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RuvAB Acts at Arrested Replication Forks

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Summary

Replication arrest leads to the occurrence of DNA double-stranded breaks (DSB). We studied the mechanism of DSB formation by direct measure of the amount of in vivo linear DNA in *Escherichia coli* cells that lack the RecBCD recombination complex and by genetic means. The RuvABC proteins, which catalyze migration and cleavage of Holliday junctions, are responsible for the occurrence of DSBs at arrested replication forks. In cells proficient for RecBC, RuvAB is uncoupled from RuvC and DSBs may be prevented. This may be explained if a Holliday junction forms upon replication fork arrest, by annealing of the two nascent strands. RecBCD may act on the double-stranded tail prior to the cleavage of the RuvAB-bound junction by RuvC to rescue the blocked replication fork without breakage.

Introduction

DNA double-stranded breaks (DSBs) occur in all living organisms. They are of multiple origin and can be caused, for instance, by different chemical and physical agents. It was recently discovered that replication arrest induces DSBs in *Escherichia coli* (Michel et al., 1997). A similar process may take place in organisms other than bacteria, as can be inferred from studies in mutated cells, deficient in replication or in homologous recombination. In proliferating vertebrate cells, DSBs were proposed to form during replication and the recombination protein Rad51 plays an essential role in their repair (Sonoda et al., 1998). Replication-induced recombination events were also reported in yeast where certain replication mutants accumulate recombination intermediates (Zou and Rothstein, 1997). In the absence of recombinational repair, DSBs lead to cell death. However, the mechanism of formation of DSBs occurring upon replication blockage is unknown.

In the present work, we searched for the functions responsible for chromosomal breakage at stalled replication forks. We took advantage of the observation that *E. coli* mutants lacking a replicative helicase (Rep or DnaB) and the RecBCD recombination complex accumulate linear DNA (Michel et al., 1997). Mutations in the *rep* gene reduce the rate of movement of *E. coli* chromosomal replication forks, and hence presumably cause frequent replication pauses (Lane and Denhardt, 1975; Colasanti and Denhardt, 1987). The RecBCD complex is essential for recombinational repair of DSBs in *E. coli* (Wang and Smith, 1983; reviewed in Kowalczykowski et al., 1994; Myers and Stahl, 1994). Briefly, in vitro, this complex binds specifically to DNA double-stranded ends, then proceeds to unwind while simultaneously degrading the DNA (Taylor and Smith, 1985; Dixon and Kowalczykowski, 1993). Upon encountering a specific site named χ, the polarity of degradation is switched from 3′→5′ to 5′→3′ (Anderson and Kowalczykowski, 1997a). This leads to the production of a 3′ single-stranded DNA, which is bound by RecA and invades a homologous molecule (Anderson and Kowalczykowski, 1997b). Mutants deficient for the Rep replicative helicase and RecBCD are not viable because of the accumulation of DSBs that are triggered by the arrest of replication forks and are not repaired (Michel et al., 1997). We isolated here mutations that restore the viability of a *rep recB*TS *recC*TS strain at restrictive temperature and found that they also suppress the occurrence of DSBs. These mutations inactivate the *ruvAB* operon.

RuvA and RuvB proteins participate in the late steps of homologous recombination, in concert with a third protein named RuvC, as deduced from genetic analyses (Benson et al., 1988, 1991; Shinagawa et al., 1988). This was confirmed by biochemical analyses, since (1) RuvA is a DNA-binding protein specific for Holliday junctions, (2) RuvB is an ATP-dependent helicase that, in the presence of RuvA, catalyses branch migration of Holliday junctions, and (3) RuvC is an endonuclease specific for Holliday junctions, which introduces symmetrical strand cleavage across the point of strand exchange (Dunderdale et al., 1991; Iwasaki et al., 1991; Parsons et al., 1992; Tsaneva et al., 1992; Bennett and West, 1996). RuvA and RuvB form a complex composed of a tetramer of RuvA and two hexamers of RuvB (Stasiak et al., 1994; Rafferty et al., 1996; Yu et al., 1997). RuvC binds to the RuvAB-DNA complex in vitro (Whitby et al., 1996; Eggleston et al., 1997; Davies and West, 1998) and presumably acts in concert with RuvAB in vivo (Lloyd, 1991; Mandal et al., 1993).

To determine whether RuvABC is also responsible for chromosome breakage in other strains than *rep* mutants, we tested *dnaB*TS and wild-type strains. Inactivation of the main *E. coli* replicative helicase, DnaB, leads to a rapid arrest of DNA replication at high temperature (Wechsler and Gross, 1971) and to the accumulation of DSBs in the absence of the RecBCD complex (Michel et al., 1997). We observed that formation of these DSBs is suppressed by inactivation of RuvC or RuvAB proteins. Furthermore, for strains containing the replicative helicases, half of the spontaneous DSBs result from RuvABC action, which suggests that replication pauses occur in wild-type strains and cause RuvABC-mediated DSBs in the absence of RecBCD.

The discovery that RuvABC acts at blocked replication forks raises the question of its mode of action on

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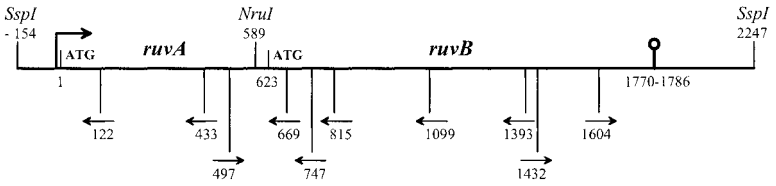

Figure 1. MudX Is Inserted in the *ruvAB* Operon in *rep recB*TS *recC*TS Thermoresistant Derivatives

Schematic representation of the *ruvAB* operon. The position of the *ruvAB* promoter is indicated by a bent arrow and that of the putative transcription terminator by a loop. The initiation codons of *ruvA* and *ruvB* are indicated (ATG). The vertical lines below show the position of the 10 MudX insertions that were determined by sequencing, the arrows pointing to the left end of Mu. The numbers indicate nucleotide positions relative to the A of the *ruvA* initiation codon, arbitrarily numbered 1. The SspI and NruI sites used for mapping by Southern hybridization are shown.

this new target. On the one hand, RuvABC may cleave replication forks directly, which would imply a new biochemical property for this complex since such a reaction was not reported so far. On the other hand, a Holliday junction, the specific target of RuvABC during homologous recombination in vivo and in vitro, may form upon replication arrest. These two alternatives were tested by genetic analyses and measures of DNA degradation in RecBC$^+$ cells. Our results are not compatible with a direct breakage of replication forks by RuvABC, whereas they are all explained if a Holliday junction forms upon replication arrest by annealing of the newly synthesized DNA strands. The formation of this Holliday junction creates a substrate for RecBCD. We propose that, in wild-type cells, the combined action of RuvAB and RecBCD mediates replication fork repair without actual breakage. This implies a new physiological role for RuvAB, distinct from its concerted action with RuvC on recombination intermediates, and a new role for RecBCD, in preventing rather than repairing DSBs.

Table 1. *ruvAB* Mutations Suppress the Thermosensitive Phenotype of *rep recB*TS *recC*TS Cells

Strain	Genotype	Cfu 42°/30°	N[a]
JJC 505	Δ*rep*::*kan recB*TS *recC*TS	5.1×10^{-6}	4
JJC 706	Δ*rep*::*kan recB*TS *recC*TS *ruvA*::Tn*10*	0.9	8
Mu insertions in *ruvA*[b]	Δ*rep*::*kan recB*TS *recC*TS *ruvA*::MudX	0.8	3
Mu insertions in *ruvB*[c]	Δ*rep*::*kan recB*TS *recC*TS *ruvB*::MudX	0.8	8
JJC 821	Δ*rep*::*kan recB*TS *recC*TS *ruvA*::Tn*10* [pGB-ruvAB]	8×10^{-6} [d]	5
JJC 820	Δ*rep*::*kan recB*TS *recC*TS Δ*ruvABC*::*cam*	0.9	5

Isolated colonies were grown in minimal medium at 30° to saturation (OD 0.8 to 1 in 24 to 30 hr). These cultures were plated on minimal medium and plates were incubated at 30° or 42° for 2 to 3 days.
[a] N: number of independent determinations.
[b] Average of the three *ruvA*::MudX insertions obtained by mutagenesis.
[c] Average of the eight *ruvB*::MudX insertions obtained by mutagenesis.
[d] In most of these clones, the *ruvAB* genes present on the plasmid were, for unknown reasons, inactivated.

