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The *E.coli* RuvAB proteins branch migrate Holliday junctions through heterologous DNA sequences in a reaction facilitated by SSB

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During genetic recombination a heteroduplex joint is formed between two homologous DNA molecules. The heteroduplex joint plays an important role in recombination since it accommodates sequence heterogeneities (mismatches, insertions or deletions) that lead to genetic variation. Two *Escherichia coli* proteins, RuvA and RuvB, promote the formation of heteroduplex DNA by catalysing the branch migration of crossovers, or Holliday junctions, which link recombining chromosomes. We show that RuvA and RuvB can promote branch migration through 1800 bp of heterologous DNA, in a reaction facilitated by the presence of *E.coli* single-stranded DNA binding (SSB) protein. Reaction intermediates, containing unpaired heteroduplex regions bound by SSB, were directly visualized by electron microscopy. In the absence of SSB, or when SSB was replaced by a single-strand binding protein from bacteriophage T4 (gene 32 protein), only limited heterologous branch migration was observed. These results show that the RuvAB proteins, which are induced as part of the SOS response to DNA damage, allow genetic recombination and the recombinational repair of DNA to occur in the presence of extensive lengths of heterology.

Keywords: *E.coli*/genetic recombination/Holliday junctions/RuvAB/SOS response

Introduction

Classical genetic studies indicate that the formation and processing of heteroduplex joints during genetic recombination leads to gene conversions and aberrant segregation events during meiosis. The formation of heteroduplex DNA requires enzymes that can catalyse the exchange of DNA strands between two recombining DNA strands. In bacteria this role is carried out by the RecA protein which promotes homologous pairing and strand exchange (DasGupta *et al.*, 1980; Cox and Lehman, 1981; West *et al.*, 1981). During heteroduplex formation, the two interacting DNA molecules are likely to contain sequence differences such as single base changes, frameshifts and even large insertions or deletions. The inclusion of such changes into heteroduplex DNA is a central aspect of the process of genetic recombination which leads to genetic variation. The ability of RecA to promote the exchange of strands between duplex DNA molecules that contain heterologies is limited to tens of base pairs *in vitro* (Hahn *et al.*, 1988; Morel *et al.*, 1994). Evidence exists, however, showing that recombination can lead to the inclusion of significant lengths of heterologous DNA (Lichten and Fox, 1984), indicating that other proteins are necessary for the bypass of large insertions and deletions.

In vitro studies show that the RuvA and RuvB proteins act upon Holliday junctions made by RecA and promote branch migration leading to the extension of heteroduplex DNA (Shiba *et al.*, 1991; Tsaneva *et al.*, 1992a,b; Müller *et al.*, 1993a). The RuvA protein interacts directly with RuvB and facilitates the binding of RuvB to DNA (Shiba *et al.*, 1991; Müller *et al.*, 1993b; Mitchell and West, 1994). Since RuvA binds specifically to Holliday junctions, it is thought to act as a specificity factor that targets RuvB to the junction (Iwasaki *et al.*, 1992; Parsons *et al.*, 1992; Parsons and West, 1993). Once targeted, the RuvB ATPase, a hexameric ring protein that exhibits DNA helicase activity, provides the motor that drives branch migration (Iwasaki *et al.*, 1989; Tsaneva *et al.*, 1993; Mitchell and West, 1994; Stasiak *et al.*, 1994; Tsaneva and West, 1994; Adams and West, 1995). Electron microscopic observations and DNase I protection studies indicate that RuvAB form a tripartite complex on the Holliday junction in which RuvA binds the crossover and is sandwiched between two rings of RuvB (Hiom and West, 1995; Parsons *et al.*, 1995). The two RuvB rings are thought to promote branch migration by a reaction that involves transient strand separation (Tsaneva *et al.*, 1993; Adams and West, 1995; Parsons *et al.*, 1995). In addition to their role in branch migration, the RuvAB proteins are able to dissociate RecA from duplex DNA and may play an auxiliary role in the recycling of RecA protein (Adams *et al.*, 1994).

Since RuvAB do not promote the pairing of two DNA molecules, it is likely that they act after RecA has initiated homologous pairing and strand exchange. Their role in recombination and the recombinational repair of DNA therefore appears to be in the extension of heteroduplex DNA. By catalysing branch migration, RuvAB may play an important role in the bypass of DNA lesions and sequence heterologies. The proteins have been shown to catalyse branch migration through irradiated DNA containing many DNA photoproducts (Tsaneva *et al.*, 1992b). Moreover, the combined action of RecA, RuvA, RuvB and SSB has been shown to lead to the bypass of heterologous inserts in reactions involving three strands of DNA (Iype *et al.*, 1994).

The fate of heteroduplex DNA also appears to play an important role in speciation since analyses of interspecies matings between *Escherichia coli* and *Salmonella typhimurium* indicate that species barriers are primarily recombinational in origin (Rayssiguier *et al.*, 1989; Matic

