart. However, in contrast with mutants previously shown to undergo replication fork reversal, RecBC protein is not required for viability in a *priA* context, as *priA* and *recB* mutations have only slightly additive effects. We propose that replication fork reversal does not improve the viability of *priA* mutants because RecBC-processed reversed forks are not efficiently used for restart in the absence of PriA.

Forks can be reversed in at least two different ways: a RecA-dependent way in *dnaBts* mutants, and independently of RecA in Pol III and *rep* mutants (Seigneur *et al*, 2000). Homologous recombination is not required for RuvABC-dependent breakage in a *priA* mutant, indicating that similarly to *rep* and Pol III mutants, fork reversal is catalysed here by an unknown, RecA-independent mechanism (Table 3).

SOS induction is strongly dependent on RecBC in *priA* mutants, in agreement with the previously reported observation that a *recB* mutation decreases significantly the percentage of filamented cells in a *priA* culture (McCool & Sandler, 2001). As *priA* mutants are deficient for RecBC- and RecA-mediated recombination (Kogoma *et al*, 1996; Sandler *et al*, 1996), recombination intermediates formed by RecBC and RecA, and not stabilized by PriA, may be unstable, which would cause a constant re-formation of RecA filaments and constitutive expression of the SOS response. Nonproductive, SOS-inducing recombination intermediates presumably form in *priA* mutants during repair of any accidental DNA double-strand breaks and at reversed forks (Fig 1, last steps of pathway C). Accordingly, in the *priA300* mutant, in which replication restart is functional because only the helicase function of PriA is inactivated, replication fork reversal and chronic SOS induction are not observed (Zavitz & Marians, 1992; Grompone *et al*, 2002).

Conclusion

Replication arrest is an important source of genome instability in all organisms (reviewed in Michel, 2000; Carr, 2002; Kolodner *et al*, 2002; Huang & Koshland, 2003; Khakhar *et al*, 2003). The

Table 4 | SOS induction is RecB dependent in *priA* mutant

Strain	Relevant genotype	β -Galactosidase (Miller units)	N
JJC2255	<i>lacZ sfiA::MudAp^RlacZ</i>	29.3 $\pm$ 11.2	2
JJC2267	<i>lacZ sfiA::MudAp^RlacZ priA</i>	375.9 $\pm$ 56.6	4
JJC2274	<i>lacZ sfiA::MudAp^RlacZ priA recB</i>	33.3 $\pm$ 12.5	8
JJC2275	<i>lacZ sfiA::MudAp^RlacZ priA recF</i>	171.6 $\pm$ 36.9	7

N is the number of determinations. Names of pAM-*priA* plasmid-carrying strains are indicated. The plasmid was segregated at the onset of the experiment.

precise origin of spontaneous replication arrest is unknown and presumably depends on the organism and on growth conditions. Theoretically, replication may be inactivated by DNA damage (Rangarajan et al, 2002), by roadblocks such as DNA-bound transcriptional and/or translational apparatus (French, 1992), or, considering the high level of spontaneous transcriptional or translational errors, by epigenetic inactivation of a replisome component (Parker, 1989; Kurland, 1992). In bacteria, different recombination proteins are required depending on the cause of arrest; for example, in contrast with cells that undergo replication fork reversal, UV-treated cells require RecA and RecFOR for replication restart, and not RecBC (Khidhir et al, 1985; Rangarajan et al, 2002). The observation of replication fork reversal in cells in which replication restart is compromised suggests that replication blocks, such as DNA-bound proteins and/or epigenetic polymerase inactivation, are important contributors to spontaneous replication inactivation. In the absence of efficient replication restart, the consequence of these blocks is fork reversal.

METHODS

Strains and plasmids. Strains used are described in supplementary Table S1 online. The strain background is JJC40, which is an *hsdR* Thr⁺ Pro⁺ derivative of AB1157. All strains were constructed by P1 transduction, except *recA recB* double mutants constructed by co-conjugation of these two mutations from a *recA recB* [pDWS2] Hfr strain (JJC1130; pDWS2 is a pBR322 derivative that carries the wild-type *recBCD* genes). The presence of the *priA2::kan^R* mutation was verified by PCR. All recombination mutations were checked for the level of survival after UV irradiation. The exonuclease-deficient phenotype of *recB* and *recD* mutants was checked by plating of gene II⁻ T4 phage. pAM-*priA* carries an isopropyl- β -D-thiogalactoside (IPTG)-dependent replication origin, a spectinomycin resistance gene (Spec^R) and the wild-type *priA* gene, and has been described previously (Grompone et al, 2003). To obtain plasmid cured cells, cells containing pAM-*priA* and grown overnight in Luria broth (LB) in the presence of 500 μ M IPTG and 60 μ g/ml spectinomycin were diluted 1,000-fold in minimal medium and grown for 10 h at 37 °C. This first minimal medium culture was either plated and freshly isolated plasmidless colonies were used, or was diluted again 1,000-fold and grown overnight, generating an overnight culture containing less than 0.1% plasmid-carrying cells (Grompone et al, 2003, 2004). Loss of the *priA*⁺ gene was 100% linked with the loss of the Spec^R marker and was also checked by PCR. Results obtained with isolated colonies or segregated cultures were always similar. For quantification of SOS induction, β -galactosidase was measured by the

classical Miller assay and results are expressed in Miller units (Miller, 1992). Antibiotics were used at the following concentrations: kanamycin (Kan) 50 μ g/ml, chloramphenicol (Cm) 20 μ g/ml, tetracycline (Tet) 15 μ g/ml, spectinomycin (Spec) 60 μ g/ml. Minimal medium is M63 (Miller, 1992), supplemented with 0.2% glucose and 0.2% casamino acids. Rich medium is LB.

Determination of generation times. Segregated cultures were diluted 200-fold in minimal medium and grown at 37 °C for 7 h. Appropriate dilutions were plated each hour on minimal medium to determine the number of colony-forming units (cfu) and the generation time was calculated from the exponential part of the growth curve: $G = \log 2 T / (\log N - \log N_0)$, where G is the generation time, N_0 the initial cfu, N the final cfu and T the time of growth expressed in minutes.

Measure of linear DNA by PFGE or DNA degradation. Quantification of pulsed-field gels and measures of DNA degradation were performed as described previously (Seigneur et al, 1998).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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