Results

Inactivation of RuvAB Suppresses the Lethality of *rep recB*TS *recC*TS Strains

A strain that carries *rep recB*TS *recC*TS mutations does not grow at 42°C, due to the occurrence of chromosome DSBs at this temperature (Michel et al., 1997). However, the appearance of a low proportion of thermoresistant clones suggested that the strain may acquire suppressor mutations that allow viability by preventing breakage. In order to identify the genes involved in chromosome breakage, the strain was mutagenized with the transposable element MudX (Baker et al., 1983; see Experimental Procedures). Eleven independent thermoresistant clones were isolated. One was used to determine about 200 bp of sequence next to the MudX insertion (see Experimental Procedures), which showed that the *ruvB* gene was inactivated. Southern hybridization analysis with RuvAB and Mu left end probes showed that the eleven insertions were in the *ruvAB* genes. Primers located in *ruvAB* and at the left end of MudX allowed the amplification of PCR fragments of the size expected from the Southern hybridization mapping. Ten of the fragments were used to determine the sequence of the MudX-*ruv* junctions, allowing the exact localization of the transposon insertion sites (Figure 1).

E. coli ruvAB mutants are sensitive to UV. Irradiation at 20 J/m^2 led to 20% to 50% survival for the *rep recB*TS *recC*TS strain but to only 1% to 2% survival for the thermoresistant MudX derivatives, as expected for *ruvAB* mutants.

To determine whether inactivation of the *ruvAB* genes is sufficient to suppress the thermosensitive phenotype of *rep recB*TS *recC*TS strain, a *rep recB*TS *recC*TS *ruvA60*::Tn*10* strain was constructed by P1 transduction at 30°C. Its plating efficiencies at 30°C and 42°C were similar to those of the *ruvA*::MudX, *ruvB*::MudX strains (Table 1). Introduction of a plasmid carrying the *ruvAB* operon into the *rep recB*TS *recC*TS *ruvA60*::Tn*10* strain rendered it thermosensitive for growth (Table 1). These results show that the RuvAB complex is responsible for *rep recB*TS *recC*TS lethality.

Inactivation of *ruvAB* Prevents the Formation of Linear DNA in *rep recB*TS *recC*TS Mutants

The fragmentation of the bacterial chromosome can be detected by pulse field gel electrophoresis (PFGE), since only linear chromosomes enter pulse field gels, circular and σ-shaped molecules remaining in the wells (Birren and Lai, 1993; Michel et al., 1997). To determine the extent of chromosome breakage, cells were labeled with

Table 2. *ruvAB* Mutations Prevent the Formation of Linear DNA in *rep recB*TS *recC*TS Cells

Strain	Genotype	% of Linear DNA[a] 30°C	42°C	N
JJC 40	Wild Type	4.7 ± 0.4	4.4 ± 1.2	2/3
JJC 213	Δ*rep*::*kan*	2.4 ± 0.9	2.3 ± 0.9	2/3
JJC 330	*recB*TS *recC*TS	9.1 ± 3.4	19.1 ± 5.0	3
JJC 505	Δ*rep*::*kan recB*TS *recC*TS	15.3 ± 4.7	47.3 ± 4.5	3
JJC 706	Δ*rep*::*kan recB*TS *recC*TS *ruvA*::Tn*10*	4.8 ± 2.0	12.2 ± 1.2	3
JJC 821	Δ*rep*::*kan recB*TS *recC*TS *ruvA*::Tn*10* [pGB-ruvAB]	14.5 ± 1.6	49.5 ± 2.5	3
JJC 820	Δ*rep*::*kan recB*TS *recC*TS Δ*ruvABC*::*cam*	4.6 ± 0.9	8.7 ± 1.2	3

N, number of independent determinations at each temperature. JJC 40 and JJC 213 were tested twice at 30° and three times at 42°.
[a] Determined by PFGE analysis (see Experimental Procedures). In all strains, the linear DNA migrated as 3 to 5 megabase molecules.

tritiated thymidine and gently lysed in plugs, and their DNA was analyzed by PFGE. The proportion of DNA entering pulse field gels was measured (see Experimental Procedures). Each strain was cultured in parallel for 3 hr at 30°C and 42°C, where the enzyme encoded by the *recB*TS *recC*TS genes is partially active and inactive, respectively. As previously reported, the *rep recB*TS *recC*TS cells contain almost 50% of linear DNA after 3 hr at 42°C (Table 2, JJC505). In contrast, the *rep recB*TS *recC*TS *ruvA* cells contain only 12% of linear DNA at this temperature (Table 2, JJC706). This shows that the *ruvA* mutation prevents most of the chromosomal breakage. Introduction of a plasmid carrying the *ruvAB* operon restored the high levels of linear DNA at 42° (Table 2, JJC821), whereas the vector plasmid had no significant effect (not shown). The effect of the *ruvA* mutation was noticeable also at 30°, where *rep recB*TS *recC*TS cells accumulate some linear DNA (Table 2, compare JJC505, JJC706, and JJC821). As previously reported, a very low amount of linear DNA is present in the wild-type strain or the *rep* single mutant, in which broken DNA is either degraded or repaired by the RecBCD enzyme at both temperatures (Table 2, JJC40 and JJC213). In the *recB*TS *recC*TS mutant at 42°C, higher levels of linear DNA are observed, presumably because the RecBCD-mediated repair or degradation enzyme is prevented (Table 2, JJC330). Taken together, our results show that inactivation of RuvAB leads to the suppression of both the lethality and the formation of linear chromosomes in the *rep recB*TS *recC*TS strain. The RuvAB proteins are therefore required for the occurrence of DSBs at blocked replication forks in the *rep* mutant.

The RuvAB complex catalyses the branch migration of recombination intermediates (reviewed in West, 1997). Genetic and biochemical evidence indicates that it interacts with the RuvC endonuclease (Eggleston et al., 1997). Since RuvAB is devoid of detectable nuclease activity (Parsons et al., 1992), we hypothesized that the actual breakage was due to RuvC and tested whether the occurrence of breakage and the inviability of *rep recB*TS *recC*TS strains would be suppressed by inactivation of RuvC. Two *ruvC* null mutations were constructed, one deleted for the entire *ruvABC* region (Δ*ruvABC*::*cam*) and one deleted for *ruvC* only (Δ*ruvC*::*cam*). A Δ*rep*::*kan recB*TS *recC*TS Δ*ruvABC*::*cam* strain was constructed at 30°C. Viability and chromosome breakage were compared at 30°C and 42°C. Deletion of the entire *ruvABC* region from the chromosome resulted in the suppression of lethality (Table 1, JJC820) and in a strong decrease of DSBs (Table 2), further confirming that RuvAB proteins are required for DSB formation. In contrast, the Δ*rep*::*kan recB*TS *recC*TS Δ*ruvC*::*cam* mutant could not be constructed, indicating that the strain is not viable. This explains, in retrospect, why no MudX insertion was obtained in the *ruvC* gene. Interestingly, this result suggests that the absence of RuvC is lethal for *rep recB*TS *recC*TS cells only when RuvAB is present.