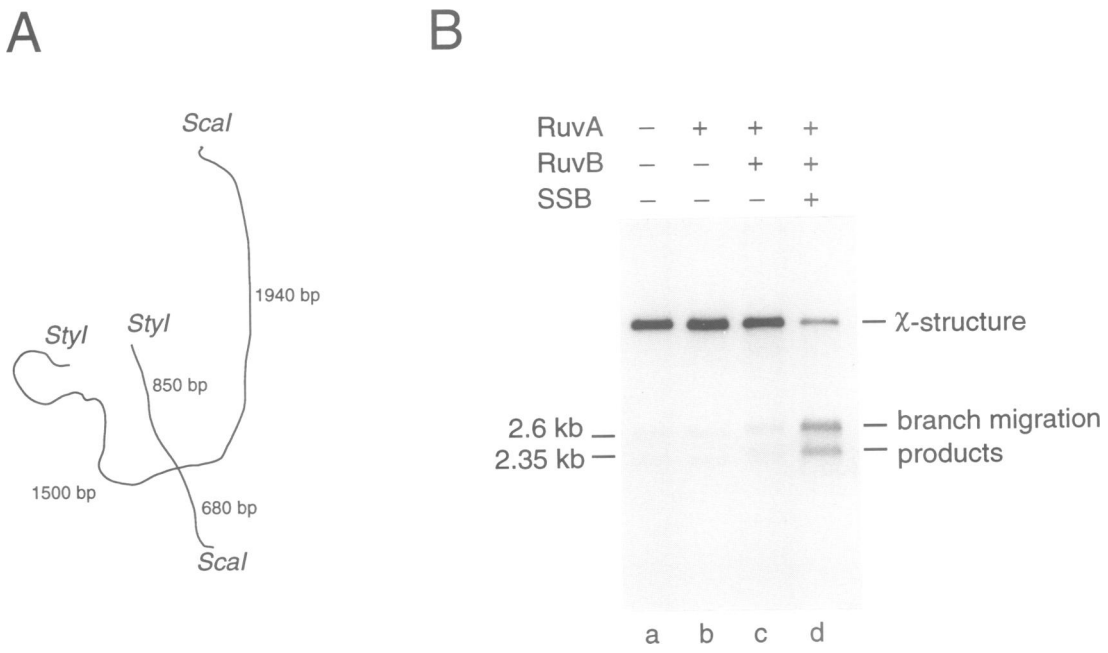

Fig. 1. RuvA and RuvB, in the presence of SSB, promote branch migration through heterologous DNA sequences. (**A**) Schematic drawing of the χ-structure indicating the approximate lengths of each of the four heterologous DNA arms. Not indicated is the region of homology (290 bp) which contains the Holliday junction (McCulloch *et al.*, 1994). The arm lengths noted on the diagram assume that the Holliday junction is located at the centre of the region of homology. (**B**) Branch migration reactions were carried out as described in Materials and methods, and contained *E.coli* RuvA, RuvB and SSB proteins as indicated. Substrates (^{32}P-labelled χ-structure) and products (^{32}P-labelled duplex DNA with single-stranded tails) were analysed by agarose gel electrophoresis followed by autoradiography. The 2.6 and 2.35 kb linear markers in lane a are by-products of the χ-structure preparation.

et al., 1995). Specifically, these studies showed that the mismatch repair system, which recognizes mispaired bases, acts as a potent inhibitor of interspecies recombination; whereas the SOS system acts as an inducible positive regulator by increasing the efficiency by which heteroduplex DNA is formed (Matic *et al.*, 1994). Induction of the SOS response leads to overproduction of several recombination proteins including RecA, RuvA and RuvB (Little and Mount, 1982; Shurvinton and Lloyd, 1982; Benson *et al.*, 1988; Shinagawa *et al.*, 1988), three proteins that play direct roles in the formation of heteroduplex DNA.

In this paper we use a well-defined biochemical system to show that RuvAB-promoted branch migration can traverse 1800 bp of heterologous DNA. The branch migration reaction occurs between two duplexes, and leads to the formation of extensive lengths of unpaired heteroduplex. The reaction is stimulated by the *E.coli* SSB protein, providing a further example of the multiple roles that this protein plays in genetic recombination and recombinational repair. These results provide biochemical support for genetic data which indicate a role for the SOS-inducible RuvA and RuvB proteins in the formation of heteroduplex DNA containing deviations from homology.

Results

The χ-structure as a model system for branch migration through heterology

In previous studies, RuvAB-mediated branch migration was demonstrated using a variety of DNA substrates. Two types of reactions have proven particularly informative: (i) fully homologous recombination intermediates made by RecA-mediated strand exchange were used to show that the combined action of RuvA and RuvB leads to their dissociation by ATP-dependent branch migration (Tsaneva *et al.*, 1992a,b; Müller *et al.*, 1993a); and (ii) small synthetic junctions produced by annealing short oligonucleotides were used to demonstrate specific binding of the Holliday junction by RuvA and RuvAB (Iwasaki *et al.*, 1992; Parsons *et al.*, 1992; Parsons and West, 1993). Moreover, RuvAB were shown to promote the ATP-dependent dissociation of small synthetic Holliday junctions containing four heterologous arms of ~25 bp in length. These results provided early evidence that RuvAB could promote branch migration through heterologous regions of DNA.

To determine whether RuvAB could promote branch migration through more extensive regions of heterology, a DNA substrate was constructed in which a Holliday junction, confined to a region of 290 bp of homology (McCulloch *et al.*, 1994), was flanked by heterologous arms with lengths of 536, 702, 1356 and 1794 bp (Figures 1A and 2). This χ-structure was recently used to demonstrate the specificity and structure of a RuvAB–Holliday junction complex (Parsons *et al.*, 1995). The χ-structures used in the following experiments were ~90% pure, although there was a small fraction of contaminating linear 2.6 and 2.35 kb species, by-products of their preparation (see Materials and methods and Figure 1B, lane a). To facilitate product analysis, the χ-structures were 3′-^{32}P-end labelled at the two *Sty*I termini (Figure 2).

Dissociation of χ-structures by RuvAB-mediated branch migration

When ^{32}P-end labelled χ-structures were incubated with RuvA, RuvB and SSB proteins, ~70% were dissociated

