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Unusual stability of recombination intermediates made by *Escherichia coli* RecA protein

Berndt Müller, Ian Burdett[1] and Stephen C.West

Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts., EN6 3LD and [1]National Institute for Medical Research, Medical Research Council, The Ridgeway, Mill Hill, London NW7 1AA, UK

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The structure and stability of recombination intermediates made by RecA protein have been investigated following deproteinization. The intermediates consist of two duplex DNA molecules connected by a junction, as visualized by electron microscopy. Although we expected the structures to be highly unstable due to branch migration of the junction, this was not the case. Instead, we found that the intermediates were stable at 37°C. At 56°C, >60% of the intermediates remained after 6 h of incubation. Only at higher temperatures was significant branch migration observed. This unexpected stability suggests that the formation of extensive lengths of heteroduplex DNA in *Escherichia coli* is likely to require the continued action of proteins, and does not occur via spontaneous branch migration. We show that heteroduplex DNA may be formed *in vitro* by ATP-dependent strand exchange catalysed by RecA protein or by the RuvA and RuvB proteins of *E.coli*.

Key words: branch migration/Holliday junctions/recombination/RuvABC proteins

Introduction

It has been proposed that Holliday junctions are intermediates in general genetic recombination (for review see Dressler and Potter, 1982). They are thought to be formed by the reciprocal exchange of strands between two duplex DNA molecules, and their movement leads to the formation of heteroduplex DNA. The rate of migration of the junction determines the length of heteroduplex DNA and is therefore responsible for gene conversion events between nearby genetic markers.

The speed of migration of Holliday junctions has been the subject of theoretical and experimental studies. Meselson developed a mathematical model based on the assumption that the rate of branch migration would be limited by rotary diffusion (Meselson, 1972). The isolation of figure-of-eight DNA molecules from replicative form G4 DNA, which could be converted into X-structures by restriction digestion, allowed Warner and colleagues to attempt to determine the rate of spontaneous branch migration experimentally (Thompson *et al.*, 1976; Warner *et al.*, 1978). Both studies concluded that the step rate (i.e. the rate of movement through one base pair) was rapid and in the order of several thousand base pairs per second at physiological temperatures. Since branch migration would be expected to progress by a random walk mechanism, this step rate would translate into a movement of a third of the junctions through at least 2000 bp in 10 min (at 30°C) (Warner *et al.*, 1978). This led to the conclusion that spontaneous branch migration could account for the formation of heteroduplex DNA during recombination.

The RecA protein of *E.coli* plays a central role in genetic recombination and catalyses homologous pairing and strand exchange reactions *in vitro* (Cox and Lehman, 1987; Radding, 1982; West, 1992). Using two duplex DNA molecules, one of which contained a short region of single-stranded DNA, it was shown that RecA protein formed heteroduplex DNA via a Holliday junction-like intermediate, as observed by electron microscopy of deproteinized samples and by enzymatic analyses (DasGupta *et al.*, 1981; Müller *et al.*, 1990; West *et al.*, 1983). These results provided biochemical support for the existence of Holliday junctions during recombination. However, recent studies of the way in which RecA protein promotes recombination reactions between single- and double-stranded DNA substrates (i.e. reactions involving three DNA strands), suggest that the intermediates contain a region of triple-stranded DNA (Hsieh *et al.*, 1990; Menetski *et al.*, 1990; Rao *et al.*, 1990, 1991; Umlauf *et al.*, 1990). The formation of triple-stranded DNA during RecA-mediated recombination had previously been suggested by Howard-Flanders *et al.* (1984). Surprisingly, the triplex structures were found to be stable following removal of RecA protein (Hsieh *et al.*, 1990; Rao *et al.*, 1990, 1991).

Since it is likely that RecA-mediated recombination reactions involving three or four strands of DNA proceed by similar mechanisms, we have investigated the structure and stability of intermediates formed during strand exchange between two duplex DNA molecules. We find that recombination intermediates that contain four DNA strands are stable following deproteinization and that spontaneous branch migration does not occur at the expected rates. These results will be discussed in the context of two alternative models for the structure of recombination intermediates.

