RuvAB is essential for replication forks reversal in certain replication mutants

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Inactivated replication forks may be reversed by the annealing of leading- and lagging-strand ends, resulting in the formation of a Holliday junction (HJ) adjacent to a DNA double-strand end. In *Escherichia coli* mutants deficient for double-strand end processing, resolution of the HJ by RuvABC leads to fork breakage, a reaction that we can directly quantify. Here we used the HJ-specific resolvase RuvA to test a putative role of the RuvAB helicase in replication fork reversal (RFR). We show that the RuvAB complex is required for the formation of a RuvA substrate in the polymerase III mutants *dnaEts* and *holD*, affected for the Pol III catalytic subunit and clamp loader, and in the helicase mutant *rep*. This finding reveals that the recombination enzyme RuvAB targets forks *in vivo* and we propose that it directly converts forks into HJs. In contrast, RFR occurs in the absence of RuvAB in the *dnaNts* mutant, affected for the processivity clamp of Pol III, and in the *priA* mutant, defective for replication restart. This suggests alternative pathways of RFR.

The EMBO Journal (2006) 25, 596–604. doi:10.1038/
sj.emboj.7600941; Published online 19 January 2006

Subject Categories: genome stability & dynamics; microbiology
& pathogens

Keywords: DNA repair; genome stability; helicase;
recombination; replication restart

Introduction

Replication fork progression may be impeded by the encounter of obstacles such as DNA-bound proteins, or by a transient deprivation of an essential replication component. The consequences of replication fork arrest has been the subject of extensive studies in the past years and replication fork arrest is now recognized as an important source of DNA rearrangements in all organisms (Michel, 2000; Carr, 2002; Kolodner *et al*, 2002). A growing number of proteins known to parti-

cipate in homologous recombination, DNA repair or replication restart have been proposed to act at blocked forks *in vivo*, and some of them directly bind Y-shaped DNA molecules that mimic fork structures *in vitro* (Jones and Nakai, 2000; Sandler and Mariani, 2000; McGlynn and Lloyd, 2002; Hishida *et al*, 2004; Flores *et al*, 2005; Heller and Mariani, 2005a). The rules that govern the action of different fork-binding proteins at inactivated replication forks *in vivo* presumably depend on the conditions of fork arrest and are still largely unknown.

Replication fork arrest leads in several *Escherichia coli* replication mutants to a reaction called replication fork reversal (RFR; Seigneur *et al*, 1998; reviewed in Michel *et al*, 2004). This reaction involves the annealing of leading- and lagging-strand ends, to form a Holliday junction (HJ) adjacent to a DNA double-strand end (Figure 1A). In *E. coli*, DNA double-strand ends are processed by the recombinase-exonuclease RecBCD and HJs by the resolvase RuvABC (Kuzminov, 1999). In the absence of RecBCD, resolution of the RFR-made HJ by RuvABC leads to fork breakage (Figure 1E). The RFR model was based on direct quantitative analysis of fork breakage and supported by genetic data. To date, the reaction was proposed to occur in: (i) two helicase mutants, the *rep* mutant defective for an accessory replicative helicase and *dnaBts* cells, in which the main replicative helicase is inactivated at high temperature (Seigneur *et al*, 1998); (ii) three Pol III mutants, *dnaEts*, *dnaNts* and *holD*^{Q10am}, respectively, affected for the catalytic Pol III subunit, the processivity clamp and one of the subunits of the clamp loader (Flores *et al*, 2001; Grompone *et al*, 2002) and (iii) finally in the *priA* mutant defective for the main replication restart pathway (Grompone *et al*, 2004a).

Although the processing of reversed forks by recombination proteins is well understood (Figure 1B–D), less is known about the mechanism of their formation. *In vitro*, both the homologous recombination protein RecA and the helicase RecG can reverse a particular fork structure (McGlynn and Lloyd, 2000; Robu *et al*, 2001, 2004). *In vivo*, RecA is required for RFR in the *dnaBts* mutant affected for the replicative helicase (Seigneur *et al*, 2000), but not in other replication mutants (Seigneur *et al*, 2000; Flores *et al*, 2001; Grompone *et al*, 2002, 2004a). RecG has been proposed to promote RFR at replication forks blocked by a UV lesion (McGlynn and Lloyd, 2002), a reaction which is now controversial (Donaldson *et al*, 2004; Wang, 2005). To date, in most conditions of replication inactivation, the mechanism by which a blocked replication fork is converted into an HJ remains unknown.

RuvA, RuvB and RuvC proteins form two complexes: a RuvAB complex with helicase and branch migration activities and a RuvABC complex that resolves HJs (reviewed in West, 1997). Forks are not broken when the RuvABC complex is inactive, because there is no other HJ resolvase in wild-type *E. coli*. This requirement for RuvABC for HJ resolution prevented us from testing whether the RuvAB complex

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Figure 1 The RFR model (adapted from Seigneur *et al*, 1998). In the first step (A), the replication fork is arrested by inactivation of *dnaE* or *dnaN* protein, causing fork reversal. The reversed fork forms a four-arm structure (HJ, two alternative representations of this structure are shown, open X and parallel stacked X). RecBC is essential for the resetting of a fork, either by RecA-dependent homologous recombination (B, C) or by DNA degradation (B–D). In the absence of RecBCD (E), resolution of the HJ causes chromosome linearization. We use here the RusA resolvase to test a putative role of RuvAB in reversing forks. Continuous lines: parental chromosome. Dashed lines: newly synthesized strands. Circle: RuvAB. Incised circle: RecBCD.