DSBs Occurring in the *dnaB*TS Strain Are Dependent on the RuvABC Proteins

Inactivation of the *E. coli* main replicative helicase, the DnaB protein, blocks the replication fork and leads to formation of chromosomal DSBs (Michel et al., 1997). When DSBs cannot be repaired, as in the strains that carry a *recB* mutation, linear DNA accumulates. This is shown by PFGE analysis for *dnaB*TS strains held at restrictive temperature (Table 3, JJC767 and JJC774). We examined the effect of *ruv* mutations on chromosomal breakage in a *dnaB*TS *recB* background. In contrast to the *ruv* proficient strain, the amount of linear DNA was low in the strains lacking either RuvC or the three RuvABC proteins at 42° (Table 3, JJC775 and JJC800; it should be noted that, for unknown reasons, the amount of linear DNA in *dnaB*TS *recB ruvC* strains is higher at 30°C than at 42°C). RuvC introduced on a plasmid restored the occurrence of DSBs in *dnaB*TS Δ*ruvC*::*cam recB* mutant (Table 3, JJC824), whereas the vector plasmid had no effect (not shown). This indicates that DSBs in a *dnaB* mutant are formed in the presence of the RuvC protein.

A plasmid encoding RuvC or two plasmids encoding RuvAB and RuvC were introduced in the *dnaB*TS Δ*ruvABC*::*cam recB*::Tn*10* mutant, and DSBs were analysed. The presence of the plasmid carrying the *ruvC* gene (DnaBTS RuvAB$^-$ RecB$^-$ cells) did not affect the amount of linear DNA significantly while the presence of the three RuvABC proteins restored a level of linear DNA similar to that of the *dnaB*TS *recB* strain (Table 3). Taken together, these results show that DSB formation in a *dnaB*TS *recB* strain is suppressed by the inactivation of either *ruvAB* or *ruvC* genes.

Table 3. *ruvAB* and *ruvC* Mutations Prevent the Formation of Linear DNA in *dnaB*TS *recB*

Strain	Genotype	% of Linear DNA[a] 30°C	42°C	N
JJC 767	*dnaB*TS	4.2 ± 1.0	12.6 ± 1.5	4
JJC 774	*danB*TS *recB*::Tn*10*	29.3 ± 4.3	66.7 ± 3.4	3
JJC 800	*dnaB*TS *recB*::Tn*10* Δ*ruvC*::*cam*	15.4 ± 1.9	8.6 ± 2.0	3
JJC 824	*dnaB*TS *recB*::Tn*10* Δ*ruvC*::*cam* [pBR-ruvC]	17.6 ± 0.1	50.0 ± 2.2	2
JJC 775	*dnaB*TS *recB*::Tn*10* Δ*ruv ABC*::*cam*	18.3 ± 4.2	10.0 ± 3.0	4
JJC 823	*dnaB*TS *recB*::Tn*10* Δ*ruvABC*::*cam* [pBR-ruvC]	12.0 ± 5.8	11.3 ± 5.6	2
JJC 822	*dnaB*TS *recB*::Tn*10* Δ*ruvABC*::*cam* [pGB-ruvAB] [pBRruvC]	26.4 ± 3.7	61.4 ± 5.8	3

N, number of independent determinations at each temperature.
[a] In all strains, the linear DNA migrated as 3 to 5 megabase molecules.

DSBs in *recB* Cells Proficient for Replicative Helicases Depend in Part on RuvABC

We observed that in the *dnaB*TS *recB* background, under conditions where DnaB is active (30°C), the inactivation of RuvABC or RuvC causes a reduction of the amount of linear DNA of about 2-fold (Table 3). This suggests that in strains proficient for the replicative helicases, an important fraction of the DSBs that occur in *recB* mutants may be dependent on RuvABC. We compared the level of DSBs in *recB*, *recB ruvC*, and *recB ruvABC* strains at 30°C and 42°C (Table 4). The total amount of linear DNA was somewhat higher at 42°C than at 30°C in all *recB* strains. At both temperatures the amount of linear DNA decreased about 2-fold upon inactivation of *ruvABC* or *ruvC*. This experiment shows that spontaneous chromosomal breakage is of dual origin, Ruv-dependent and Ruv-independent.

DNA Degradation in *recA* Mutants Is Not Affected by *rep* or *ruvA* Mutations

While the *dnaB*TS mutation is lethal at high temperatures regardless of the presence of recombination mutations,

Table 4. Part of the *recB*-Dependent Linear DNA Is *ruvABC* Dependent in Strains Proficient for Replicative Helicases

Strain	Genotype	% of Linear DNA[a] 30°C	42°C	N
JJC 40	Wild Type	4.7 ± 0.4	4.4 ± 1.2	2/3
JJC 315[b]	*recB*::Tn*10*	25.3 ± 6.4	39.2 ± 6.9	5
JJC 806	*recB*::Tn*10* Δ*ruvC*::*cam*	11.7 ± 0.8	15.0 ± 4.5	3
JJC 813	*recB*::Tn*10* Δ*ruvABC*::*cam*	14.9 ± 2.6	20.1 ± 1.2	3

N, number of independent determinations at each temperature. JJC 40 was tested twice at 30° and three times at 42°.
[a] In all strains, the linear DNA migrated as 3 to 5 megabase molecules.
[b] At 42°C, the amount of linear DNA was higher in the *recB* null mutant than in the *recB*TS *recC*TS strain JJC330 (Table 2), probably because of residual RecBCD activity in the TS mutant.

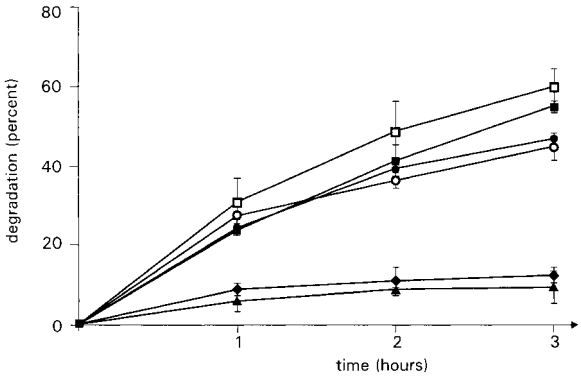

Figure 2. DNA Degradation in *recA* Strains Is Not Significantly Affected by *rep* or *ruvAB* Mutations

DNA degradation was determined as described in Experimental Procedures. Cells containing the plasmid pBRara-recA, carrying the *recA* gene under the control of the *araC* promoter were used. In these cells the *recA* gene is expressed in the presence of arabinose (RecA+) and repressed in the presence of glucose (*recA*). Results are the average of two or three experiments, standard deviations are shown. JJC744 arabinose (wild-type) (closed triangle); JJC742 arabinose (*rep*) (closed diamond); JJC744 glucose (*recA*) (closed circle); JJC742 glucose (*recA rep*) (closed square); JJC745 glucose (*recA ruvAB*) (open circle); and JJC743 glucose (*recA rep ruvAB*) (open square). DNA degradation was also measured in *recA* and *rep recA* strains cells with no plasmid; results were the same as in cells containing pBRara-recA grown in the presence of glucose (data not shown).

the *rep* mutation allows genetic analyses in different backgrounds, *rep* mutants were therefore used for further studies. DSBs require RecA and RecBCD for repair, regardless of their origin (Krasin and Hutchinson, 1977; Sargentini and Smith, 1986; Leach et al., 1997; reviewed in Lloyd and Low, 1996). Consequently, mutants that suffer DSBs require RecA and RecBCD for viability (*polA*, *dam*, *lig* mutants; reviewed in Kuzminov, 1995). In addition, in such strains the accumulation of linear molecules upon inactivation of RecBCD correlates with a high level of DNA degradation upon inactivation of RecA due to the action of exonuclease V on these linear molecules (Monk and Kinross, 1972; reviewed in Kuzminov, 1995). In contrast, the RecA protein is not essential in *rep* mutants (Uzest et al., 1995; Michel et al., 1997), which is at odds with the lethality of the *rep recBC* combination. Interestingly, the viability of the *rep recA* mutant relies on DNA degradation, since *rep recA recD* triple mutants are not viable (Uzest et al., 1995) and *recD* mutations abolish only the exonuclease V activity of RecBCD. We tested whether the linear molecules detected in the *rep recBC* conditions are degraded in *rep recA* cells (Figure 2). There was little DNA degradation in *rep* and wild-type strains. As previously reported, *recA* mutants suffered a high level of DNA degradation (Skarstad and Boye, 1993). Surprisingly, this DNA degradation was not significantly increased by the *rep* mutation. In addition, inactivation of RuvA did not modify the level of DNA degradation in these strains (Figure 2). These results show that, in contrast to DSBs formed in other RecBC-dependent mutants, those formed in *rep* mutants produce little or no DNA degradation in a *recA*