Our findings suggest that recombination intermediates made by RecA protein require the actions of other proteins for further processing. We show that strand exchange resumes upon re-addition of stoichiometric amounts of RecA protein and occurs at the normal rate. In addition, the SOS-regulated recombination/repair proteins RuvA and RuvB (Benson *et al.*, 1988; Shinagawa *et al.*, 1988; Shurvinton and Lloyd, 1982) also convert recombination intermediates into heteroduplex products by strand exchange. A different form of processing is carried out by the RuvC protein, a junction-specific endonuclease that catalyses the resolution of intermediates into mature recombinant products (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991).

Results

Purification and visualization of recombination intermediates

To produce protein-free recombination intermediates containing four DNA strands, we utilized the well characterized RecA-mediated strand exchange reaction between gapped circular ϕX174 duplex DNA (gDNA) and homologous ^{32}P-labelled linear duplex DNA (Figure 1A). A time course of strand exchange, as observed by agarose gel electrophoresis, is shown in Figure 1B. After 20 min, the majority of the ^{32}P-labelled DNA migrated slowly through the gel, at a position characteristic of a recombination intermediate (Figure 1B, lane d). To confirm that this slow mobility form consisted of the two reaction partners (gDNA and linear duplex DNA), we analysed them in a double labelling experiment. Intermediates prepared from ^{3}H-labelled gDNA and ^{32}P-labelled linear duplex DNA were excised from an agarose gel and their content assayed by liquid scintillation counting. The intermediates contained 1.08 ± 0.07 molecules of ^{3}H-labelled gDNA per molecule of ^{32}P-labelled linear duplex DNA (mean of four measurements; data not shown).

To study the structure and properties of the recombination intermediates, we stopped a large scale strand exchange reaction after 20 min and deproteinized the sample by treatment with SDS, EDTA and proteinase K. The DNA was then purified by gel filtration chromatography through Sepharose CL-2B. After this treatment, we were unable to detect any RecA protein by SDS−PAGE and by Western blotting with anti-RecA antibodies (detection limit 0.1 ng RecA protein) (data not shown). Quantitatively, this indicates that the recombination intermediates (93 pmol) contained <2.6 fmol of RecA protein, or <1 RecA monomer per recombination intermediate. Based on the time course shown in Figure 1B, we assumed that the junction point would be situated ~2−3 kb (approximately half the size of the ϕX174 genome) away from each DNA end.

Using a protein-free spreading method, we analysed the DNA preparation by electron microscopy. We observed unreacted gDNA (Figure 2A), linear DNA (not shown) and more complex structures formed by RecA-mediated homologous pairing and strand exchange (Figure 2B−D). The structure of 138 recombination intermediates was analysed and three different classes were found: (i) recombination intermediates which were joined by structures that resembled classical representations of Holliday junctions (33%) (Figure 2B); (ii) structures in which the two DNA molecules appeared to have their DNA sequences aligned over ~60−300 bp (11%) (Figure 2C and D) and (iii) complexes that were so highly entangled (56%) that it was not possible to characterize the nature of the joint.

Although it was intriguing to observe structures that contained regions in which two interacting DNA molecules appeared to be aligned (Figure 2C and D), the fine detail of this region could not be determined. The alignment could be a consequence of the interwinding of the two DNA molecules in the vicinity of the Holliday junction. Alternatively, it may represent a stretch of multi-stranded DNA in which the two duplexes are paired, as proposed by Howard-Flanders and colleagues (Howard-Flanders *et al.*, 1984).