could be able to bind blocked replication forks *in vivo* and to convert them into HJs. To address this question, we took advantage of the RusA protein (Mandal *et al*, 1993; Mahdi *et al*, 1996). RusA is a resolvase encoded by a cryptic *E. coli* prophage; it specifically resolves HJs *in vitro* (Sharples *et al*, 1994). *In vivo*, RusA is able to resolve HJs in *E. coli* as well as in heterologous organisms such as yeast and mammalian cells (Doe *et al*, 2000; Saintigny *et al*, 2002). The *rusA* gene is not expressed in wild-type *E. coli* due to the lack of a functional promoter, but mutations such as *rus-1* activate the expression of a functional RusA protein and suppress the homologous recombination defect of *ruvAB* or *ruvABC* mutants (Mandal *et al*, 1993; Mahdi *et al*, 1996). In addition to HJs made by homologous recombination, RusA cleaves reversed forks in the helicase mutant *dnaBts*, in which RFR requires RecA (Seigneur *et al*, 2000).

In this work, we used the RusA HJ resolvase to test in different replication mutants whether RFR occurs in the absence of RuvAB. We first compared in detail two Pol III mutants, *dnaNts* affected for the processivity clamp and *dnaEts* affected for the polymerase subunit. RusA cleaves forks in *dnaNts ruvABC* mutant cells, indicating that reversal of *dnaNts*-blocked forks does not require RuvAB. In contrast, RusA is not able to cleave blocked forks in *dnaEts ruvAB* mutants, indicating that the RuvAB helicase is required for the conversion of *dnaEts*-blocked forks into HJs. The analysis of the other replication mutants in which RFR occurs confirms that RuvAB is required for fork reversal in some but not all of them.

Figure 2 RusA cleaves forks in the absence of RuvABC in a *dnaNts* mutant, but not in a *dnaEts* mutant. The histograms indicate the percentage of linear DNA in cultures propagated for 3 h at restrictive temperature (42°C) or semipermissive temperature (37°C). Bars indicate standard deviations. Strain genotypes are indicated below the blocks (full genotypes are described in Supplementary Table S1). (A) RusA cleaves blocked forks in the *dnaNts recBC ruvABC* mutant. Hatched blocks 37°C, white blocks 42°C: *Ruv+*, JJC1221 (*dnaNts recBC*); *ruv-*, JJC1323 (*dnaNts recBC ruvABC*); *ruv- rus-1*, JJC2648 (*dnaNts recBC ruvABC rus-1*). (B) RusA does not cleave blocked forks in the *dnaEts recBC ruvABC* mutant. White blocks, *dnaEts* strains, 42°C: *Ruv+*, JJC1983 (*dnaEts recBCts*); *ruv-*, JJC1541 (*dnaEts recBCts ruvABC*); *ruv- rus-1*, JJC2624/JJC2671 (*dnaEts recBCts ruvABC rus-1*). Gray block, DnaE⁺ *recBCts ruvABC rus-1* mutant at 42°C (JJC2712/JJC2641).

Results

RFR occurs in *dnaNts ruvABC*

Replication fork breakage is catalyzed in Pol III_{ts} mutants by the action of the HJ resolvase RuvABC on reversed forks (Grompone *et al*, 2002). Quantification of the amount of chromosomal DNA able to enter pulse-field gels is used to measure fork breakage (Seigneur *et al*, 1998; see Materials and methods). As only fully linear DNA is able to enter pulse-field gels, breakage of both replication forks in a circular chromosome is needed for a measurable linearization. Since the presence of RecB and RecC proteins prevents forks cleavage (Figure 1; Michel *et al* (2004) and references therein), RuvABC-dependent breakage can only be measured in cells in which either *recB* or *recC* is inactivated (see Supplementary Table S1 for strain constructions). In the *dnaNts recB* mutant, a high level of fork breakage occurs at 37°C (62.3%), which is lower when DnaN is fully inactivated at 42°C (36.7%) and is largely abolished by *ruvABC* inactivation (8.1% at 37°C; Figure 2A and Supplementary Table S2). The lower level of fork breakage in the *dnaNts recB* mutant at 42°C compared to 37°C has been previously described and is specific for this mutant (Grompone *et al*, 2002). Reasons for this observation are presently unknown (a partial DnaN activity may be required for RFR, or the DnaN mutant protein may block access to the replication fork at 42°C; Grompone *et al*, 2002).

In order to test whether the helicase action of the RuvAB complex is required for the conversion of inactivated replica-