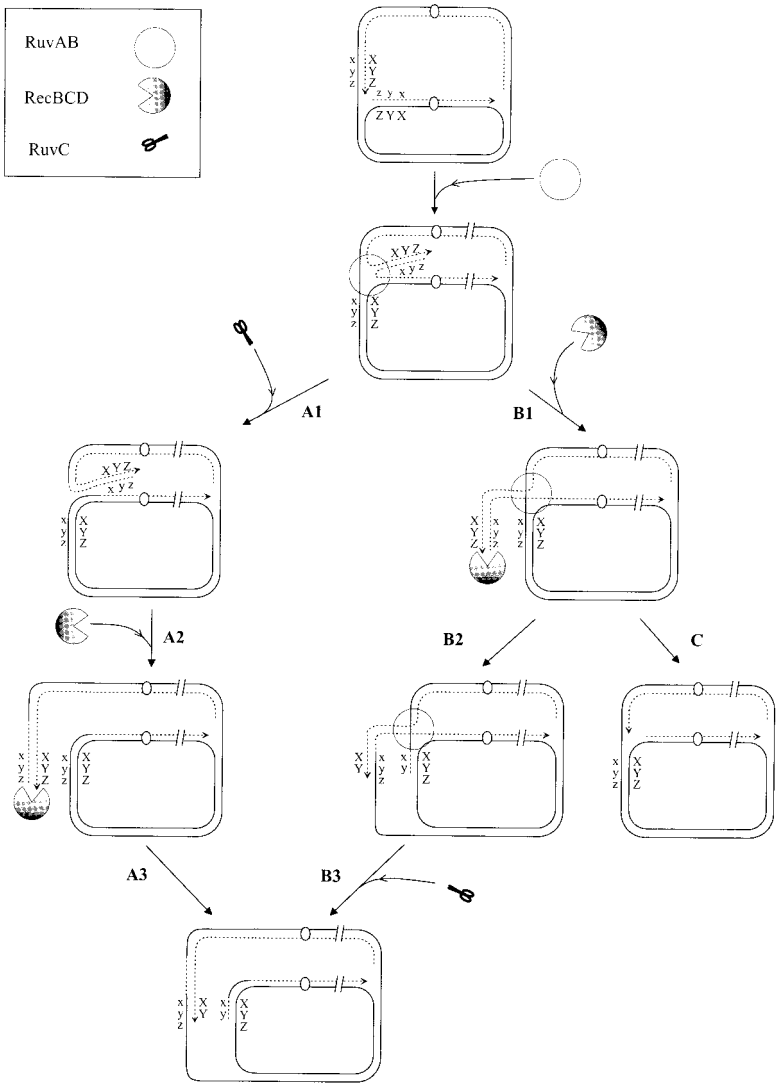

Figure 3. Model for RuvAB/RecBCD-Mediated Rescue of Blocked Replication Forks

Continuous and discontinuous lines represent the template and the newly synthesized strand of the chromosome, respectively. The arrow indicates the 3′ end of the growing strand. In the first step the replication fork is blocked and the two newly synthesized strands anneal, forming a Holliday junction that is stabilized by RuvAB binding.

Pathway A: (A1) RuvC resolves the RuvAB-bound junction. (A2) RecBCD binds to the double-stranded end. (A3) The double-stranded break is repaired by RecBCD/RecA-mediated homologous recombination. If the same strands are exchanged at both Holliday junctions, (patch type of event) a replication fork is reconstituted on a monomeric chromosome (shown here). Resolution using two strands at one junction and the two other strands at the other junction (splice type of event) leads to the reconstitution of a replication fork on a dimeric chromosome (not shown).

Pathway B: (B1) RecBCD binds to the double-stranded tail. (B2) Degradation has taken place up to the first CHI site (between locus yY and zZ) and is followed by a genetic exchange mediated by RecA (an exchange between the lagging strand and the leading strand template is shown). (B3) RuvC resolves the first Holliday junction bound by RuvAB. As in pathway A, the outcome, monomeric or dimeric chromosome, depends on the strands used for the two resolution reactions.

Pathway C: RecBCD-mediated degradation of the tail progresses up to the RuvAB-bound Holliday junction. Replication can restart when RecBCD has displaced the RuvAB complex.

background. Furthermore, this raises an apparent paradox since the viability of the *rep recA* strain actually relies on exonuclease V activity.

A Model for RuvABC-Dependent DSB Formation in *rep recBC* Mutants

We have shown here that the presence of the three proteins, RuvA, RuvB, and RuvC, is required for DSB formation at arrested replication forks in *recB* strains. Since, of these proteins, only RuvC possesses an endonuclease activity (Parsons et al., 1992), the RuvAB complex somehow allows the RuvC endonuclease to operate on arrested replication forks. However, three observations are not explained by a model in which the arrested replication forks would be simply broken by the RuvABC complex: (1) repair of a broken chromosome requires RecA, and the *rep recA* strain is viable; (2) DNA breakage leads to DNA degradation in the presence of exonuclease V, and such DNA degradation was not detected; (3) the action of RuvABC implies similar phenotypes for cells lacking any of these three proteins, and we found that inactivation of RuvAB rescues the viability of *rep recB*TS *recC*TS strain at 42°C, whereas inactivation of RuvC renders this strain inviable at any temperature. The later observation gives a clue on the mechanism of RuvABC-mediated DSB formation. The requirement for RecBCD in *rep ruvC* strains indicates that the RecBCD target, a double-stranded end, exists in the absence of RuvC. In contrast, the *rep ruvABC* strains is viable in the absence of RecBCD, which indicates that this double-stranded end requires RuvAB for its formation. The double-stranded end recognized by RecBCD in *rep* strains is therefore created by the RuvAB complex, which is devoid of endonuclease activity, and not by the RuvC endonuclease. To account for this observation, we propose that a Holliday junction is formed at arrested replication forks by annealing of the two newly synthesized strands (Figure 3). The junction may form spontaneously after disassembly of the replisome and be stabilized by RuvAB binding (Figure 3, first step). The resulting structure could be recognized by RuvC or by RecBCD (Figure 3A and 3B). Resolution by RuvC would release a chromosome arm that requires the combined action of RecBCD and RecA for repair (Figure 3A). In

the absence of recombinational repair, breakage of both replication forks would lead to the formation of linear molecules, accounting for the observed linearization of chromosomes in the *rep* and *dnaB* strains that lack RecBCD. The formation or the stabilization by RuvAB of a double-stranded tail may allow RecBCD to act prior to RuvC (Figure 3B). RecBCD possesses two activities that could promote the formation of a viable chromosome by processing of this double-stranded tail (Figure 3B and 3C). In a *recA*$^+$ strain, the encounter with a χ site would initiate recombination with the homologous region on the chromosome, restoring a replication fork (Figure 3B). In a *recA*$^-$ strains, the exonuclease V function of RecBCD would degrade the duplex DNA and remove RuvAB (Figure 3C). This accounts for the observation that although the *rep recA* strain requires a potent exonuclease to survive, it does not degrade its DNA significantly more than *recA* strains. In *rep recA* cells, degradation of only the double-stranded tail would be sufficient to restore a viable chromosome, and this tail is not expected to be more than a few kilobases. Formation of a Holliday junction accounts for the observation that in *rep* strains RecBCD is essential only in the presence of RuvAB, regardless of the presence of RuvC. Nevertheless, in cells lacking RecBCD, RuvC action on the Holliday junction leads to chromosome breakage (Figure 3A).