Thermal stability of the protein-free recombination intermediates

To determine the thermal stability of the deproteinized intermediates, samples similar to those used for electron microscopy were incubated for 10 min at different temperatures prior to analysis by agarose gel electrophoresis. When the intermediates were incubated in a buffer containing 15 mM $MgCl_2$, they were stable at 22, 37, 56, 66 and 72°C (Figure 3A, lanes a−e). At 80°C (lane f) many

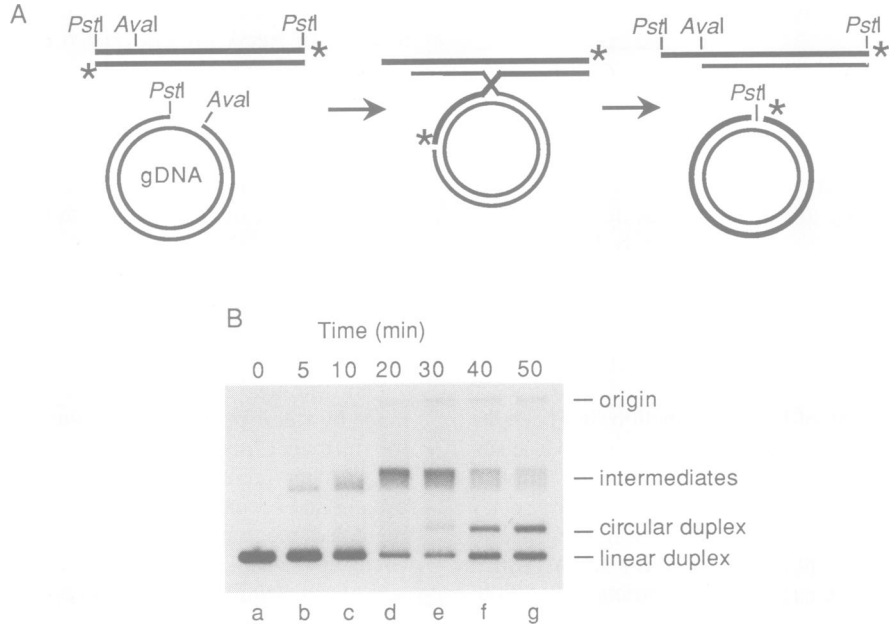

Fig. 1. Strand exchange between duplex DNA molecules by RecA protein. (**A**) Schematic drawing showing the reaction substrates [gapped circular ϕX174 DNA (gDNA) and homologous linear duplex DNA; left], intermediates (centre) and products (nicked circular and gapped linear DNA; right). 3′-^{32}P labels are indicated with an asterisk. (**B**) Time course of RecA-mediated reaction (110 μl) between gDNA and linear duplex DNA. Reaction conditions were as described in Materials and methods. Aliquots (15 μl) were stopped at the indicated times and analysed by agarose gel electrophoresis.

intermediates dissociated by branch migration, as observed by the formation of run-off products (^{32}P-labelled nicked circular and ^{32}P-labelled linear duplex DNA). At 87°C and 95°C, the DNA was denatured, as indicated by the formation of aggregates which failed to enter the gel (lanes g and h). To permit quantitative analysis of their dissociation by branch migration, ^{32}P-labelled DNA was excised from an agarose gel and the amount of radioactivity determined by scintillation counting as described in Materials and methods. In this and subsequent analyses, the fraction of ^{32}P-labelled intermediates will be expressed as a percentage of total ^{32}P-labelled DNA. The quantification of the experiment of Figure 3A is shown in Figure 3B.

The unexpected thermal stability of the intermediates was also observed during prolonged incubation, and >90% of the intermediates remained after 6 h of incubation at 37°C [in 20 mM Tris−HCl (pH 7.5) and 15 mM $MgCl_2$] (Figure 3C). However, prolonged incubation at 56, 66 and 72°C did with time lead to the dissociation of a significant number of the intermediates (Figure 3C), although a fraction did not dissociate even after extended incubation at 66 and 72°C. These results may indicate that some intermediates