tion forks into HJs, we used *ruvABC rus-1* mutant cells, which lack RuvABC and express the RusA resolvase. *dnaNts recB* (RuvABC active), *dnaNts recB ruvABC* (no active resolvase) and *dnaNts recB ruvABC rus-1* cells (RusA active) were compared. A high level of DNA breakage was also observed at 37°C in the *ruvABC rus-1* mutant context, that is, when RusA is active (59.9%), indicating that HJs are formed at *dnaNts*-blocked forks in the absence of RuvAB (Figure 2A and Supplementary Table S2). Similar results were obtained when the RecBC complex is inactivated at high temperature by *recBts* and *recCts* mutations (called here *recBCts*) rather than a *recB* null mutation (Supplementary Table S2). It should be noted that 38% linear DNA forms in the *recBCts ruvABC rus-1* mutant in which replication is not affected by a mutation (JJC2712; Figure 2B and Supplementary Table S2). The origin of spontaneous chromosome breakage is unknown. It may result from spontaneous replication arrest and resolution by RusA of Ruv-independent RFR occurring at these arrested replication forks. In addition, the level of linear DNA in replication-proficient cells is higher in *ruvABC* mutants when RusA is expressed (38.1% of linear DNA in *recBCts ruvABC rus-1* cells at 42°C versus 20% in *recB ruvABC* mutant; Seigneur *et al*, 1998), presumably because RusA resolves recombination intermediates that would otherwise prevent some broken chromosomes from entering pulse-field gels. Nevertheless, the use of the *rus-1* allele and the comparison of *DnaN⁺* and *dnaNts* cells allow us to conclude that at *dnaNts*-blocked forks RFR does not require RuvABC. The occurrence of RFR in the absence of RuvAB suggests alternative pathways of RFR, but it does not exclude the involvement of RuvAB in RFR in the *dnaNts* mutant.

RFR does not occur in *dnaEts ruvABC*

The *rus-1* allele was similarly used to test whether HJs are still formed at blocked forks in the *dnaEts* mutant when RuvABC proteins are absent. In contrast with the high level of fork breakage in the *dnaEts recBCts* mutant (64%; Figure 2B and Supplementary Table S3), the level of chromosome breakage in *dnaEts recBCts ruvABC rus-1* was not significantly different from that in a *DnaE⁺ recBCts ruvABC rus-1* mutant (37.2 versus 38%; Figure 2B and Supplementary Table S3). Similar results were observed when RecBCD was inactivated by a *recB* null mutation (*recB268::Tn10*, JJC2647; Supplementary Table S3). Consequently, the comparison of *DnaE⁺* and *dnaEts* cells indicates that *DnaE* inactivation does not cause fork breakage when RusA is active and RuvABC absent. Similar results were obtained when only RuvA and RuvB were absent (*ruvA60::Tn10* mutation, J1JC2725; Supplementary Table S3). The absence of RusA-dependent fork breakage in the *dnaEts recBCts ruvAB(C) rus-1* mutant did not result from a peculiar inhibition of the RusA protein in this mutant, as the *rus-1* allele fully suppressed the sensitivity to UV irradiation caused by *ruvAB* and *ruvABC* mutations in this background as in all mutants (data not shown).

In order to ascertain that the lack of *dnaEts*-induced fork breakage in a *ruvABC rus-1* context results from the absence of RuvABC, a plasmid carrying functional *ruvABC* genes was introduced in the *ruvABC* mutant (the low copy plasmid routinely used for complementation experiments, pGB-*ruvABC*, has no deleterious effect in contrast with high copy plasmids carrying *ruvABC* genes; Seigneur *et al*, 1998;

Grompone *et al*, 2002; Lopez *et al*, 2005). Expression of RuvABC from pGB-*ruvABC* in the *dnaEts recBCts ruvABC rus-1* mutant restored more than 60% of linear DNA, whereas the plasmid vector had no effect (Figure 2B and Supplementary Table S3). This result confirms that the inactivation of *ruvABC* is responsible for the lower level of fork breakage *dnaEts recBCts ruvABC rus-1* cells compared to *dnaEts recBCts*. The plasmid pGB-*ruvABC*, which expresses only RuvA and RuvB (Seigneur *et al*, 1998), was also tested. It was previously reported that cells that express RuvAB and RusA but not RuvC are unable to resolve HJ, presumably because, as shown *in vitro*, RuvAB prevents RusA action by masking the HJ (Chan *et al*, 1997). Accordingly, when pGB-*ruvABC* was introduced in *dnaEts recBCts ruvABC rus-1* cells, it prevented homologous recombination at 30°C (rendering cells UV sensitive) and did not restore fork breakage (measured by pulse-field gel quantification, data not shown). In conclusion, the lack of *dnaEts*-induced fork breakage when RusA is active and RuvAB inactive indicates that RFR requires a functional RuvAB complex in the *dnaEts* mutant, in contrast with the *dnaNts* mutant.

RuvAB is required for RFR at *dnaEts*-blocked forks in *recA* or *recFOR* mutants

Previous results indicate that in both *dnaEts* and *dnaNts* mutants a futile reaction takes place at blocked forks prior to RFR: RecA binds to the fork with the help of the pre-synaptic proteins RecQ, RecJ and RecFOR, and the resulting recombination intermediate is then undone by the UvrD helicase (Flores *et al*, 2005). In order to determine whether the requirement for RuvAB for RFR is dependent on this 'RecA-binding/RecA-removal' reaction, RusA-catalyzed fork breakage was tested in *recF*, *recO* and *recA* backgrounds (Figure 3, and Supplementary Tables S4 and S5). As in the *RecA^{FOR}* context, the level of DNA breakage measured in a *dnaEts recBCts ruvABC rus-1* context (RusA active and *ruvABC* inactive) was significantly lower (31–37%) than in the respective *RuvABC⁺* isogenic strain (55–65%), and not significantly different from the *DnaE⁺ recBCts ruvABC rus-1 recA* (or *recO*) mutant (32.5–41%; Figure 3A, and Supplementary Tables S4 and S5). Expression of RuvABC from a plasmid in *dnaEts recBCts ruvABC rus-1 recF* (*recO*) cells restored a high level of fork breakage, indicating that the low level of breakage results from the absence of RuvABC (Figure 3B and Supplementary Table S5). These results show that RuvAB is required for the occurrence of RusA-catalyzed fork breakage, and hence for the formation of reversed forks in the *dnaEts* mutant, regardless of the presence of RecFOR or RecA. Similarly, breakage in a *dnaEts recBCts recF uvrD* mutant (60.5%) was decreased to 42% by the combination of *ruvABC rus-1* mutations, a level similar to that of the *DnaE⁺ recBCts recO uvrD ruvABC rus-1* mutant (JJC2722; Supplementary Table S5). This observation shows that, in a *uvrD recFOR* context also, RusA does not act at forks inactivated by the *dnaEts* mutation when the RuvAB complex is absent. We conclude that RuvAB is required for RFR in the *dnaEts* mutant regardless of the 'RecA-binding/RecA-removal' reaction.