Exonuclease V Is Essential in *rep ruvC* Strains

The RuvC protein could not be inactivated in a *rep recB*TS *recC*TS strain at any temperature. We observed that in this strain, as in all strains carrying the two thermosensitive *recBC* mutations, the recombination activity of RecBC is not significantly affected at 30°C, while the exonuclease V activity is abolished. This suggests that the RecBCD function that is essential in *rep ruvC* strains is the exonuclease V activity. To confirm this observation, the well characterized *recD* mutation, which abolishes only exonuclease V activity of RecBCD, was used. We tested the viability of *rep ruvC recD* triple mutants. The *ruvC* mutation could not be introduced in a *rep recD* strain by P1 transduction, except in the presence of pGBTS-rep, a plasmid carrying the *rep* gene on a thermosensitive replicon (see Experimental Procedures). pGBTS-rep could not be eliminated by segregation at high temperature from the resulting strain, showing that the *rep recD ruvC* combination is lethal (data not shown). This indicates that RuvC and exonuclease V can replace each other to fulfill an essential role in *rep* strains. In contrast, the *rep recD ruvABC* strain was viable, as expected from the viability of JJC820 (*rep recB*TS *recC*TS *ruvABC*). Therefore, the lethality of the *rep ruvC recD* combination is due to the presence of RuvAB. We propose that the exonuclease V action of RecBCD may displace RuvAB from Holliday junctions in the absence of RuvC (Figure 3C).

ruvAB Mutations Restore the Viability of *rep recA recD* Mutants

rep mutants that lack RecA, like those that lack RuvC, rely on exonuclease V for viability (see above; Uzest et al., 1995). If RuvAB acts prior to RecA and RecD, a *ruvA* mutation should also restore the viability of the *rep recA recD* strain. To construct a *rep recA recD ruvA* strain, we made use of a plasmid carrying the *recA* gene under the control of the *araC* promoter, named here pGBara-recA (see Experimental Procedures; Boudsocq et al., 1997). In *recA* strains harboring this plasmid, the *recA* gene is expressed in the presence of arabinose and repressed in the presence of glucose. JJC748 (*rep recA recD* [pGBara-recA]) and JJC827 (*rep recA recD ruvA* [pGBara-recA]) were constructed. Strains were grown in arabinose-containing medium (RecA$^+$), and plating efficiencies were compared on plates containing arabinose or glucose (Table 5). The plating efficiency of *rep recA recD* (pGBara-recA) cells was greatly reduced on glucose (RecA$^-$), consistent with the observation that *rep recA recD* strains could not be constructed. In contrast, the *ruvA* derivative formed colonies on both media with equal efficiency (Table 5, JJC827). As expected, the plating efficiency of the *rep recA* and *rep recA ruvA* control strains was the same on both media (Table 5, JJC825 and JJC826). This experiment shows that inactivation of the *ruvAB* operon restores the viability of the *rep recA recD* mutant.

Table 5. *ruvAB* Mutations Suppress the Lethality of *rep recD recA* cells

Strain	Genotype	Cfu Glucose/ Arabinose	N
JJC 748	Δ*rep*::*kan* Δ*recA*::*cam* *recD*1013 [pGBara-recA]	4.9 10^{-4}	10
JJC 827	Δ*rep*::*kan* Δ*recA*::*cam* *recD*1013 *ruvA*::Tn*10* [pGBara-recA]	1.0	10
JJC 825	Δ*rep*::*kan* Δ*recA*::*cam* [pGBara-recA]	1.0	5
JJC 826	Δ*rep*::*kan* Δ*recA*::*cam* *ruvA*::TN*10* [pGBara-recA]	1.2	5

Isolated colonies were grown in LBT medium containing 0.2% arabinose at 37° to saturation, i.e., overnight for the *ruvA*$^+$ strains and up to 3 days for the *ruvA*$^-$ strains. These cultures were plated on rich medium containing either 0.2% arabinose or 1% glucose and plates were incubated at 37° for 24 hr to 3 days. N, number of independent determinations.

In conclusion, the model shown on Figure 3 implies that *rep* cells lacking RecA or RuvC (required for pathways A and B) rely on exonuclease V for viability (pathway 2C) and that this requirement is relieved by RuvAB inactivation. These predictions were all verified.

Discussion

In this work, we show that the occurrence of DSBs at arrested replication forks depends on the integrity of the RuvABC proteins since (1) mutations in the *ruvAB* operon reduce chromosome breakage in *rep recB*TS *recC*TS and in *dnaB*TS *recB* strains to the background level and (2) a *ruvC* null mutation also abolishes breakage in a *dnaBTS recB* strain. Furthermore, about half of the spontaneous DSBs occurring in a *recB* single mutant are also dependent on RuvABC, which suggests that replication pauses occur in wild-type cells and are acted upon by RuvABC. This leads us to propose a new role for the RuvAB proteins in vivo, distinct from their role

in the processing of homologous recombination intermediates. Importantly, our data indicate that in *recBC*$^+$ cells, the RuvAB complex may be uncoupled from RuvC and rather acts in concert with RecBCD to prevent breakage. The marked difference in the effects of *ruvAB* and *ruvC* mutations in *rep recD* strains, which is difficult to reconcile with a concomitant action of the RuvABC proteins on replication forks, is expected if the exonuclease V activity is required only in the presence of RuvAB and in the absence of RuvC, as diagramed in Figure 3. The viability of the *rep recA* strain and the absence of specific DNA degradation in this strain are difficult to reconcile with a requirement for recombinational repair or degradation of broken chromosomes in *rep* backgrounds. They are explained if DNA breakage is actually avoided in cells proficient for RecBCD, by the formation of a Holliday junction that allows the reconstitution of a replication fork with limited DNA degradation. Our results lead to a new concept, the concerted action of recombination proteins to rescue blocked replication forks.

Formation of a Holliday Junction upon Replication Arrest

Formation of a Holliday junction by annealing of the two newly synthesized strands was previously proposed to occur upon encounter of a replication fork with a DNA lesion in mammalian cells (Higgins et al., 1976). In this model, the leading strand polymerase is blocked by a lesion while lagging strand synthesis progresses further. Pairing of the nascent strands therefore allows DNA synthesis from the 3′ end of the leading strand, the lagging strand being used as a template. More than 20 years later, this strand switching model is still used to explain translesion synthesis in *E. coli* (Koffel-Schwartz et al., 1996), yeast (TorresRamos et al., 1997), or mammalian cells (CordeiroStone et al., 1997) and to account for recombination-dependent replication in vivo and in vitro (Formosa and Alberts, 1986; Zou and Rothstein, 1997).

Annealing of the newly synthesized strands at a blocked replication fork was also proposed to occur upon arrest of a helicase at a replication terminator (*Ter* site) of the *E. coli* chromosome (Louarn et al., 1991). This reaction may occur if the disassembly of replication proteins upon helicase arrest allows local melting of the two newly synthesized strands and their annealing. This may be stimulated by supercoiling constraints. Alternatively, transient binding of RuvAB to the arrested replication fork may facilitate formation of the Holliday junction. Once formed and bound by RuvAB, the junction could be either destroyed by branch migration toward the chromosome terminus or translated away from the site of helicase arrest by branch migration toward the replication origin. This generates a double-stranded tail, hence an entry point for the RecBCD enzyme (Figure 3). RecBCD, which travels on DNA at rates up to 1000 bp/s and therefore more rapidly than RuvAB (Roman and Kowalczykowski, 1989; Roman et al., 1992), may reach the RuvAB-DNA complex prior to encounter with a χ site and dissociate this complex. This would allow restoration of a replication fork without any breakage or recombination event. Alternatively, RecBCD might encounter a χ site and initiate homologous recombination (Anderson and Kowalczykowski, 1997a, 1997b). The distance between two χ sites is on the average 5 kb when RecBCD progresses on the *E. coli* chromosome toward the replication origin (Blattner et al., 1997) and the position of the first χ site encountered by RecBCD would be determined fortuitously by that of the replication arrest.