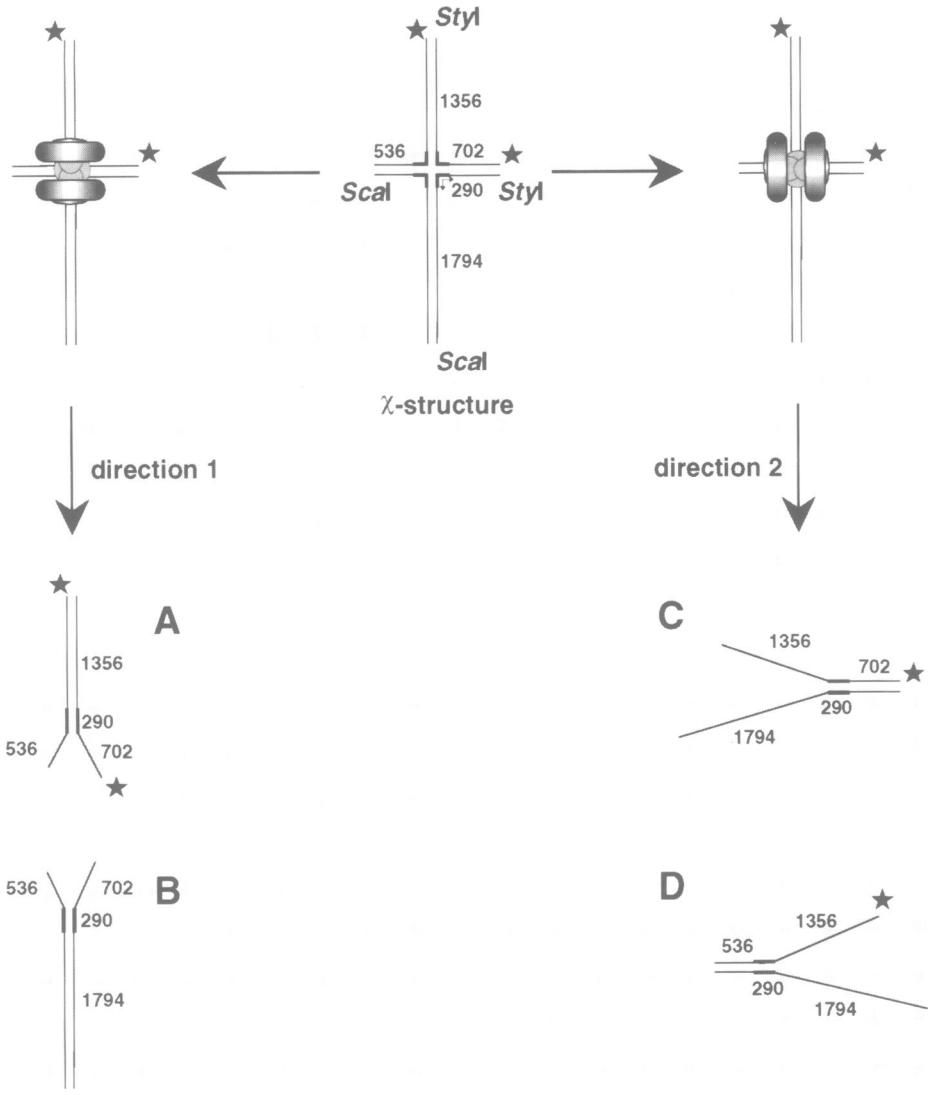

Fig. 2. Scheme indicating the expected products of bidirectional branch migration. The χ-structure was 3′-^{32}P-end labelled at the two *Sty*I sites as indicated by stars. Dissociation by branch migration in direction 1 leads to the formation of two flayed arm products, one molecule (**A**) contains two ^{32}P-labels (the longest strand is 2348 nucleotides) whereas the other molecule (**B**) is unlabelled (2786 nucleotides). Branch migration in direction 2, leads to two ^{32}P-labelled products with total lengths of 2786 (**C**) and 2620 (**D**) nucleotides. The direction of branch migration depends on which pair of arms are bound by the RuvB rings, as indicated.

to form ^{32}P-labelled products which migrated slightly more slowly than the contaminating 2.6 and 2.35 kb species, as determined by agarose gel electrophoresis (Figure 1B, lane d). Previous studies have shown that RuvAB-mediated branch migration can occur in two possible directions, dependent on which pair of arms are bound by the RuvB rings (Hiom and West, 1995; Parsons *et al.*, 1992, 1995) as shown schematically in Figure 2. Branch migration in direction 1 will lead to the formation of products A and B. Product A will contain two ^{32}P-end labels whereas product B will be unlabelled. Branch migration in direction 2 will form two ^{32}P-labelled products (C and D) although these products are unlikely to separate on the gel. Since product A is mainly duplex DNA with the longest strand being 2348 nucleotides, and products C and D contain extensive flayed single-stranded tails with the longest strands being 2786 and 2620 nucleotides respectively, the labelled bands observed by gel electrophoresis are consistent with branch migration in direction 1 (lower band) and direction 2 (upper band). Since the bands are of approximately equal intensities, branch migration appears to have occurred with similar efficiencies in the two possible directions. Consistent with their expected flayed-arm structure, all ^{32}P-labelled products were sensitive to treatment with S1 nuclease (data not shown). The branch migration reaction required both RuvA and RuvB, since dissociation was not observed in the absence of either protein (Figure 1B, lane b; data not shown). In the absence of SSB protein, only a small fraction of the χ-structures (~10%) were dissociated (lane c). These results show that the RuvA and RuvB proteins, in the presence of SSB, are capable of promoting branch migration through 1794 bp of heterologous DNA.

SSB protein stimulates branch migration through heterologous DNA sequences

To quantify the effect of SSB on the branch migration reaction, assays were carried out in which the concentration

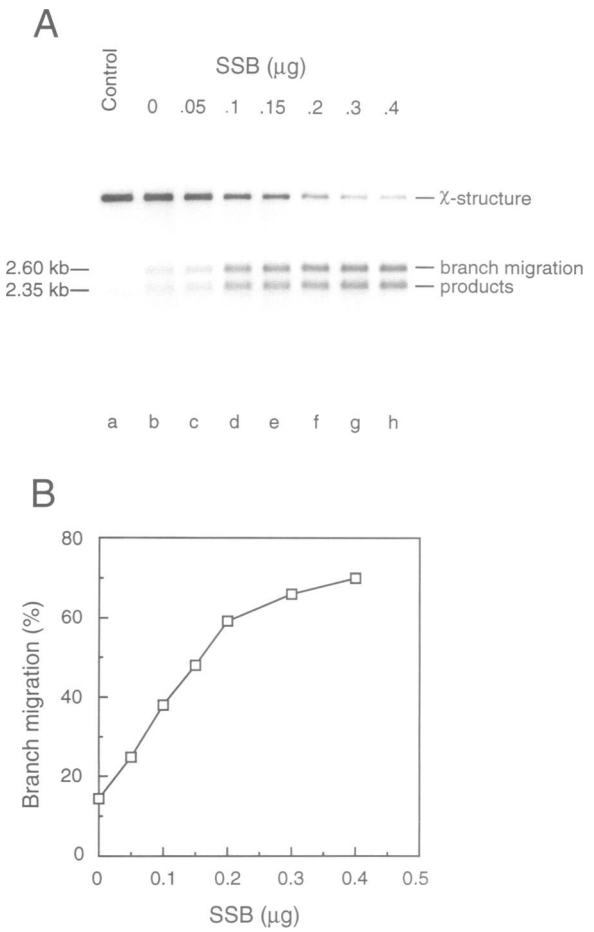

Fig. 3. SSB stimulation of RuvAB-mediated branch migration through heterologous DNA sequences. Reaction mixtures, set up as described in Materials and methods, containing RuvA, RuvB and the χ-structure, were supplemented with the indicated amounts of SSB protein. Following incubation for 60 min, reactions were stopped, deproteinized and the ^{32}P-labelled products were analysed by agarose gel electrophoresis. (**A**) Autoradiograph of the gel. Lane a, control lacking protein; lanes b–h, RuvAB reactions with the indicated amounts of SSB protein. (**B**) ^{32}P-labelled products were quantified by phosphorimaging. Backgrounds observed in the absence of proteins (typically ~10%) have been subtracted. Each data point represents the average of several experiments, including the experiment shown in (A).

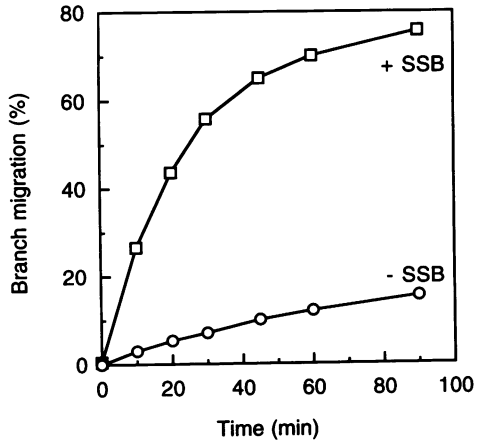

Fig. 4. Time-course of RuvAB-mediated branch migration through heterologous DNA in the absence and presence of SSB protein. Two large-scale reactions (180 μl) containing 0.45 μg ^{32}P-labelled χ-structure DNA were set up in either the absence (○) or presence (□) of SSB protein (4.5 μg). After incubation for 2 min at 37°C, RuvA (2.25 μg) and RuvB (4.5 μg) were added to initiate branch migration. The incubation was continued and at various time points after RuvAB addition (designated 0 min), 20 μl aliquots were removed for analysis. The samples were deproteinized and the products analysed by electrophoresis as described in Materials and methods. Band intensities were quantified using a phosphorimager. The amount of ^{32}P-labelled DNA product is expressed as a percentage of the total ^{32}P-labelled DNA. Backgrounds (10%) observed in the absence of protein have been subtracted.

of SSB protein was varied, and the conversion of ^{32}P-labelled χ-structure into flayed-arm product was analysed by agarose gel electrophoresis (Figure 3A). In the absence of SSB protein, the combined action of RuvA and RuvB resulted in dissociation of 17% of the DNA substrate (Figure 3A, lane b). However, addition of SSB led to stimulation of the branch migration activity until a plateau was reached at ~70% dissociation (Figure 3A, lanes c–h). The results of several such experiments were quantified and are shown in Figure 3B. The level of SSB at which maximal activity was observed corresponded to one tetramer of SSB per 30 nucleotides of DNA substrate. Given that the flayed-arm products are only partially unpaired this result indicates a requirement for saturating levels of SSB protein.