Preventing RFR is not lethal in *dnaEts* at 37°C

dnaEts cells can propagate at 37°C, but with a reduced ability to form colonies (Figure 4A; Grompone *et al*, 2002). *dnaEts*

Figure 3 RuvA does not cleave forks in *dnaEts recBCts ruvABC rus-1* mutants regardless of the presence or absence of RecA or RecF. The histograms indicate the percentage of linear DNA in cultures propagated for 3 h at restrictive temperature (42°C). Bars indicate standard deviations. Strain genotypes are indicated below the blocks (full genotypes are described in Supplementary Table S1). (A) *recA* mutants. White blocks, *dnaEts*: Ruv⁺, JJC1394 (*dnaEts recBC recA*); *ruv*⁺, JJC1396 (*dnaEts recBC recA ruvABC*); *ruv*⁻ *rus-1*, JJC2663 (*dnaEts recBC recA ruvABC rus-1*). Gray blocks, *DnaE*⁺:Ruv⁺, JJC1105 (*recBC recA*); *ruv*⁻, JJC1152 (*recBC recA ruvABC*); *ruv*⁻ *rus-1*, JJC1282 (*recBC recA ruvABC rus-1*). (B) *recF(O)* mutants. White blocks, *dnaEts*: Ruv⁺, JJC1476 (*dnaEts recBCts recF*); *ruv*⁻, JJC2217 (*dnaEts recBCts recF ruvABC*); *ruv*⁻ *rus-1*, JJC2625 (*dnaEts recBCts recF ruvABC rus-1*). Gray block, *DnaE*⁺ *recBCts ruvABC rus-1 recO* (JJC2685).

cells are chronically induced for the SOS response and their viability is strongly improved by mutations that abolish or decrease SOS induction, such as *lexAind*, *recA*, *recFOR* or *recQ* mutations (Flores et al, 2005; Figure 4). The *dnaEts ruvABC* mutant is not viable at 37°C (Figure 4A) and its lethality is suppressed by the inactivation of either *recA* or *recFOR* (Figure 4). However, the lethality of the *dnaEts ruvABC* mutant is not suppressed by the sole inactivation of the SOS induction, since *dnaEts lexAind ruvABC* and *dnaEts recQ ruvABC* mutants are killed upon shift to 37°C (data not shown). As RecFOR and RecA are required for recombinational gap repair as well as SOS induction, we propose that their inactivation alleviates the need for RuvABC by preventing recombinational gap repair (allowing, e.g., gap filling by a polymerase). Indeed, gaps may form during chromosome replication in the *dnaEts* mutant as a consequence of defects in lagging-strand synthesis, and recombine in a RecA- and RecFOR-dependent way, forming HJs that render RuvABC essential for viability. If RFR were required for the viability of the *dnaEts* mutant at 37°C, *dnaEts ruvABC recF* and *dnaEts ruvABC recA* strains would not be viable because of the lack of RFR. Since these mutants can propagate at 37°C, we conclude that the *dnaEts* mutant does not require RFR for growth at this temperature.

We tested whether the inactivation of *ruvABC*, which suppresses fork breakage, suppresses the requirement for RecBC for growth of *dnaEts* cells at 37°C. To avoid the lethal

Figure 4 RFR is not required for growth of the *dnaEts* mutant at semipermissive temperature. Cells propagated at 30°C for 2 h were shifted to 37°C and appropriate dilutions were plated at the indicated times; plates were counted after 48-h incubation at 30°C. (A) Inactivation of *recA* allows growth of the *dnaEts ruvABC* mutant at 37°C: JJC1954, *dnaEts* (circles); JJC2024, *dnaEts recA* (crosses); JJC2745, *dnaEts recA ruvABC* (stars); JJC2654, *dnaEts ruvABC* (triangles); JJC2650 *dnaEts recB* (squares). (B) Inactivation of *recF* allows growth of the *dnaEts ruvABC* mutant: JJC2750, *dnaEts recF* (circles); JJC2758, *dnaEts recF ruvABC* (triangles). Inactivation of *ruvABC* improves growth of the *dnaEts recF recB* mutant: JJC2864, *dnaEts recF ruvABC recB* (crosses); JJC2807, *dnaEts recF recB* (squares).

effect of gap repair in *dnaEts* cells that lack RuvABC, the question was addressed in a *recF* background. The inactivation of *ruvABC* allowed a slow growth of the *dnaEts recF recB* mutant at 37°C in liquid medium (Figure 4B) and allowed colony formation (data not shown). This result supports the idea that RuvAB is responsible for the requirement for RecB upon replication impairment.