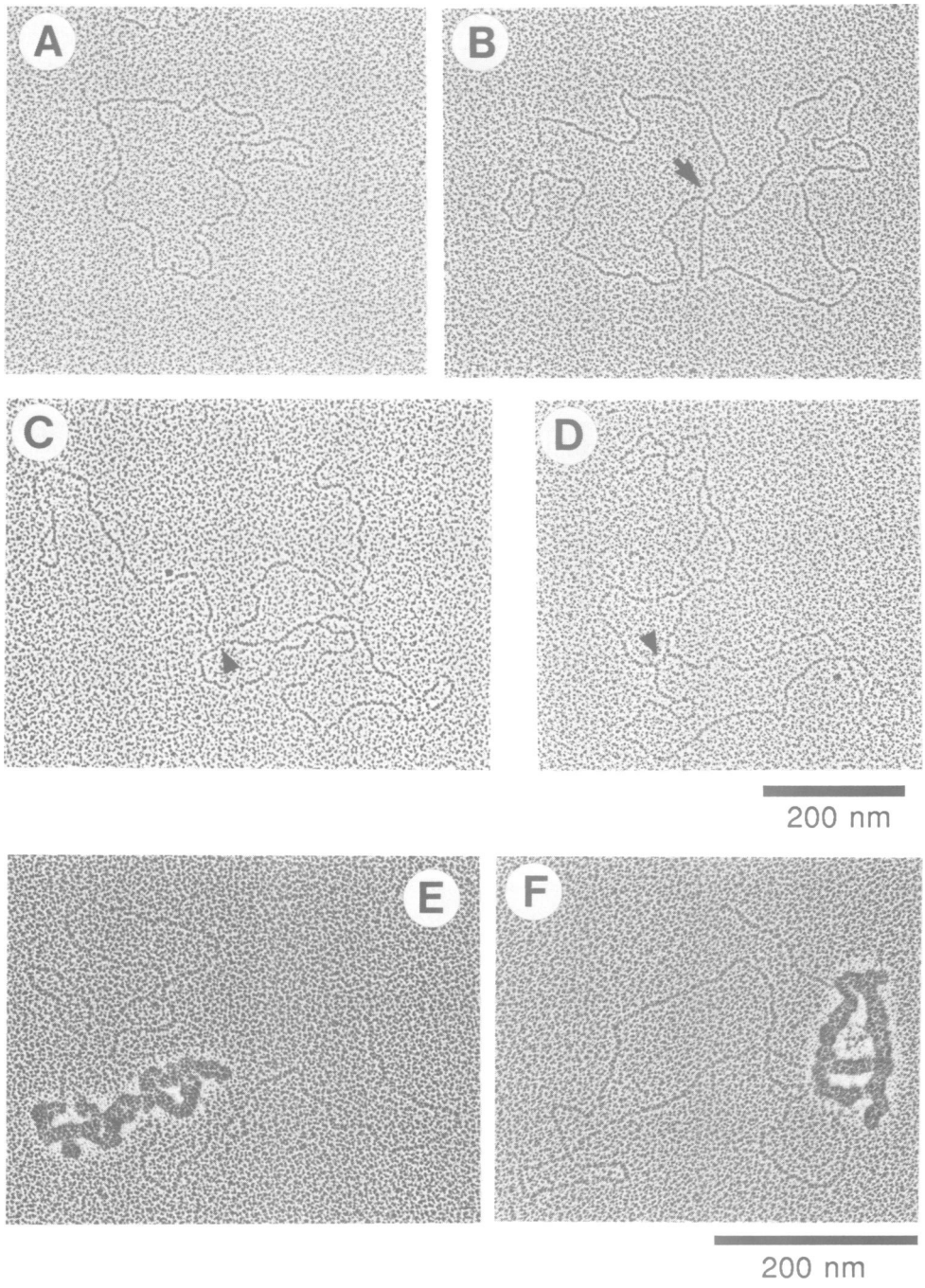

Fig. 2. (**A**−**D**) Electron micrographs of protein-free recombination intermediates. (A) Gapped circular duplex DNA. (B) Recombination intermediate with a classical Holliday junction (→). (C and D) Intermediates containing a region where the DNA molecules are aligned (▲). (**E** and **F**) Electron micrographs of nucleoprotein filaments formed between RecA protein and the recombination intermediates. Samples were prepared as described in Materials and methods.