Direct visualization of branch migration through DNA heterologies

Time courses of branch migration reactions catalysed by RuvAB were analysed in the absence and presence of SSB (Figure 4). To visualize directly the intermediates and products, aliquots were taken from branch migration reactions carried out in the presence of SSB and ATP, and were spread for electron microscopy. The photographs presented in Figure 5 show the substrate DNA (A), intermediates of the reaction (B–D) and the products (E). The micrograph in panel A shows a χ-structure in the absence of any added proteins. Two long arms and two shorter arms can be seen radiating from the Holliday junction. Panel B shows a molecule in which the DNA lies in a square-planar configuration with the proteins resembling the tripartite RuvAB complex observed previously (Parsons *et al.*, 1995). Molecules interpreted as early intermediates of the branch migration reaction can be seen in panel C. In this photograph, protein clustering was observed at the junction point, indicative of regions of DNA unwound by RuvAB and bound by SSB protein. The micrograph indicates that increased binding by SSB to two of the arms is associated with a shortening in length of the other arms. Due to the nature of SSB binding, RuvAB complexes at the Holliday junction were not observed. Panel D shows a molecule at a later stage of branch migration in which long regions of DNA were covered by SSB. The DNA exhibits a twisted nature, presumably resulting from rotation of the DNA during unwinding. Also, SSB was seen to bind to one terminus of the χ-structure. However, this was only observed at a low frequency. Panel E shows a region of a specimen in which two products of the reaction are

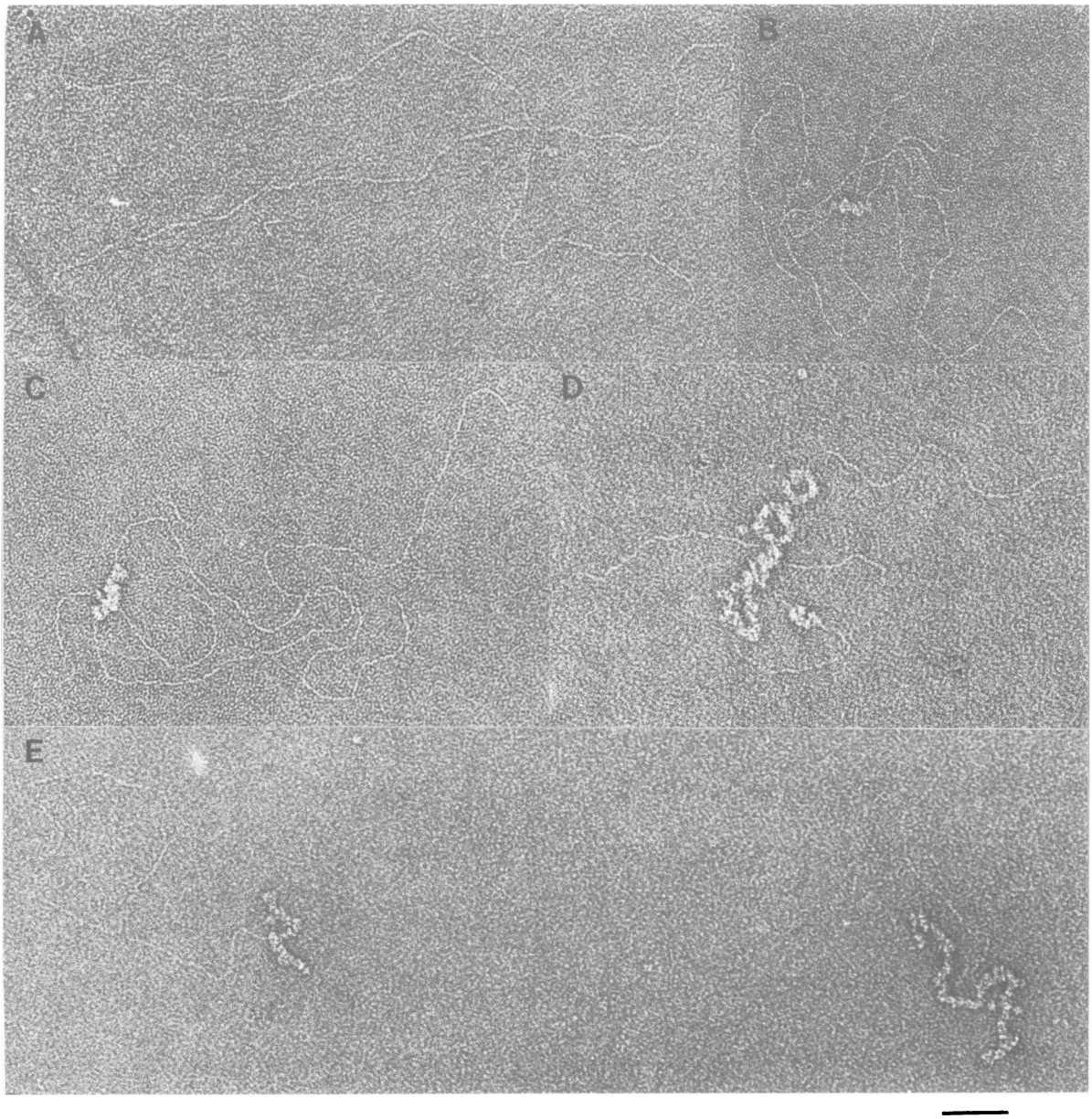

Fig. 5. Electron microscopic visualization of RuvAB-mediated branch migration reactions in the presence of SSB protein. Branch migration reactions were carried out as described in Materials and methods, and at various times samples were fixed by treatment with glutaraldehyde. Protein–DNA complexes were then visualized by electron microscopy following negative staining with uranyl acetate. (**A**) χ-structure without added proteins; (**B–E**) RuvAB–SSB–χ-structure complexes observed after 5 min (**B**, **C** and **E**) or 20 min (**D**) incubation at 37°C. Bar = 50 nm.

seen. The molecule on the left exhibits a long double-stranded region with short SSB-covered flayed tails. In contrast, the molecule on the right has a short duplex region and relatively long SSB-covered tails. Presumably these two molecules represent the products of branch migration in the two possible directions (see Figure 2). The electron microscopic visualization of reaction intermediates and products confirmed that RuvAB and SSB co-operate to promote branch migration through heterologous DNA.