RecA, RecG and UvrD are not required for RFR in *dnaNts*

As RFR is RecA-dependent in the *dnaBts* mutant and RuvAB-dependent in *dnaEts* mutant, we tested a possible redundant role of RuvAB and RecA for the catalysis of RFR in the *dnaNts* mutant by measuring chromosome breakage in a *dnaNts* mutant that lacks both. As RecFOR can be required for RecA binding to block forks, the *recO* mutation was also tested. A high level of RuvA-catalyzed fork breakage was observed in *dnaNts recB ruvABC rus-1 recA* (or *recO*) mutants (66–71%; Table I), indicating that *dnaNts*-blocked forks are reversed in the absence of both RuvAB and RecA (or both RuvABC and RecFOR).

RuvAB and RecG share the property of being able to migrate HJs *in vitro* and *in vivo* (Lloyd and Sharples, 1993a). RecG has also been shown to reverse fork-like structures *in vitro*, and possibly *in vivo* (McGlynn and Lloyd, 2000; Robu et al, 2004). We tested whether RecG could be the helicase that reverses *dnaNts*-blocked forks in

Table 1 RecA, RecG and UvrD are not required for RFR in *dnaNts*

Strain	Relevant genotype	Percentage linear chromosome			N
		30°C	37°C	42°C	
JJC1319 ^a	<i>dnaNts recB recA</i>	41 ± 6	65.3 ± 5.8	41.7 ± 6.3	3/3/4
JJC1420 ^a	<i>dnaNts recB recA ruvABC</i>	33.6 ± 3	22.2 ± 4.6	24.3 ± 3.7	2/4/3
JJC2665	<i>dnaNts recB recA ruvABC rus-1</i>	ND	66.4 ± 1.2	44.8 ± 4.5	2/2
JJC2681	<i>dnaNts recBCts recA ruvABC rus-1</i>	ND	68 ± 2.5	53.6 ± 4.2	4/3
JJC2339 ^a	<i>dnaNts recBCts recO</i>	31.8	66.7 ± 6.4	46.5 ± 4.5	1/4/3
JJC2766	<i>dnaNts recBCts recO ruvABC</i>	9.6	12.8 ± 4.1	12.7 ± 1.3	1/3/3
JJC2680	<i>dnaNts recBCts recO ruvABC rus-1</i>	ND	71.3 ± 3.6	45.4 ± 0.6	4/2
JJC2021 (pBR-Gam)	<i>dnaNts recA</i> (pBR-Gam +)	25.1	67.5 ± 5	47.7 ± 3.9	1/7/3
JJC2966/JJC2975 (pBR-Gam)	<i>dnaNts recA recG ruvABC rus-1</i> (pBR-Gam +)	29.7 ± 0.3	58.7 ± 2.7	42.1 ± 1.4	2/8/5
JJC2978 (pBR-Gam)	<i>dnaNts recA recG ruvABC</i> (pBR-Gam +)	23.4	18.5 ± 3.7	12.3 ± 0.5	1/3/3
JJC2981 (pBR-Gam)	<i>recA recG ruvABC rus-1</i> (pBR-Gam +)	16.5	29.3 ± 0.8	23.9 ± 1.8	1/3/3
JJC2354/2374 ^a	<i>dnaNts recBCts recO uvrD</i>	23.3 ± 3.7	67.1 ± 4.2	49.7 ± 5.3	2/13/7
JJC2412 ^a	<i>dnaNts recBCts recO uvrD ruvABC</i>	12.3 ± 3	6.7 ± 4.2	10 ± 5.4	2/2/2
JJC2741/JJC2729	<i>dnaNts recBCts recO uvrD ruvABC rus-1</i>	24.3 ± 0.8	67.9 ± 5.1	53.2 ± 4.1	2/4/4

^aResults are the average of values previously published (Grompone *et al*, 2002; Flores *et al*, 2004, 2005) and values obtained in this work. New values did not differ from previous ones. ND = not defined.

the *ruvAB recA* context, but we observed that RusA-mediated fork breakage occurs in *dnaNts* cells that lack RecA, RuvAB and RecG (58%; Table 1). This indicates that forks are reversed by yet another activity. Finally, RusA-catalyzed fork cleavage occurs in the *dnaNts* mutant when RecFOR, UvrD and RuvABC are all inactive (JJC2741/JJC2729; Table 1). Therefore, these experiments did not allow identification of the RFR pathway that operates at *dnaNts*-blocked forks.

RuvAB is required for RFR in *hoID*^{Q10am} and *rep* mutants, but not in the *priA* mutant