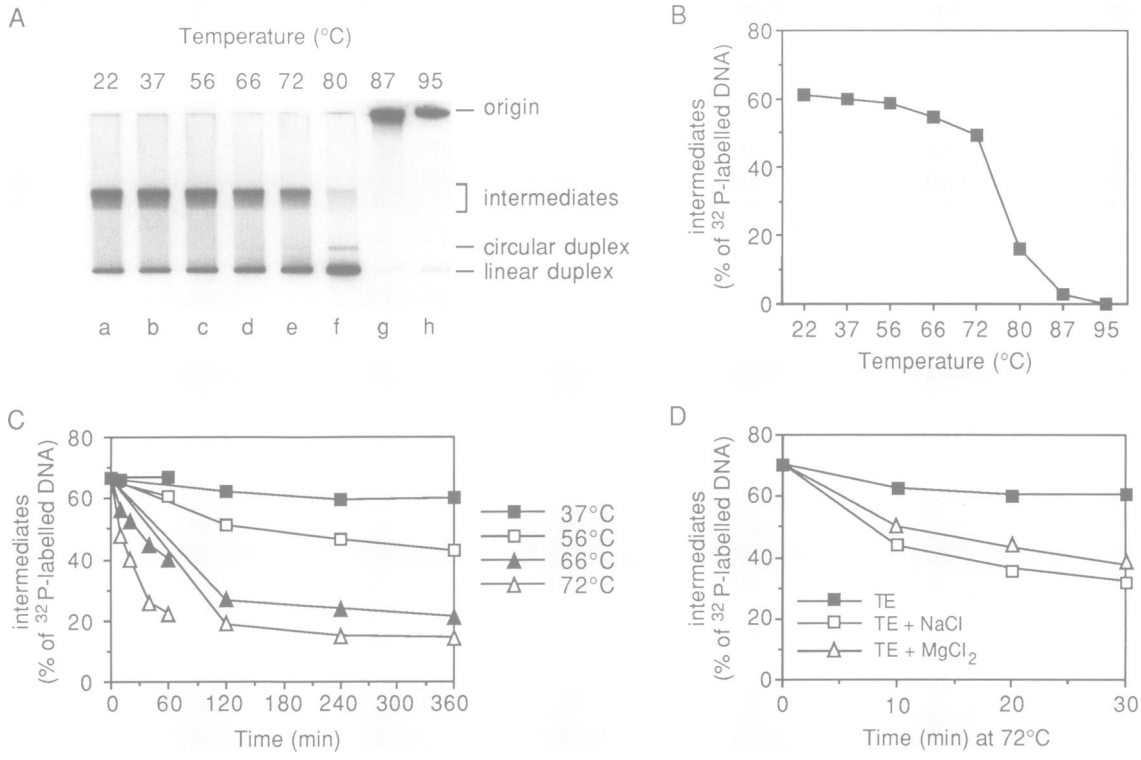

Fig. 3. Thermal stabilities of protein-free recombination intermediates. (**A**) DNA intermediates (200 μl at 8.5 μM) were deproteinized as described in Materials and methods, and aliquots (25 μl) were incubated for 10 min at the indicated temperatures, chilled on ice and analysed by agarose gel electrophoresis. (**B**) For the gel shown in (A), the percentage of ^{32}P-labelled DNA in the form of intermediates was quantified as described in Materials and methods. (**C**) Deproteinized intermediates (400 μl at 9.2 μM) were isolated as described. Aliquots (50–90 μl) were incubated at 37°C (■), 56°C (□), 66°C (▲) and 72 °C (△). At the indicated times, samples (15 μl) were removed, chilled on ice and analysed as described for (B). For the 0 min time point, samples were immediately chilled on ice. The graph shows two independent time courses at each temperature. For incubations exceeding 60 min, the reaction mixtures were covered with paraffin oil. (**D**) Deproteinized intermediates (250 μl at 7 μM) were isolated as described except that 10 mM Tris–HCl (pH 7.5), 1 mM EDTA was used as elution buffer. The intermediates were left unchanged (■) or supplemented with 100 mM NaCl (□), or 16 mM $MgCl_2$ (△) and subdivided into aliquots (20 μl). The aliquots were incubated at 72°C for the times indicated, chilled on ice and analysed as described for (**B**). For the 0 min time point, intermediates in TE were immediately chilled on ice.