In these studies, the RuvAB–SSB branch migration reaction was found to exhibit asynchrony. When samples were taken after 5 min, reaction intermediates and products were both observed; more products were seen after 20 min. However, many molecules were observed which either had not initiated branch migration or were at different stages of the reaction (Figure 5; data not shown). The reason for the asynchronous nature of the reaction is unknown.

Specificity of SSB-mediated stimulation

To determine whether SSB specifically stimulates RuvAB-mediated branch migration, its effect was compared with that of a bacteriophage-encoded single-stranded DNA binding protein (T4 gene 32 protein; gp32) (Figure 6A). In contrast to the concentration-dependent stimulation of final product formation observed with SSB protein, only a limited stimulation was observed using moderate concentrations of gp32. Higher levels of gp32 did not stimulate the branch migration reaction. Similar results were obtained using purified human RPA protein (data not shown). When competition reactions were carried out by

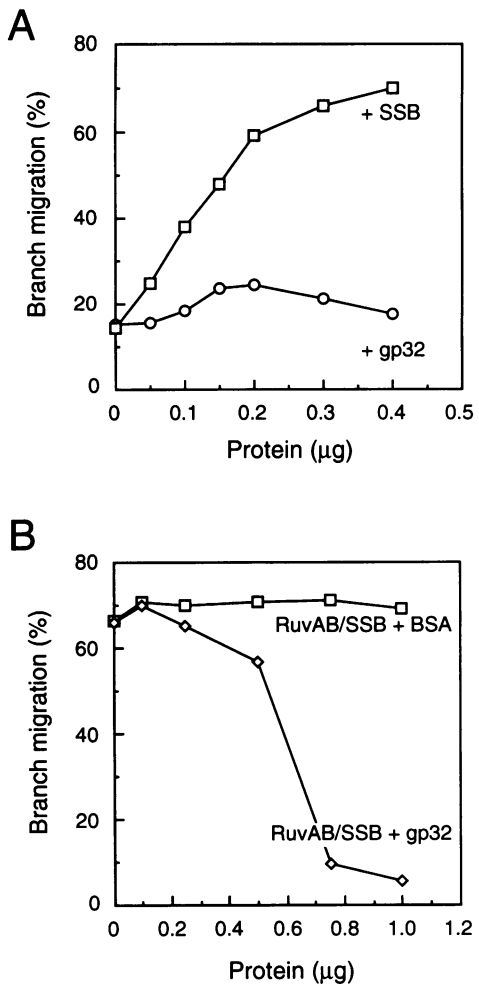

Fig. 6. Specificity for *E.coli* SSB protein. (**A**) Standard branch migration reactions containing χ-structure DNA and RuvAB were supplemented with varying amounts of either SSB protein (□) or bacteriophage T4 gp32 (○). Incubation was for 60 min at 37°C. ^{32}P-labelled products were analysed by gel electrophoresis as described in Materials and methods. Each data point represents the average of several experiments (>6). Backgrounds observed in the absence of protein (~10%) have been subtracted. (**B**) Standard branch migration reactions containing the χ-structure, RuvAB and SSB, together with the indicated amounts of T4 gp32 protein (◇) or BSA (□), were incubated at 37°C for 60 min as described in Materials and methods. ^{32}P-labelled products were analysed as described above.

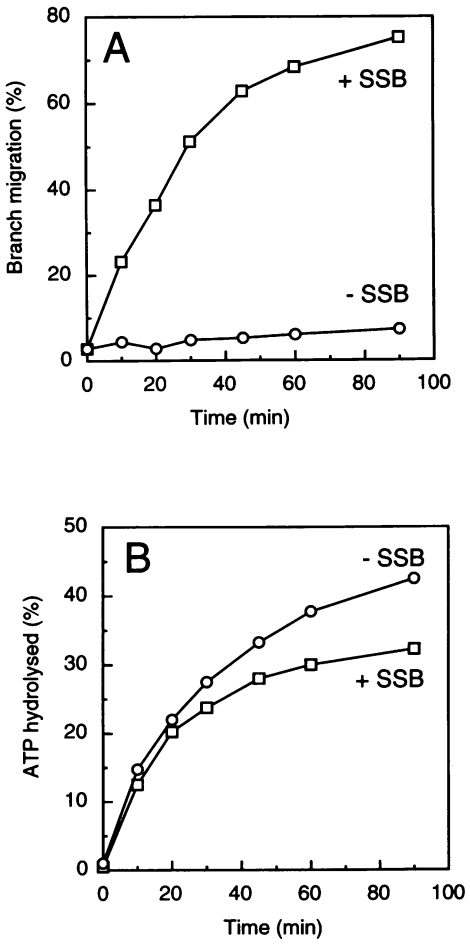

Fig. 7. Effect of SSB on ATP hydrolysis by RuvAB during branch migration through heterology. Large scale reactions were set up with or without SSB and supplemented with [α-^{32}P]ATP as described in Materials and methods. Incubation conditions were as described in the legend to Figure 3. At the indicated times, 20 μl samples were stopped and (**A**) the amount of branch migration was determined by agarose gel electrophoresis; and (**B**) the amount of ATP hydrolysed was determined by thin-layer chromatography as described in Materials and methods. Background levels of ADP observed in the absence of protein (6%) have been subtracted.

adding various amounts of gp32 to branch migration reactions containing RuvA, RuvB and SSB, moderate concentrations of gp32 were found to inhibit the formation of branch migration products (Figure 6B). Levels of gp32 in excess of the SSB concentration completely blocked branch migration. In contrast, addition of bovine serum albumin (BSA) to control reactions did not adversely affect branch migration. The specificity of the reaction for SSB may be due to the mode of binding that SSB exhibits with single-stranded DNA, and this requirement may not be met by other single-stranded DNA binding proteins such as gp32.

Effect of SSB protein on ATPase activity of RuvAB

Branch migration through heterology occurs by a reaction that is thermodynamically unfavourable due to loss of base stacking. It therefore requires energy input. To analyse the relationship between heterologous branch migration and ATP hydrolysis by RuvAB, the amounts of ATP hydrolysed during on-going branch migration reactions were determined in the presence and absence of SSB protein. At various times aliquots were removed and assayed to determine (i) the extent of dissociation of the χ-structure; and (ii) the amount of ATP hydrolysed. The data are presented in Figure 7A and B. The amounts of ATP hydrolysed over the first 30 min were remarkably similar despite the difference in the percentage of χ-structures dissociated. These results indicate that ATP hydrolysis is not directly coupled to branch migration through DNA heterology. At later time points, ATP hydrolysis in the presence of SSB was slightly lower relative to that observed in its absence, presumably because SSB bound to the unpaired single-stranded products, inhibiting subsequent binding and ssDNA-dependent ATP hydrolysis by RuvAB.