Based on the occurrence of RuvABC-dependent fork breakage in a *recBC* mutant context, RFR was proposed to occur in several replication mutants. The *hoID*^{Q10am} mutant carries an amber mutation in the gene encoding the HoID protein, one of the polypeptides of the Pol III clamp loader. This mutant was isolated in a background in which the mutation is poorly suppressed (Flores *et al*, 2001). In the AB1157 background used here, the amber mutation is only partially suppressed also since the *hoID*^{Q10am} mutant is lethal when RecBC is inactivated and the *hoID*^{Q10am} *recBCts* mutant suffers fork breakage at 42°C (Figure 5 and Supplementary Table S6). RFR also occurs in the *priA* mutant, in which spontaneously arrested replication forks persist because of the inactivation of the main replication restart pathway (Grompone *et al*, 2004a) and in the *rep* mutant (Seigneur *et al*, 1998). The role of the Rep helicase *in vivo* is not entirely elucidated; it is proposed to remove obstacles in front of replication forks and to participate in replication restart (Seigneur *et al*, 1998; Sandler, 2000; Heller and Mariani, 2005b). In *hoID*^{Q10am}, *priA* and *rep* mutants, RFR was shown to be RecA-independent and the molecular mechanism of the reaction is still unknown (Seigneur *et al*, 2000; Flores *et al*, 2001; Grompone *et al*, 2004a). To test whether RuvAB is required for RFR in these three mutants, *hoID*^{Q10am}, *rep* and *priA* mutations were combined with the *recBCts ruvABC rus-1* mutations. As these combinations of mutations affect growth, strain constructions were made in the presence of a complementing plasmid with

Figure 5 RusA cleaves forks in *hoID recBCts ruvABC rus-1* mutants and *rep recBCts ruvABC rus-1*, but not in *priA recB ruvABC rus-1* cells. The histograms indicate the percentage of linear DNA in cultures propagated for 3 h at 42°C. Bars indicate standard deviations. Strain genotypes are indicated below the blocks (full genotypes are described in Supplementary Table S1). White blocks Ruv⁺, JJC2716 cured of pAM-*hoID*⁺ (*hoID recBCts*), JJC1401 cured of pAM-*priA*⁺ (*priA recB*), JJC505 and JJC790 (*rep recBCts*). Gray blocks, *ruvABC rus-1*, JJC2689 cured of pAM-*hoID*⁺ (*hoID recBCts ruvABC rus-1*), JJC2667 cured of pAM-*priA*⁺ (*priA recB ruvABC rus-1*) and JJC2730 cured of pAM-*rep*⁺ (*rep recBCts ruvABC rus-1*).

conditional replication origin (see Materials and methods). Therefore, mutants were constructed in *HoID*⁺, *PriA*⁺ or *Rep*⁺ backgrounds, respectively, and *hoID*^{Q10am}-, *priA*- or *rep*-deficient cells were isolated after segregation of the plasmid (in addition, these mutants were propagated on minimal medium to further prevent the appearance of suppressor mutations).

Measures of fork breakage by pulse-field gels showed that RusA did not cause breakage in the *hoID*^{Q10am} *recBCts ruvABC rus-1* mutant (28.9 versus 51.5% in RuvABC + cells; Figure 5 and Supplementary Table S6). Therefore, similarly to *dnaNts*-blocked forks, *hoID*^{Q10am}-blocked forks are not converted to HJs in the absence of RuvAB. In contrast, the level of DNA breakage was similar in *priA recB* and *priA recB ruvABC rus-1* cells, indicating that HJs form at *priA*-inactivated forks in the absence of RuvAB (Figure 5 and Supplementary Table S6). Finally, the level of fork breakage in *rep recBCts ruvABC rus-1*

cells (47.6%) was lower than in *rep recBCts* cells (67.7%), indicating that RuvAB is involved in the formation of reversed forks at *rep*-blocked forks. Nevertheless, this level was higher than in *Rep⁺ recBCts ruvABC rus-1* cells (38.8%), suggesting that in the absence of *ruvAB*, a low level of RFR still occurs at *rep*-blocked forks (Figure 5 and Supplementary Table S6). The analysis of *holD*^{Q10am}, *priA* and *rep* mutants confirms that the action of RuvAB at blocked forks depends on the cause of replication inactivation.

Discussion

In this work, we show that, in addition to its central role in HJ branch migration, RuvAB participates in the formation of HJs at blocked replication forks. This finding reveals a new role for the RuvAB complex during chromosome dynamics, in the rescue of inactivated replication forks. Interestingly, RuvAB is not required in certain replication mutants for the conversion of blocked replication forks into HJs, which underlines that the processing of blocked replication is a complex reaction that depends on the cause of replication inactivation.

RFR has been proposed in *E. coli*, *Saccharomyces cerevisiae* and mammalian cells (Higgins *et al.*, 1976; Seigneur *et al.*, 1998; Sogo *et al.*, 2002; Subramanian and Griffith, 2005), but little is known on the mechanism of the reaction. RuvAB is the paradigm of HJ-binding enzymes and its functional homologue in eukaryotes remains to be clearly identified (Kaliraman *et al.*, 2001; Gaillard *et al.*, 2003; Osman *et al.*, 2003; Liu *et al.*, 2004). We used here the HJ-specific RusA resolvase to investigate the role of RuvAB in the formation of reversed forks because of the high specificity of RusA for four-way junctions (Bolt and Lloyd, 2002; Rafferty *et al.*, 2003). In two different Pol III mutants, the RusA resolvase does not act at blocked forks in the absence of RuvAB or RuvABC, which points to a need for RuvAB to convert a blocked fork into a HJ. Indeed, it is unlikely that forks could be reversed and protected from RusA in these mutants. RusA is able to resolve HJs made: (i) by homologous recombination, (ii) at *dnaBts*-blocked forks reversed by RecA and (iii) at *dnaNts*- and *priA*-blocked forks reversed by an unknown

activity. Furthermore, it resolves HJs in heterologous environments such as yeast or mammalian cells, where apparently none of the endogenous protein prevents its action (Doe *et al.*, 2000; Saintigny *et al.*, 2002). Consequently, we favor a model according to which RusA does not cleave forks in the absence of RuvAB because RuvAB itself catalyzes the conversion of replication forks into HJs.