were more stable than others and reflect the different DNA forms observed by electron microscopy. To determine the influence of cations on the stability of the intermediates, incubations were performed at 72°C in either 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA (TE), TE supplemented with 100 mM NaCl or TE supplemented with 16 mM $MgCl_2$ (Figure 3D). We observed that the branch migration observed at 72°C was dependent upon the presence of either $MgCl_2$ or NaCl.

These results show that recombination intermediates made by RecA protein are unexpectedly stable and only show significant branch migration at elevated temperatures.

Re-initiation of strand exchange upon re-addition of RecA protein

To test whether strand exchange could be re-initiated, we added RecA protein and ATP back to the Sepharose-purified DNA fraction that contained the recombination intermediates, and incubated the mixture at 37°C. A time course of the reaction is presented in Figure 4A. We found that the recombination intermediates were converted into ^{32}P-labelled nicked circular and ^{32}P-labelled linear duplex DNA products (lanes a–f). These products are similar to those formed by the action of RecA protein on gapped and linear duplex DNA, the substrates used to prepare the recombination intermediates (as shown in Figure 1). However, the time course of the reaction was faster, and product formation occurred within 20 min of the re-addition of RecA protein (Figure 4A, lane c). This indicated that they were formed by RecA-mediated branch migration of the junction. In addition to the conversion of intermediates into products by RecA protein, we also observed the formation of DNA aggregates that could not enter the gel (Figure 4A, lanes c–f). These aggregates were most probably due to complex RecA-mediated interactions between recombination intermediates, linear duplex DNA and gDNA.

Re-initiation of strand exchange required stoichiometric amounts of RecA protein (>1 RecA monomer/4 nucleotides), as shown in Figure 4B. To determine whether the binding of RecA protein to the recombination intermediates was initiated at the single-stranded gap, intermediates were pre-incubated with a single-strand specific nuclease, exonuclease VII (Chase and Richardson, 1974), prior to the addition of RecA protein. The action of exonuclease VII prevented branch migration, showing that the single-stranded region served as the initiation site for RecA binding (data not shown).

To determine whether RecA-mediated branch migration could be distinguished from the complete recombination reaction (i.e. pairing and strand exchange), we compared the requirements of the two reactions in parallel. We found that both reactions required ATP, and this could not be