Discussion

To determine whether the RuvAB proteins promote branch migration between two duplexes that shared little DNA homology, recombination intermediates containing extensive heterologies were prepared *in vivo* using the XerC-*cer* site-specific recombination system (Blakely *et al.*, 1993; McCulloch *et al.*, 1994). Using the plasmid pSD115 which contains two directly repeated *cer* sites, intramolecular recombination was initiated by expression of XerC, leading to the formation of figure-8 molecules in which 2.60 and 2.35 kb circular duplexes were joined by a Holliday junction (McCulloch *et al.*, 1994). After restriction enzyme cleavage, the resultant χ-structures comprise a Holliday junction, contained within a 290 bp region of homology, flanked by four heterologous arms ranging in size from 536 to 1794 bp. Remarkably, despite the presence of almost total heterology, RuvA and RuvB were able to promote branch migration until the χ-structures were physically dissociated. To bring about complete dissociation, it was necessary for RuvAB to promote branch migration through either 700 or 1800 bp of heterologous DNA, dependent on the directionality of the reaction. Branch migration through such a length of heterology far exceeds that observed with RecA protein, since efficient strand exchange between heterologous duplex DNA molecules by RecA does not exceed more than a few tens of base pairs *in vitro* (Hahn *et al.*, 1988; Morel *et al.*, 1994).

Current understanding of the structure of the branch migration complex indicates that two RuvB hexameric rings (which possess DNA helicase activity) are targeted to the Holliday junction by RuvA. Within the RuvAB–Holliday junction complex, the junction assumes an unfolded square structure as shown in Figure 8A (Parsons *et al.*, 1995). Branch migration is thought to be a consequence of the two RuvB rings applying equal and opposite forces to the DNA, thereby driving the DNA into and out of the RuvAB complex. This reaction is likely to involve localized strand separation within the RuvB hexamer. When branch migration occurs between two homologous duplexes, DNA unwinding will be followed by rewinding (i.e. base pairing) of the heteroduplexes. The results presented in this paper with heterologous DNA substrates show that unwinding can be uncoupled from rewinding. Although ~10–20% of the χ-structures could be dissociated by RuvAB alone, the reaction was stimulated by the presence of the *E.coli* SSB protein. SSB protein presumably binds to the single-stranded DNA as it exits from the RuvAB complex (Figure 8B and C). In these experiments, the DNA substrate becomes fully dissociated and takes the form of duplex molecules with flayed single-stranded tails (Figure 8D). However, branch migration *in vivo* would be expected to continue into regions of homology, leading to base pairing and the formation of heteroduplex products containing single-stranded loops.

The mechanism by which SSB facilitates RuvAB-mediated branch migration through heterology is currently unknown. SSB may play a direct role by binding and removing the single-stranded DNA as it is produced by the branch migration motor. Alternatively, since SSB binds the products of unwinding, it could help provide

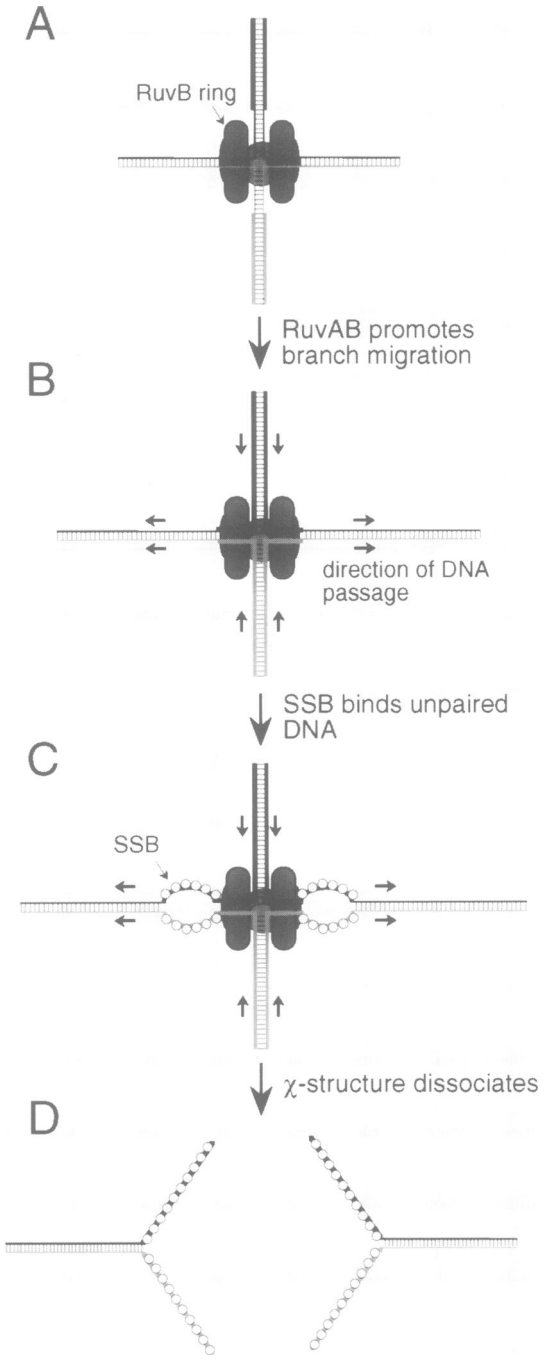

Fig. 8. Schematic diagram indicating the role of SSB protein in RuvAB-mediated dissociation of the χ-structure by branch migration through heterology. In this diagram, the RuvA protein and RuvB hexameric rings are shown. The thickened lines represent regions of duplex DNA that are heterologous to each other. (**A**) Specific recognition of the Holliday junction leads to assembly of the tripartite RuvAB complex on DNA. In this complex, the DNA is unfolded and RuvA protein is sandwiched between the two hexameric rings of RuvB. (**B**) RuvAB promote ATP-dependent branch migration, initially through homologous DNA (thin lines). The direction of strand passage is indicated by the arrows. The branch migration reaction is thought to involve localized strand separation within the RuvB rings. Branch migration then proceeds into the heterologous DNA regions (thick lines), but in this case the DNA strands cannot reassociate after passage through RuvB. (**C**) Unpaired single-strands of DNA are bound by SSB protein after they pass out of the RuvAB complex. (**D**) The reaction products are pairs of partially duplex DNA molecules which contain single-stranded tails covered by SSB.

directionality to the reaction. Finally, the free energy of SSB binding could drive the formation of unpaired DNA (a reaction that is energetically unfavourable). This latter point may be particularly important since there is currently little evidence to suggest that ATP hydrolysis, which is required for branch migration, is directly coupled to strand exchange.

RuvAB-mediated branch migration was stimulated to a lesser extent by gp32 or human RPA, than by *E.coli* SSB. These results could be interpreted as an indication of specific contacts between RuvAB and SSB. However, the way in which SSB associates with single-stranded DNA is known to be complex since SSB exhibits several distinct DNA binding modes dependent on solution conditions (Lohman *et al.*, 1988; Lohman and Ferrari, 1994). In the absence of any data to support specific interactions, we propose that SSB provides a positive stimulatory effect on branch migration by binding DNA in a particular mode which helps facilitate branch migration.