Extensive biochemical and structural studies have shown that RuvA proteins form tetramers that bind HJ in preference to any other substrate (reviewed in West, 1997). Two tetramers assemble as an octamer that sandwiches a HJ (reviewed in West, 1998). RuvB forms hexamers that target double-strand DNA on each side of the RuvA octamer and pull on DNA strands, causing branch migration of the RuvA-bound HJ (Stasiak *et al.*, 1994). RuvA and RuvB also bind and unwind double-stranded Y-DNA molecules *in vitro*, although less efficiently than HJs (Lloyd and Sharples, 1993b; Hiom *et al.*, 1996; McGlynn and Lloyd, 2001). In contrast, RuvC is specific for HJs as it does not act at RuvAB-bound Y-structures (Benson and West, 1994). *In vitro*, the nature of the RuvA–RuvB complex formed on Y-molecules seems to depend on the experimental conditions. It was originally observed that incubation of Y-DNA molecules with RuvA and RuvB leads to the formation of a complex composed of one RuvA tetramer and only one RuvB hexamer bound to one of the three branches (Hiom *et al.*, 1996). Such RuvA–RuvB complexes could catalyze branch migration (George *et al.*, 2000). Accordingly, a RuvA mutant protein that has lost the capacity to form octamers binds HJ poorly, but still binds Y-DNA as efficiently as wild-type RuvA protein, again forming a complex with one RuvA tetramer and one RuvB hexamer per Y-molecule (Privezentzev *et al.*, 2005). In contrast, in a different study, two RuvB hexamers were proposed to bind to Y-molecules in the presence of RuvA (McGlynn and Lloyd, 2001). On a Y-molecule, the action of one RuvB hexamer bound to the template strand will promote RFR (Figure 6), whereas the action of two RuvB hexamers bound to the leading and lagging strand arms will unwind the template strands (McGlynn and Lloyd, 2001). Whatever the reasons for the reports of different complexes *in vitro*, the action of RuvAB at blocked forks *in vivo* is likely to be strongly

Figure 6 Model of action of RuvAB at blocked forks. In the first step, a RuvA tetramer binds to the fork and drives the assembly of a RuvB hexamer on the template strands. The translocase action of this RuvB hexamer pulls the leading and lagging strands into the RuvA complex (direction of migration of DNA is indicated by arrows) and results in the formation of a HJ. This HJ is bound by a second RuvB hexamer forming a *bona fide* branch migration complex (direction of translocation of DNA is indicated by arrows; it is unclear at present whether the active form of the branch migration complex *in vivo* carries one or, as drawn here, two tetramers of RuvA). The HJ is then resolved by RuvC resulting in a cleaved replication fork. For simplicity, RuvABC and DNA are shown alone, but the complexity of the replication fork factory suggests that additional proteins could play a role in this process.

influenced by the protein environment and our results indicate that this action leads to RFR. Consequently, our results are best accounted for by the model shown in Figure 6. We propose that conversion of replication forks to HJs can occur by the assembly of a tetramer of RuvA on a three-arm replication fork, followed by the binding of a single hexamer of RuvB to the template-strand arm. The helicase activity of the RuvB hexamer would convert the three-arm fork into a four-way structure to which a second RuvB hexamer and a second RuvA tetramer can bind. Next, in this *bona fide* branch migration complex, RuvC can resolve the junction. *In vivo*, the lagging-strand arm is likely to be single-stranded and covered by single-strand DNA-binding protein (SSB). In agreement, RuvA binds single-strand DNA more efficiently than double-strand DNA *in vitro*, and SSB can stimulate RuvAB activity (Shiba *et al*, 1991; Parsons *et al*, 1995). In support of the idea that SSB might be an important structural determinant of the fate of blocked forks, SSB strongly influences the helicase activity of several fork-binding proteins *in vitro* (Jones and Nakai, 1999; Cadman and McGlynn, 2004; Heller and Marians, 2005b).

Our results underline the existence of several pathways for RFR, which presumably depend on the structure of the fork and/or the nature of the replication proteins that remain bound after inactivation. RuvAB may reverse all types of blocked forks, and its action would not be detectable in the *dnaNts* and *priA* mutants because of the existence of another pathway of RFR. Alternatively, RuvAB may reverse forks only in *dnaEts* and *holD* mutants, either because it is targeted specifically to blocked forks in these mutants, or because it is prevented from binding to *dnaNts*- or *priA*-blocked forks. The action of RuvAB at *dnaEts*-blocked forks is independent of the presence of RecA or presynaptic recombination proteins (RecFOR, RecB), which are likely to be present in the vicinity of recombination intermediates during homologous recombination. RuvAB is therefore not targeted to forks by its homologous recombination partners. Replication in bacteria takes place at localized, highly ordered, multi-protein complexes called 'replication factories' (Lemon and Grossman, 1998; Lau *et al*, 2003; Molina and Skarstad, 2004); it is conceivable that RuvAB binding to blocked forks is controlled by interactions with a protein that belongs to the replication factory. To date, the precise constituents of replication factory and, in turn, the control of the accessibility of replication forks to different repair proteins are still largely unknown (Sherratt, 2003).