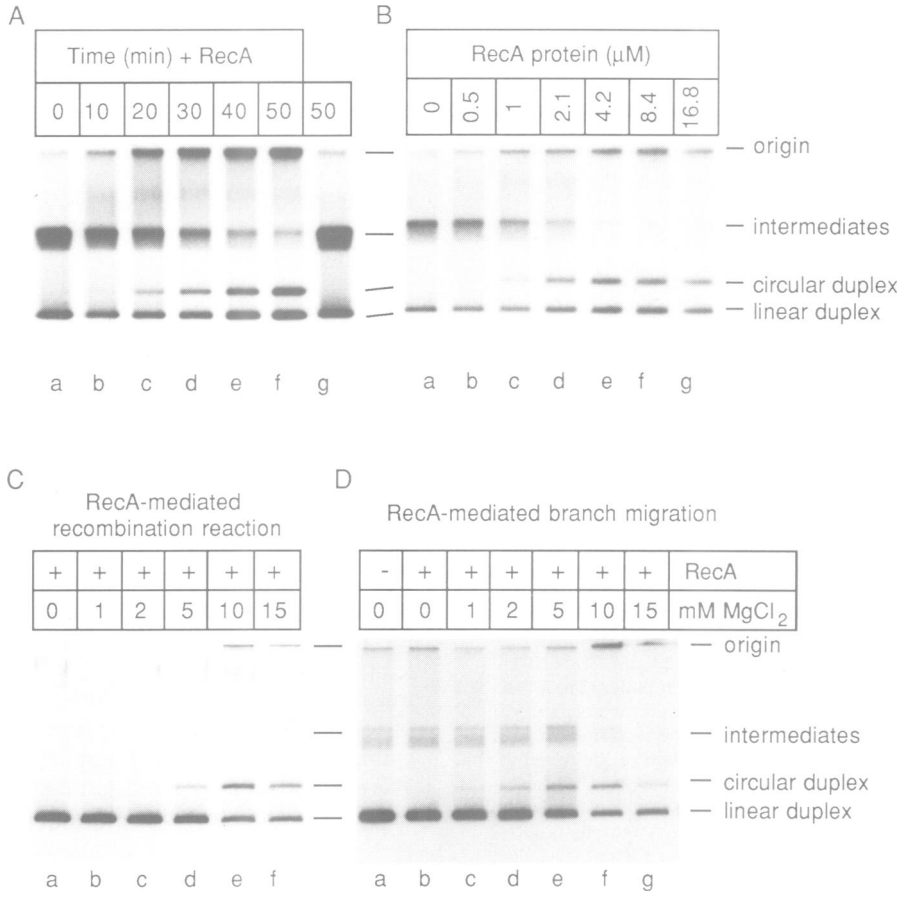

Fig. 4. Re-initiation of strand exchange by the addition of RecA protein to the recombination intermediates. (**A**) Time course. Recombination intermediates were deproteinized after 20 min of incubation as described in Materials and methods. 175 μl were supplemented with ATP (2 mM), creatine phosphokinase (6 units/ml), phosphocreatine (20 mM) and RecA protein (3.5 μM). The final DNA concentration was 7.6 μM (200 μl total volume). Lanes a–f: aliquots (25 μl) were stopped at the times indicated and analysed by agarose gel electrophoresis. Lane g: intermediates incubated for 50 min without RecA protein. (**B**) Requirement for RecA protein. Intermediates were deproteinized as described in Materials and methods and supplemented with ATP, creatine phosphokinase and phosphocreatine as described above. The final DNA concentration was 7.6 μM (400 μl total volume). Aliquots (50 μl) were supplemented with RecA protein as indicated and incubated for 60 min at 37°C. Then, the reactions were stopped and analysed by agarose gel electrophoresis. (**C**) Magnesium requirement of the RecA-mediated strand exchange reaction between gDNA and homologous linear duplex DNA. gDNA (5.2 μM), linear DNA (2.9 μM) and RecA protein (6.5 μM) were pre-mixed in 20 mM Tris–HCl (pH 7.5), 2 mM ATP, 2 mM dithiothreitol and 100 μg/ml BSA, and aliquots (20 μl) were adjusted to the indicated $MgCl_2$ concentrations. After 60 min at 37°C, reactions were stopped and the products analysed by agarose gel electrophoresis. (**D**) Magnesium requirement for re-initiation of strand exchange by RecA protein. Intermediates (200 μl at 8.1 μM) were purified as described in Materials and methods and aliquots (20 μl) were supplemented with 2 mM ATP and the indicated amounts of $MgCl_2$. Reactions were incubated for 60 min at 37°C following addition of RecA (6.5 μM), and analysed by agarose gel electrophoresis.

replaced by either ADP or ATPγS (data not shown). However, the magnesium requirements were different. The complete recombination reaction required 5–15 mM $MgCl_2$ (Figure 4C, lanes d–f), whereas RecA-mediated branch migration occurred at concentrations as low as 1 or 2 mM (Figure 4D, lanes c and d). The requirement for high levels of magnesium during RecA-mediated recombination reflects the need for the formation of large DNA aggregates for homologous pairing (Rusche *et al.*, 1985; Tsang *et al.*, 1985).

When re-initiation reactions were visualized by electron microscopy following fixation with glutaraldehyde, we observed the formation of a RecA nucleoprotein filament on each recombination intermediate (Figure 2E and F). However, although these experiments were carried out with excess RecA protein (2 RecA monomers/nucleotide), we found that only a part of each intermediate was covered by RecA protein, while much of the DNA appeared protein-free.