In recent electron microscopic studies, RuvA and RuvB proteins were found to form a tripartite complex on the χ-structure (Parsons *et al.*, 1995). To stabilize the complex, a non-hydrolysable analogue of ATP, ATPγS, was used since this does not support branch migration. In the experiments described in the present work, reactions were carried out in the presence of ATP and SSB. Reaction intermediates which appeared to contain loops of single-stranded DNA bound by SSB were observed by electron microscopy. These intermediates were representative of DNA molecules physically undergoing RuvAB-mediated branch migration at the time of glutaraldehyde fixation. The electron microscopy also showed that the reaction is highly asynchronous, with DNA products and intermediates visualized in each sample taken over a time-course. The reason for this is unknown. It is possible that the system lacks a component, or alternatively, the reaction may exhibit a low processivity, possibly caused by the RuvAB complex dissociating after branch migrating only a few hundred base pairs.

In conclusion, we have observed that the RuvA and RuvB proteins promote branch migration through extensive lengths of heterologous DNA, in a reaction that was stimulated by SSB protein. These proteins are therefore likely to provide an important and possibly unique function in genetic recombination and the recombinational repair of damaged DNA.

Materials and methods

Proteins

The *E.coli* RuvA and RuvB proteins were purified to homogeneity as described (Tsaneva *et al.*, 1992a). Protein concentrations were determined using BSA as standard (Bradford, 1976). *E.coli* SSB protein and T4 gene 32 protein were obtained from Pharmacia, and DNA polymerase I (Klenow fragment), S1 nuclease and BSA were from Gibco BRL. RPA protein, purified from HeLa cells (Kenny *et al.*, 1990), was a gift from M.Shivji and R.Wood (ICRF).

Bacterial strains and plasmids

Escherichia coli RM40 and plasmid pSD115 (McCulloch *et al.*, 1994) were a gift from D.Sherratt (University of Oxford). RM40 contains the *xerC* gene under control of the *lac* promoter. It was grown at 37°C with aeration in Luria broth supplemented with 50 μg/ml diaminopimelic acid. Glucose (2%) was included to repress the chromosomal *lac* promoter. Plasmid pSD115 contains two directly repeated *cer* sites. It was transformed into RM40 and maintained by growth in the presence of carbenicillin.

Preparation of χ-structures

RM40 carrying pSD115 was grown to an OD_{650} of ~0.4–0.6. To initiate XerC-induced site-specific recombination, IPTG was added to 1 mM and growth was continued for 1 h at 37°C. The cells were then harvested and chilled on ice. Plasmid DNA was prepared using Qiagen mega kits. The resulting DNA consisted of a mixture of non-recombined pSD115 DNA, figure-8 DNA, and 2.6 and 2.35 kb recombinant products (McCulloch *et al.*, 1994). The DNA was cut by incubation with *Sty*I and *Sca*I for 18 h at 37°C in 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol and 100 μg/ml BSA. The DNA was concentrated by ethanol precipitation and most of the non-recombined pSD115 plasmid DNA, and the 2.6 and 2.35 kbp products, were then removed by two successive rounds of sedimentation through 35 ml 5–20% neutral sucrose gradients (in 10 mM Tris–HCl, pH 7.5, 1 M NaCl, 10 mM EDTA) at 25 000 r.p.m. for 25 h in a Beckman SW28 rotor at 4°C. Fractions containing the purified χ-structures were identified by agarose gel electrophoresis and peak fractions were pooled, ethanol precipitated and resuspended in 10 mM Tris–HCl, pH 8.0, 1 mM EDTA (TE). Typically, the final preparation was >90% χ-structures with a small fraction of contaminating linear 2.6 and 2.35 kb species.

DNA preparations were 3′-^{32}P-end labelled at the *Sty*I termini using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. The reaction was stopped by addition of EDTA, the enzyme heat-inactivated and the DNA ethanol-precipitated, followed by dialysis against TE. DNA concentrations were quantified by measuring the absorbance at 260 nm.

Branch migration assay

Unless stated otherwise, standard reaction mixtures (20 μl) contained 3′-^{32}P-end labelled χ-structure DNA (50 ng), RuvA (0.25 μg), RuvB (0.5 μg) and SSB (0.5 μg) in 20 mM triethanolamine–HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM ATP and 1 mM dithiothreitol. Incubation was for 60 min at 37°C. Reactions were stopped by addition of EDTA to 20 mM and SDS to 1%. Products were analysed by electrophoresis at 6 V/cm through 1% (w/v) agarose gels in TAE (40 mM Tris–acetate, pH 8.5, 1 mM EDTA), at room temperature with buffer recirculation. The gels were dried and exposed to Kodak XAR5 film to obtain autoradiographs. In addition, each gel was quantified using a Molecular Dynamics Model 425E PhosphorImager with ImageQuant software.

ATPase assay

Large-scale reaction mixtures (180 μl) were set up as described for the branch migration assays, except that the ATP concentration was reduced to 0.5 mM. The reaction was also supplemented with 4 μCi [α-^{32}P]ATP. At set times, samples (20 μl) were taken and the reactions were stopped by addition of EDTA to 20 mM. Aliquots (1 μl) were then spotted onto CEL300 PEI/UV_{254} (Polygram) thin-layer chromatography plates which were developed in 1 M formic acid and 0.5 M LiCl. The percentage of [α-^{32}P]ATP hydrolysed to [α-^{32}P]ADP was determined by phosphor-imaging and quantification.

Electron microscopy

Samples were fixed by addition of glutaraldehyde to 0.2% followed by incubation for 15 min at 37°C. For spreading and adsorption, DNA–protein complexes were diluted to 1 μg/ml in 5 mM magnesium acetate, pH 7.0. Complexes were visualized after staining with 2% uranyl acetate. Images were recorded under minimal dose conditions at ×35 000 magnification on a Philips CM12 electron microscope.

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References

Adams,D.E. and West,S.C. (1995) Unwinding of closed circular DNA by the *Escherichia coli* RuvA and RuvB recombination/repair proteins. *J. Mol. Biol.*, **247**, 404–417.

Adams,D.E., Tsaneva,I.R. and West,S.C. (1994) Dissociation of RecA filaments from duplex DNA by the RuvA and RuvB DNA repair proteins. *Proc. Natl Acad. Sci. USA*, **91**, 9901–9905.

Benson,F.E., Illing,G.T., Sharples,G.J. and Lloyd,R.G. (1988) Nucleotide sequencing of the *ruv* region of *E.coli* K-12 reveals a LexA regulated operon encoding two genes. *Nucleic Acids Res.*, **16**, 1541–1550.

Blakely,G., May,G., McCulloch,R., Arciszewska,L.K., Burke,M., Lovett,S.T. and Sherratt,D.J. (1993) Two related recombinases are required for site-specific recombination at *dif* and *cer* in *Escherichia coli* K12. *Cell*, **75**, 351–361.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.

Cox,M.M. and Lehman,I.R. (1981) RecA protein of *E.coli* promotes branch migration, a kinetically distinct phase of DNA strand exchange. *Proc. Natl Acad. Sci. USA*, **78**, 3433–3437.

DasGupta,C., Shibata,T., Cunningham,R.P. and Radding,C.M. (1980) The topology of homologous pairing promoted by RecA protein. *Cell*, **22**, 437–446.