Although RuvAB is nearly ubiquitous in prokaryotes (Rocha *et al*, 2005), its functional homologue(s) in unicellular or pluricellular eukaryotes has not been clearly identified so far. Several eukaryote proteins have been shown to recognize HJs *in vitro*, and the role of these enzymes in homologous recombination is the subject of intense research (Kaliraman *et al*, 2001; Gaillard *et al*, 2003; Osman *et al*, 2003; Liu *et al*, 2004). We show here that in *E. coli* the enzyme that catalyzes branch migration of HJs is also required for their formation at certain blocked forks, in a reaction that is dependent on the cellular context. The dual function of the prokaryotic RuvAB complex in homologous recombination and replication fork processing strengthens the links between these two processes and may provide clues for understanding the roles of its functional homologues in other organisms.

Materials and methods

Strains and plasmids

The strain background is JJC40, which is a *hsdR* Thr⁺ Pro⁺ derivative of AB1157 (*leu-6 thi-1, his-4, lacY1, galK2, ara-14, xylS, mtl-1, tsx-33, rpsL31* and *supE44*). Most of the strains were constructed by P1 transduction. Antibiotics were used at the following concentrations: ampicillin 20 µg/ml for chromosome-carried genes and 50 µg/ml for plasmids, tetracycline 10 µg/ml, chloramphenicol 10 µg/ml, spectinomycin 60 µg/l, kanamycin 50 µg/ml and rifampicin 50 µg/ml. Details of strain construction and strains genotypes are described in Supplementary Table S1. All mutants were checked for thermosensitivity at 42°C, for UV sensitivity (suppression of the sensitivity of *ruvAB* and *ruvABC* mutants to UV irradiation ascertains the functionality of *RusA* in all backgrounds) and for exonuclease V activity (the exonuclease V action of RecBC is inactivated at all temperatures in the *recBCts* mutant and *exo V*-deficient cells allow the growth of T4 gpII mutant phages). The plating efficiency at 37°C was systematically measured (*recBCts* and *recB* mutation prevented growth at 37°C for all mutants except *priA* (Grompone *et al*, 2004b)). The mutator phenotype of *uvrD* mutants was tested by plating overnight cultures on rifampicin plates. In addition, the presence of insertional mutations was routinely checked by PCR amplification of the corresponding gene.

The low copy vector pGB2 carries the pSC101 replicon (Churchward *et al*, 1984). Its derivatives pGB-*ruvAB* (Seigneur *et al*, 1998) and pGB-*ruvABC* (Seigneur *et al*, 2000) have been described. *rep recBCts ruvABC rus-1* cells were constructed in the presence of the pGBts-*rep* plasmid (Michel *et al*, 2000). Plasmidless cells were isolated by propagation at 42°C in liquid medium for 2 h and then plating at 30°C. The *HolD*⁺ and *PriA*⁺ derivatives of the IPTG-dependent plasmid pAM34 (Gil and Bouche, 1991) have been described (Flores *et al*, 2001; Grompone *et al*, 2004b; Veaute *et al*, 2005) and were used for construction of *holD*^{Q10am} and *priA* mutants. Plasmidless cells were isolated by propagation in liquid medium devoid of IPTG for 2 h, followed by plating at 30°C on a medium devoid of IPTG. Plasmidless cells were always propagated on minimal medium. They formed smaller colonies than plasmid-containing cells and were checked for their sensitivity to UV irradiation and for their sensitivity to rich medium (*priA* mutants have a decreased plating efficiency on rich compared to minimum medium).

Growth curves

Overnight cultures grown at 30°C were diluted to OD 0.001 or 0.002 in Luria Broth medium containing additional thymine (LBT), grown at 30°C for 2 h and then shifted to 37°C. Aliquots were taken and dilutions were plated on LB every hour. Plates were incubated for 48 h at 30°C.

Measure of linear DNA by PFGE

Quantification of pulse-field gels was performed as described previously (Seigneur *et al*, 1998). Briefly, for chromosome labeling, cells were grown in minimal medium in the presence of tritiated thymidine and deoxyadenosine for 3 h at 30°C; then, part of the culture was shifted to 37 or 42°C for three more hours. Cells were collected, washed and embedded in agarose plugs. Gentle lysis was performed in plugs, which were then used for pulse-field gel electrophoresis (to avoid DNA damaging during PFGE, the apparatus was routinely washed with 0.1% SDS). The proportion of migrating DNA was determined by cutting each lane in slices and counting the tritium present in the wells and in the gel slices. Results were compared by a KHI2 test and were considered as (i) highly significantly different when $P < 0.001$ (values that differ by more than 10% were always found highly significantly different, as, for example, the levels of chromosome breakage in *dnaEts recBCts ruvABC rus-1* and *dnaEts recBCts* cells at 42°C), (ii) significantly different when $0.001 < P < 0.05$ (*rep recBCts* and *rep recBCts ruvABC rus-1* cells, which differ by 10%, were found significantly different) and (iii) not significantly different when $P > 0.05$ (values that differ by less than 10% were found not significantly different, as, for example, the lack of effect of *recG ruvABC rus-1* mutations in *dnaNts recA* (pBR-Gam)).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Dr David Leach, Dr Philippe Noirot and Dr Patrice Polard for very helpful reading of the manuscript. We also thank Nicolas

Sanchez for excellent technical assistance. This work was supported, in part, by the ACI 'Microbiologie 2003' and the ACI 'Biologie Cellulaire, Moléculaire et Structurale 2004' of the Ministère de la Recherche Française. BM is on the CNRS staff.

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