The Double Role of RuvAB in *E. coli*

In our model, the target of RuvC during replication is the same as during recombination, a RuvAB-bound Holliday junction. Extensive in vitro studies of the properties of the RuvABC proteins have led to the conclusion that they bind Holliday junctions in preference to any other DNA substrate (Lloyd and Sharples, 1993; Benson and West, 1994; reviewed in West, 1997). The view that Holliday junctions are the preferential target of RuvABC proteins is further supported by detailed structural analysis of these proteins (Ariyoshi et al., 1994; Rafferty et al., 1996; Yu et al., 1997; Hargreaves et al., 1998; reviewed in Rice et al., 1997). Therefore, it is not surprising that the structure cleaved by RuvC at a replication fork is a RuvAB-bound Holliday junction. However, during replication, RuvAB provides an entry point for RecBCD and thereby fulfills a new function in vivo that can be separated from its interaction with RuvC to resolve junctions. Interestingly, the *ruvA* and *ruvB* genes form an SOS-inducible operon, whereas RuvC is not under SOS control (Sharples and Lloyd, 1991; Takahagi et al., 1991). The reason for this difference in the control of the expression of proteins that act in concert on recombination intermediates is unclear. The specific action of RuvAB on blocked replication forks may provide an explanation. In the *rep* mutants that suffer from frequent replication pauses, SOS is slightly but significantly derepressed (Ossanna and Mount, 1989; our unpublished results). The higher expression of the *ruvAB* operon without concomitant increase in the level of RuvC protein might favor the rescue of blocked replication forks by the combined action of RuvAB and RecBCD, without breakage. Formation of a Holliday junction might prevent breakage of the naked replication fork by single-stranded endonucleases. If the two newly synthesized chromosomes are pulled apart during the process of replication (Scott Gordon et al., 1997), the occurrence of a DSB could lead to a release of the broken arm, rendering its repair difficult and possibly favoring chromosomal rearrangements.

RuvABC-Dependent Breakage and Genome Stability

E. coli strains that depend on RecBC for growth (*polA*, *lig*, and *dam* mutants) also require RecA (Monk and Kinross, 1972; Condra and Pauling, 1982; Wang and Smith, 1986). In all cases tested, these strains are also hyper-rec for recombination between two copies of the same gene placed several hundred kilobases apart on the *E. coli* chromosome (Zieg et al., 1978; reviewed in Kuzminov, 1995). The hyper-rec phenotype is attributed to the occurrence of DSBs that are sometimes repaired

Table 6. Strains and Plasmids

Strain	Relevant Genotype	Reference or Origin
CAG5050	*lacZ8305*::Mu *cts*62 MudX	Baker et al., 1983
PC8	*dnaB*8	B. Backman
SK129 (JJC330)	*recB270, recC*271 (AB1157 background)	S. Kushner
N2101	*recB*268::Tn*10*	R.G. Lloyd
GY9731	*RecA938*::*cam* (AB1157 background)	R. Devoret
GY9701	*RecA938*::*cam*, [miniF *recA*]	R. Devoret
FR559	*mutS*::Tn*5*	M. Radman
N2057	*ruvA*60::Tn*10*	R.G. Lloyd
JJC40	Wild type (AB1157, *hsdR*)	Laboratory stock
JJC213	Δ*rep*::*kan*	Uzest et al. (1995)
JJC273	*recD*::Tn*10*	Uzest et al. (1995)
JJC304	Δ*rep*::*kan recD 1013*	Uzest et al. (1995)
JJC315	*recB268*::Tn*10*	Michel et al. (1997)
JJC390	Δ*rep*::*kan recA938*::*cam*	Michel et al. (1997)
JJC494	Δ*rep*::*kan ruvA60*::Tn*10*	P1 JJC213 * N2057
JJC505	Δ*rep*::*kan recB270 recC271*	Michel et al. (1997)
JJC700	*recB270 recC271 ruvA60*::Tn*10*	P1 N2057 * SK129
JJC706	Δ*rep*::*kan recB270 recC271* Δ*ruvA60*::Tn*10*	P1 JJC213 * JJC700
JJC730	*recD*::Tn*10 mutS*::Tn5	P1 FR559 * JJC273
JJC742	Δ*rep*::*kan recA938*::*cam* [pBRara-recA]	JJC390 + [pBRara-recA]
JJC743	Δ*rep*::*kan recA938*::*cam ruvA60*::Tn*10* [pBRara-recA]	P1 N2057 * JJC742
JJC744	*recA938*::*cam* [pBRara-recA]	GY9731 + [pBRara-recA]
JJC745	*recA938*::*cam ruvA60*::Tn*10* [pBRara-recA]	P1 N2057 * JJC744
JJC746	Δ*rep*::*kan recD1013*, [pGBara-recA]	JJC304 + [pGBara-recA]
JJC748	Δ*rep*::*kan recD1013 recA938*::*cam* [pGBara-recA]	P1 GY9701 * JJC746
JJC753	*recB270 recC271* Δ*ruvABC*::*cam*	P1 Δ*ruvABC*::*cam* de JJC730 * SK129
JJC754	Δ*ruvABC*::*cam*	P1 Δ*ruvABC*::*cam* de JJC730 * JJC40
JJC767	*dnaB*TS	P1 PC8 * JJC40
JJC768	*dnaB*TS Δ*ruvABC*::*cam*	P1 JJC754 * JJC767
JJC774	*dnaB*TS *recB268*::Tn*10*	P1 JJC777 * JJC767
JJC775	*dnaB*TS Δ*ruvABC*::*cam recB268*::Tn*10*	P1 JJC777 * JJC768
JJC777	*recB268*::Tn*10* [pDWS2]	JJC315 + [pDWS2]
JJC783	Δ*ruvC*::*cam*	P1 [JJC730 Δ*ruvC*::*cam*] * JJC40
JJC784	*recB*270 *recC*271 Δ*ruvC*::*cam*	P1 [JJC730 Δ*ruvC*::*cam*] * JJC330
JJC785	*dnaB*TS Δ*ruvC*::*cam*	P1 [JJC730 Δ*ruvC*::*cam*] * JJC767
JJC800	*dnaB*TS Δ*ruvC*::*cam recB268*::Tn*10*	P1 JJC777 * JJC785
JJC802	Δ*rep*::*kan recD*::Tn*10*	P1 JJC273 * JJC213
JJC806	Δ*ruvC*::*cam recB268*::Tn*10*	P1 JJC777 * JJC783
JJC807	Δ*ruvC*::*cam recD*::Tn*10*	P1 JJC273 * JJC783
JJC811	Δ*rep*::*kan* Δ*ruvC*::*cam*	P1 JJC783 * JJC213
JJC812	Δ*rep*::*kan* Δ*ruvABC*::*cam*	P1 JJC754 * JJC213
JJC820	Δ*rep*::*kan recB270 recC*271 Δ*ruvABC*::*cam*	P1 JJC213 * JJC753
JJC821	Δ*rep*::*kan recB*270 *recC*271 *ruvA60*::Tn*10* [pGBruvAB]	JJC706 [pGBruvAB]
JJC822	*dnaB*TS Δ*ruvABC*::*cam recB268*::Tn*10* [pBRruvC] [pGBruvAB]	JJC775 [pBRruvC] [pGBruvAB]
JJC823	*dnaB*TS Δ*ruvABC*::*cam recB268*::Tn*10* [pBRruvC]	JJC775 [pBRruvC]
JJC824	*dnaB*TS Δ*ruvC*::*cam recB268*::Tn*10* [pBRruvC]	JJC800 [pBRruvC]
JJC825	Δ*rep*::*kan recA938*::*cam* [pGBara-recA]	P1 GY9701 * JJC828
JJC826	Δ*rep*::*kan recA938*::*cam ruvA60*::Tn*10* [pGBara-recA]	P1 GY9701 * JJC829
JJC827	Δ*rep*::*kan recA938*::*cam ruvA60*::Tn*10 recD*1013 [pGBara-recA]	P1 N2057 * JJC748
JJC828	Δ*rep*::*kan* [pGBara-recA]	JJC213 [pGBara-recA]
JJC829	Δ*rep*::*kan ruvA60*::Tn*10*[pGBara-recA]	JJC494 [pGBara-recA]
JJC830	*dnaB*TS Δ*ruvABC*::*cam recB268*::Tn*10* [pBR322]	JJC775 [pBR322]
JJC831	*dnaB*TS Δ*ruvABC*::*cam recB268*::Tn*10* [pBR322] [pGB2]	JJC775 [pBR322] [pGB2]
JJC832	*dnaB*TS Δ*ruvC*::*cam recB268*::Tn*10* [pBR322]	JJC800 [pBR322]