Hahn,T.R., West,S.C. and Howard-Flanders,P. (1988) RecA-mediated strand exchange reactions between duplex DNA molecules containing damaged bases, deletions and insertions. *J. Biol. Chem.*, **263**, 7431–7436.

Hiom,K. and West,S.C. (1995) The mechanism of branch migration in homologous recombination: Assembly of a RuvAB-Holliday junction complex *in vitro*. *Cell*, **80**, 787–793.

Iwasaki,H., Shiba,T., Makino,K., Nakata,A. and Shinagawa,H. (1989) Overproduction, purification, and ATPase activity of the *Escherichia coli* RuvB protein involved in DNA repair. *J. Bacteriol.*, **171**, 5276–5280.

Iwasaki,H., Takahagi,M., Nakata,A. and Shinagawa,H. (1992) *Escherichia coli* RuvA and RuvB proteins specifically interact with Holliday junctions and promote branch migration. *Genes Dev.*, **6**, 2214–2220.

Iype,L.E., Wood,E.A., Inman,R.B. and Cox,M.M. (1994) RuvA and RuvB proteins facilitate the bypass of heterologous DNA insertions during RecA protein-mediated strand exchange. *J. Biol. Chem.*, **269**, 24967–24978.

Kenny,M.K., Schlegel,U., Furneaux,H. and Hurwitz,J. (1990) The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. *J. Biol. Chem.*, **265**, 7693–7700.

Lichten,M. and Fox,M.S. (1984) Evidence for inclusion of regions of nonhomology in heteroduplex products of bacteriophage lambda recombination. *Proc. Natl Acad. Sci. USA*, **81**, 7180–7184.

Little,J.W. and Mount,D.W. (1982) The SOS regulatory system of *E.coli*. *Cell*, **29**, 11–22.

Lohman,T.M. and Ferrari,M.E. (1994) *Escherichia coli* single stranded DNA binding protein: multiple DNA-binding modes and cooperativities. *Annu. Rev. Biochem.*, **63**, 527–570.

Lohman,T.M., Bujalowski,W. and Overman,L.B. (1988) *E.coli* single strand binding protein: A new look at helix-destabilizing proteins. *Trends Biochem. Sci.*, **13**, 250–255.

McCulloch,R., Coggins,L.W., Colloms,S.D. and Sherratt,D.J. (1994) Xer-mediated site-specific recombination at *cer* generates Holliday junctions *in vivo*. *EMBO J.*, **13**, 1844–1855.

Matic,I., Radman,M. and Rayssiguier,C. (1994) Structure of recombinants from conjugational crosses between *Escherichia coli* donor and mismatch repair deficient *Salmonella typhimurium* recipients. *Genetics*, **136**, 17–26.

Matic,I., Rayssiguier,C. and Radman,M. (1995) Interspecies gene exchange in bacteria: the role of SOS and mismatch repair systems in evolution of species. *Cell*, **80**, 507–515.

Mitchell,A.H. and West,S.C. (1994) Hexameric rings of *Escherichia coli* RuvB protein: Cooperative assembly, processivity, and ATPase activity. *J. Mol. Biol.*, **243**, 208–215.

Morel,P., Stasiak,A., Ehrlich,S.D. and Cassuto,E. (1994) Effect of length and location of heterologous sequences on RecA-mediated strand exchange. *J. Biol. Chem.*, **269**, 19830–19835.

Müller,B., Tsaneva,I.R. and West,S.C. (1993a) Branch migration of Holliday junctions promoted by the *Escherichia coli* RuvA and RuvB proteins: I. Comparison of the RuvAB- and RuvB-mediated reactions. *J. Biol. Chem.*, **268**, 17179–17184.

Müller,B., Tsaneva,I.R. and West,S.C. (1993b) Branch migration of Holliday junctions promoted by the *Escherichia coli* RuvA and RuvB proteins: II. Interaction of RuvB with DNA. *J. Biol. Chem.*, **268**, 17185–17189.

Parsons,C.A. and West,S.C. (1993) Formation of a RuvAB-Holliday junction complex *in vitro*. *J. Mol. Biol.*, **232**, 397–405.

Parsons,C.A., Tsaneva,I., Lloyd,R.G. and West,S.C. (1992) Interaction of *Escherichia coli* RuvA and RuvB proteins with synthetic Holliday junctions. *Proc. Natl Acad. Sci. USA*, **89**, 5452–5456.

Parsons,C.A., Stasiak,A., Bennett,R.J. and West,S.C. (1995) Structure of a multisubunit complex that promotes DNA branch migration. *Nature*, **374**, 375–378.

Rayssiguier,C., Thaler,D.S. and Radman,M. (1989) The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch repair mutants. *Nature*, **342**, 396–401.

Shiba,T., Iwasaki,H., Nakata,A. and Shinagawa,H. (1991) SOS-inducible DNA repair proteins, RuvA and RuvB, of *Escherichia coli*: Functional interactions between RuvA and RuvB for ATP hydrolysis and renaturation of the cruciform structure in supercoiled DNA. *Proc. Natl Acad. Sci. USA*, **88**, 8445–8449.

Shinagawa,H., Makino,K., Amemura,M., Kimura,S., Iwasaki,H. and Nakata,A. (1988) Structure and regulation of the *Escherichia coli ruv* operon involved in DNA repair and recombination. *J. Bacteriol.*, **170**, 4322–4329.

Shurvinton,C.E. and Lloyd,R.G. (1982) Damage to DNA induces expression of the *ruv* gene of *Escherichia coli*. *Mol. Gen. Genet.*, **185**, 352–355.

Stasiak,A., Tsaneva,I.R., West,S.C., Benson,C.J.B., Yu,X. and Egelman,E.H. (1994) The *Escherichia coli* RuvB branch migration protein forms double hexameric rings around DNA. *Proc. Natl Acad. Sci. USA*, **91**, 7618–7622.

Tsaneva,I.R. and West,S.C. (1994) Targeted versus non-targeted DNA helicase activity of the RuvA and RuvB proteins of *Escherichia coli*. *J. Biol. Chem.*, **269**, 26552–26558.

Tsaneva,I.R., Illing,G.T., Lloyd,R.G. and West,S.C. (1992a) Purification and properties of the RuvA and RuvB proteins of *Escherichia coli*. *Mol. Gen. Genet.*, **235**, 1–10.

Tsaneva,I.R., Müller,B. and West,S.C. (1992b) ATP-dependent branch migration of Holliday junctions promoted by the RuvA and RuvB proteins of *E.coli*. *Cell*, **69**, 1171–1180.

Tsaneva,I.R., Müller,B. and West,S.C. (1993) The RuvA and RuvB proteins of *Escherichia coli* exhibit DNA helicase activity *in vitro*. *Proc. Natl Acad. Sci. USA*, **90**, 1315–1319.

West,S.C., Cassuto,E. and Howard-Flanders,P. (1981) RecA protein promotes homologous pairing and strand exchange reactions between duplex DNA molecules. *Proc. Natl Acad. Sci. USA*, **78**, 2100–2104.

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