Plasmids	Description	Origin or Reference
pACYC184	Cloning vector	
pBR322	Cloning vector	
PBR-ruvABC	pBR322 carrying *ruvABC*	This work
PBR-ΔruvABC::*cam*	pBR322 carrying Δ*ruvABC*::*cam*	This work
PBR-ruvC	pBR322 carrying *ruvC*	This work
PBR-ΔruvC::*cam*	pBR322 carrying Δ*ruvC*::*cam*	This work
pGB2	Cloning vector	Churchward et al., 1984
pGB-ruvAB	pGB2 carrying *ruvAB*	This work
p30	pBR322 carrying *recA* under the *araC* promoter	Boudsocq et al., 1997
pGBara-recA	pGB2 carrying *recA* under the *araC* promoter	This work
pGBTS		Clerget et al., 1991
pDG148		Stragier et al., 1988
pDWS2	pBR322 carrying the thy^+ $recBCD^+$ arg^+ region of *E. coli*	G. Smith
pDGrep	pDG148 carrying *rep*	This work
PGBTS-rep	pGBTS carrying *rep*	This work
Phage M13mp2		J. Messing

erroneously by homologous recombination with the ectopic gene copy. In contrast, *rep* mutants are not hyperrec in the same test (Zieg et al., 1978). The absence of increased intrachromosomal recombination supports the idea that chromosomal breakage does not normally occur in *rep* mutants. To our knowledge, the only homologous recombination events stimulated in mutants that have a defective replicative helicase (*dnaB*TS or *rep*) are those that occur between adjacent tandem repeats (Bierne et al., 1997b; Saveson and Lovett, 1997). Part of these events are RecA-independent. However, some require RecA and may occur by recombination of the double-stranded tail with the homologous chromosome. Finally, DNA breakage promotes illegitimate recombination (Bierne et al., 1997a); the combined action of RuvAB and RecBCD may protect chromosomes against this type of rearrangement by preventing breakage.

Replication Restart

The slow growth of *rep ruvAB* cells suggests that replication restart is facilitated in the *rep* single mutants by processing of the arrested fork by RuvAB. An obvious reason could be that this processing leads to the release of the blocking element, for example by modification of the topological state of the DNA in this region. This processing may also lead to the formation of a structure on which the assembly of a replisome is facilitated.

When the blocked replication fork is transformed by RuvAB in a double-stranded tail processed by recombination proteins (Figure 3B), replication restarts from a recombination intermediate. Recombination-dependent replication was originally proposed in T4 bacteriophage (reviewed in Mosig et al., 1995) and more recently in the *E. coli* chromosome (reviewed in Kogoma, 1997) and in yeast (Malkova et al., 1996). In *E. coli*, the key element for replication restart is the PriA protein. PriA initiates the assembly of a multiprotein complex, the primosome, that will allow the loading of the DnaB helicase and replication restart (Asai et al., 1994; Masai et al., 1994; Jones and Nakai, 1997). We were unable to construct a *rep priA* strain (our unpublished data), which supports the notion that PriA is required for recombination-induced replication. Importantly, the viability of *rep recA* strains suggests that a replisome can reassemble on an arrested replication fork independently of recombination events (Figure 3C). Consequently, the inviability of the *rep priA* double mutant suggests that PriA is also required for replication restart from a Y structure. This is in agreement with a proposed role for PriA and other primosome proteins in the assembly of replication forks (Jones and Nakai, 1997).

Arrested Replication Forks in Wild-Type Strains

Based on genetic and biochemical studies, Rep was proposed to facilitate chromosomal replication by removing proteins from the path of replication forks (Yancey-Wrona and Matson, 1992; Matson et al., 1994). The *rep* mutations might amplify a phenomenon that occurs in wild-type growing cells. We found a high amount of linear DNA in *recB* single mutants. About half of these DSBs were not found in the absence of RuvAB or RuvC suggesting that they result from replication blockage. DNA damage of exogenous origin could account for the remaining DSBs that are RuvABC-independent (Wang and Smith, 1983; Imlay and Linn, 1988). Paradoxically, we propose here that RuvABC-dependent chromosome breakage in *recB* mutants reflects the formation of a Holliday junction that can be processed without breakage in the presence of RecBCD. Our results suggest that such a rescue of arrested replication forks by recombination proteins is not a rare event, particularly in rapidly growing cells. Consequently, the physiological role of recombination-dependent replication (reviewed in Kogoma, 1997) in exponentially growing cells may be the restart of stalled replication forks. RuvAB would be the missing link between replication blockage and replication restart from recombination intermediates.

It was recently reported that Holliday junction recombination intermediates accumulate spontaneously during DNA replication in mitotically growing yeast and that specific replication defects led to an increase in Holliday junctions (Zou and Rothstein, 1997). In vertebrate cells, the absence of the Rad51 recombination protein induces isochromatid breaks that are likely to occur during DNA replication (Sonoda et al., 1998). These observations may suggest that processes similar to those analyzed here in bacteria also take place in higher organisms. In eucaryotes, a block in DNA replication or DNA damage induces the activation of checkpoint systems that will ensure that replication is completed before mitosis. There is a large overlap between checkpoint proteins that act in response to stalled replication forks and those that respond to DSB (reviewed in Bentley and Carr, 1997). Transformation of a blocked replication fork into a Holliday junction results in the creation of a double-stranded end, hence of the same signal as DSBs, without actual breakage.

In conclusion, we propose that (1) replication arrests occur in rapidly growing cells, (2) blocked forks are processed by homologous recombination enzymes with RuvAB acting before RecBCD, and (3) RuvAB forms or binds to a four-way structure formed by annealing of the newly synthesized strands. Further work should answer some of the questions raised by this general scheme, concerning the parameters that favor annealing of the newly synthesized strands, the way this process helps to dislodge the replication blocking structure, and which are the proteins involved in replication restart from different structures.

Experimental Procedures

Strains and Plasmids

The *E. coli* strains are listed in Table 6. All strains constructed during this work were made by P1 transduction. P1 stocks were prepared and transductions were performed as described (Miller, 1992). Antibiotics were used as described (Michel et al., 1997). The *rep* phenotype, the UV sensitive phenotype of *recA*, *recB*, *recC*, and *ruv* mutants and the exo$^-$ phenotype of *recB*, *recC*, and *recD* mutants were verified as described (Michel et al., 1997).

For all gene cloning, the fragments containing the genes of interest were obtained by long accurate PCR using the *E. coli* chromosome as a template (LAPCR, Barnes, 1994). Δ*ruvABC*::*cam*, Δ*ruvC*::*cam* deletions-insertions were first constructed on pBR322 derivatives. For Δ*ruvABC*::*cam*, a PCR fragment carrying these three genes was made from *E. coli* chromosome using 5′TAGGGATCCTGGCGCACA

GTGCCC3′ (creating a BamHI site) and 5′CGGTGGCGAAAGCTTAC GATATGG3′ (creating a HindIII site) as primers and cloned in the BamHI-HindIII sites of pBR322, leading to the plasmid pBR-ruvABC (for unknown reasons, *ruvAB* was inactive in this construct). The region of the *ruvABC* genes between two BssHII sites was replaced by the BstBI-XmnI fragment carrying the Cam^R gene of pACYC184. This eliminates the last 270 bp of *ruvC*, the orf23 gene, the *ruvA* gene, and the first 220 bp of *ruvB*. For Δ*ruvC*::*cam*, the *ruvAB* genes and orf23 were excised from the pBR-ruvABC plasmid with AseI (in orf23) and BamHI, producing the pBR-ruvC plasmid. Replacing the BssHII-NdeI (in *ruvC*) restriction fragment by the BstBI-XmnI fragment carrying the Cam^R gene of pACYC184 inactivated the *ruvC* gene. Strains carrying these inactivated genes were constructed by gene replacement. The plasmids containing the inactivated genes were cut with NdeI and PstI and used to transform a *mutS*::Tn*5 recD*::Tn*10* strain (JJC730) selecting for Cam^R. The structure of the chromosomal region and the phenotypes of the strains were verified. The inactivated genes were finally P1 transduced into a wild-type strain.

Five plasmids were constructed for complementation experiments. pBR-ruvC was made as described above. It carries an active *ruvC* gene since it restores the UV^R of *ruvC* mutants. pGB-ruvAB was constructed by cloning the *ruvAB* genes on the pGB2 cloning vector (Churchward et al., 1984). A PCR fragment carrying the *ruvAB* operon was made from the *E. coli* chromosome using 5′TCTGGATC CTTCGCTGGATATCTATC3′ (creating a BamHI site) and 5′TGCTACT GCGCGAAGCTTTGCC3′ (creating a HindIII site) as primers and cloned in the BamHI- HindIII sites of pGB2. The *ruvA* and *ruvB* genes were both active in this plasmid, as they restore the UV^R of *ruvA* and *ruvB* mutants. To construct pGBara-recA, the EcoO109I-NdeI fragment of p30 (Boudsocq et al., 1997), containing the *recA* gene under the control of the *araC* promoter, was cloned in the SmaI site of pGB2. To construct pBRara-recA, the Km^R gene inserted in the Ap^R gene in p30 was removed by Pst1 digestion. The activity and the arabinose dependence of the *recA* gene in these construct was checked: the plasmids restored the UV^R of a *recA* mutant in the presence of 0.2% arabinose and did not modify the UV^S of a *recA* mutant in the presence of 1% glucose. pDG-rep was constructed by cloning the *rep* gene on pDG148. A PCR fragment carrying the *rep* gene was made from the *E. coli* chromosome using 5′GATTGAG CAATACACATATGCGTC3′ and 5′TAAGTGCCGGATCCGATGCTGA CG3′ (creating a BamHI site) as primers and cloned in the SalI-BamHI sites of pDG148. The AvaI-BamHI fragment of pDG-rep containing the *rep* gene was cloned in the EcoRI-BamHI sites of pGB2TS to form pGBTS-rep. This plasmid is thermosensitive, as checked by its inability to be propagated at 42°C and carries an active *rep* gene, as judged by its ability to allow M13 propagation in *rep* mutants.

To test the viability of *rep recD ruvC* and *rep recB*TS *recC*TS *ruvC* strains, these mutants were constructed in the presence of pGBTS-rep. The P1 transductions were made at 30°C where the chromosomal *rep* deletion is complemented by the plasmid *rep* gene. The multiple mutants were propagated at 42°C in order to eliminate pGBTS-rep, and cells were plated at 30°C (*recB*TS *recC*TS derivative) or 37°C (*recD* derivative) on LBAT or minimal medium. When the *rep* gene was essential in the mutants, the plasmid could not be segregated and no Spc^S colony was obtained. *rep ruvA, rep ruvC, rep recB*TS *recC*TS*, rep recB*TS *recC*TS *ruvABC*, and *rep recD ruvABC* strains containing pGBTS-rep were used as controls and segregated Spc^S colonies in these experiments.

MudX Mutagenesis and Mutants Characterization

Preparation of MudX stock and MudX mutagenesis was performed as described (Baker et al., 1983). To mutate JJC505, cells were grown in LBT to OD 1 to 1.2. Aliquots of 10 ml were incubated with 2 ml of a fresh MudX stock at 30°C without agitation for 15 min and at 37°C with agitation for 30 min. Cells were plated on LBAT (Luria broth-agar-thymine) containing Cam. Microcolonies appeared in 4 to 5 days and could be streaked out on minimal medium plates containing Cam at 42°C in 3 days. For unknown reasons, no MudX mutant was obtained when the selection was performed directly on minimal medium.

The chromosome of one MudX mutant was cut with NlaIII and the restricted DNA was treated with DNA ligase and subjected to PCR amplification using the following primers: 5′CCCGAATAATCCA ATGTCC3′ and 5′GCTTGCAAGCCTGTAGTGCAAA3′ and Taq polymerase. These primers hybridize to each strands of the left end of MudX. The same primers were used to sequence the ligation product to determine the localization of the Mu insertion. PCR products used for sequencing were prepared as described in Sorokin et al. (1996). For Southern hybridization analysis, all mutant chromosomes were cut with SspI (located before *ruvA* and after *ruvB*) and NruI (located at the end of *ruvA*), see Figure 1. They were analyzed with *ruvAB* and Mu left end probes. Amplification of the MudX-Ruv junction of all mutants was performed with primers 5′CGGCATAAGCTGATTTG TG3′, 5′TCTGGATCCTTCGCTGGATATCTATC3′, and 5′TGCTACT GCGCGAAGCTTTGCC3′ that hybridize respectively to the left end of Mu, 60 bp before the beginning of RuvA, and 320 bp after the end of RuvB. The junction was sequenced from the PCR fragment using the primer 5′CGGCATAAGCTGATTTGTG3′ that hybridizes with Mu. PCR sequencing was performed with the use of Applied Biosystems PRISM dye terminators sequencing kit on the Perkin Elmer 9600 thermal cycler and analyzed in Applied Biosystems 373 DNA sequencer. Oligonucleotides were synthesized with a Beckman DNA synthesizer Oligo 1000.

Preparation of Plugs and PFGE Migration

Plugs were prepared as described (Michel et al., 1997). 1% agarose (SeaKem GTG agarose) gels were run for 48 hr at 3 V/cm (37.5 V) in 1% TAE buffer with a switch time of 500 s in a Chef DRIII apparatus. These migration conditions differ from those used in previous work (Michel et al., 1997). The resulting slight difference in the proportion of DNA that enter the gels is probably due to a higher proportion of forked DNA entering CHEF compared to FIGE gels. After migration, gels were stained with ethidium bromide and photographed. Plugs containing the chromosomes of *Saccharomyces cerevisiae*, *Hansenula wingei*, and *Schizosaccharomyces pombe* were routinely used as molecular standards (Bio-Rad). The lanes were cut into 3 mm slices using a razor blades slicer. A total of 26 slices representing 8.5 cm of migration were usually made to allow the recovery of all fragments from the migration origin to 100–200 kb fragments. The amount of tritium in the slices corresponding to the smaller fragments was generally low or null. Determination of the proportion of linear DNA by measure of total DNA and DNA in the sliced gel was as described previously (Michel et al., 1997).

DNA Degradation

As the *rep ruvA recA* strain could only be constructed by P1 transduction of *ruv* in a *rep recA* strain carrying an inducible *recA* gene, DNA degradation was measured in cells containing the plasmid pBRara-recA, carrying the *recA* gene under the control of the *araC* promoter (Boudsocq et al., 1997; see above). Degradation was measured in LB containing either 1% glucose (*recA* conditions) or 0.2% arabinose (RecA$^+$ conditions). The level of DNA breakdown was measured by labeling the DNA with [^{3}H]thymidine (NEN, England) for three generations, removing the label by several washings, and reincubating the cells in warm medium supplemented with unlabeled thymidine (40 μg/ml). Samples were withdrawn after 1, 2, and 3 hr. The amount of [^{3}H]thymidine released in the medium was determined after filtration through 0.2 μm millipore filters and scintillation counting of the filtered medium. The amount of acid-insoluble counts remaining in the cells was determined as described (Skarstad and Boye, 1993). The percentage of DNA degradation was deduced from the ratio of [^{3}H]thymidine present in the filtered medium versus total [^{3}H]thymidine. DNA degradation was also measured in *recA* and *rep recA* strains cells with no plasmid; results were similar in the cells devoid of plasmid and in cells carrying pBRara-recA grown in the presence of glucose (data not shown).

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