Processing of recombination intermediates by RuvA, RuvB and RuvC proteins of E.coli

Recent results indicate that the RuvA, RuvB and RuvC proteins of *E.coli* participate in the processing of recombination intermediates. Addition of RuvC protein to an on-going RecA-mediated strand exchange reaction was shown to result in the formation of recombinant DNA molecules (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991). We now show that deproteinized recombination intermediates serve as a substrate for RuvC protein (Figure 5, lane d). Addition of purified RuvC protein led to a decrease in the amount of recombination intermediates and to the formation of ^{32}P-labelled linear dimer DNA and ^{32}P-labelled nicked circular duplex DNA, the two products

expected from resolution (Connolly and West, 1990; Dunderdale *et al.*, 1991; Müller *et al.*, 1990).

A different form of processing was carried out by the RuvA and RuvB proteins. As shown in Figure 5, lane c, recombination intermediates were converted by the combined action of RuvA and RuvB proteins to ^{32}P-labelled nicked circular and ^{32}P-labelled linear duplex DNA. These products result from RuvAB-mediated branch migration which occurs in the presence of ATP. More detailed studies of the interaction of RuvA and RuvB proteins with recombination intermediates (Tsaneva *et al.*, 1992b) and synthetic Holliday junctions (Parsons *et al.*, 1992) are presented elsewhere.

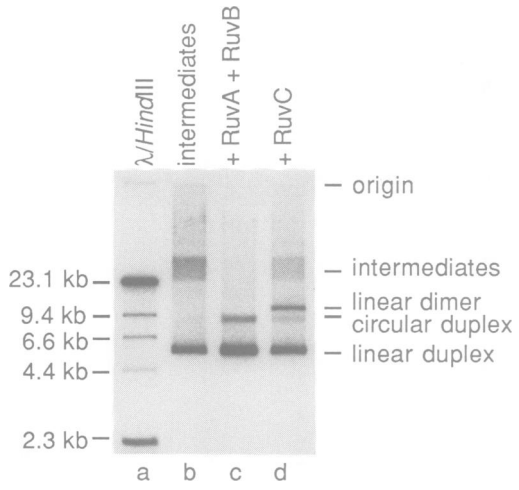

Fig. 5. Processing of recombination intermediates by RuvA, RuvB and RuvC proteins. Intermediates (220 μl at 4.8 μM) were purified as described in Materials and methods, except that 20 mM Tris–HCl (pH 7.5), 0.5 mM EDTA was used as the elution buffer. The DNA was supplemented with 15 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 100 μg/ml BSA. Aliquots (50 μl) were then supplemented with either purified RuvC protein (200 nM) or a mixture of RuvA and RuvB proteins (140 nM and 90 nM, respectively) as indicated, and incubated for 15 min at 37°C. Reaction products were analysed by agarose gel electrophoresis. Lane a: marker DNA fragments (λ DNA cleaved with *Hin*dIII and 3′ ^{32}P-end-labelled using Klenow fragment and [α-^{32}P]dATP).

Discussion

Most models for general genetic recombination propose that duplex DNA molecules are linked by a cross-over of single-strands known as a Holliday junction (Holliday, 1964; Meselson and Radding, 1975; Dressler and Potter, 1982; Szostak *et al.*, 1983). Movement of this junction, by a random process which requires no energy and occurs spontaneously, would lead to the formation of heteroduplex DNA. Using the theoretical treatment of Meselson (1972), it is possible to calculate the step rate of branch migration for DNA molecules of the length of φX174 DNA at a temperature of 37°C to be $\sim 5 \times 10^5$ bp/s. However, this treatment assumes that rotary diffusion is the limiting factor of branch migration and treats the DNA molecules as stiff cylinders. Using isolated figure-of-eight DNA molecules, Warner and colleagues determined the rate of branch migration experimentally (Thompson *et al.*, 1976; Warner *et al.*, 1978). At 10°C, they calculated a step rate of ~60 bp/s, at 30°C ~ 10 000 bp/s. At 50°C branch migration

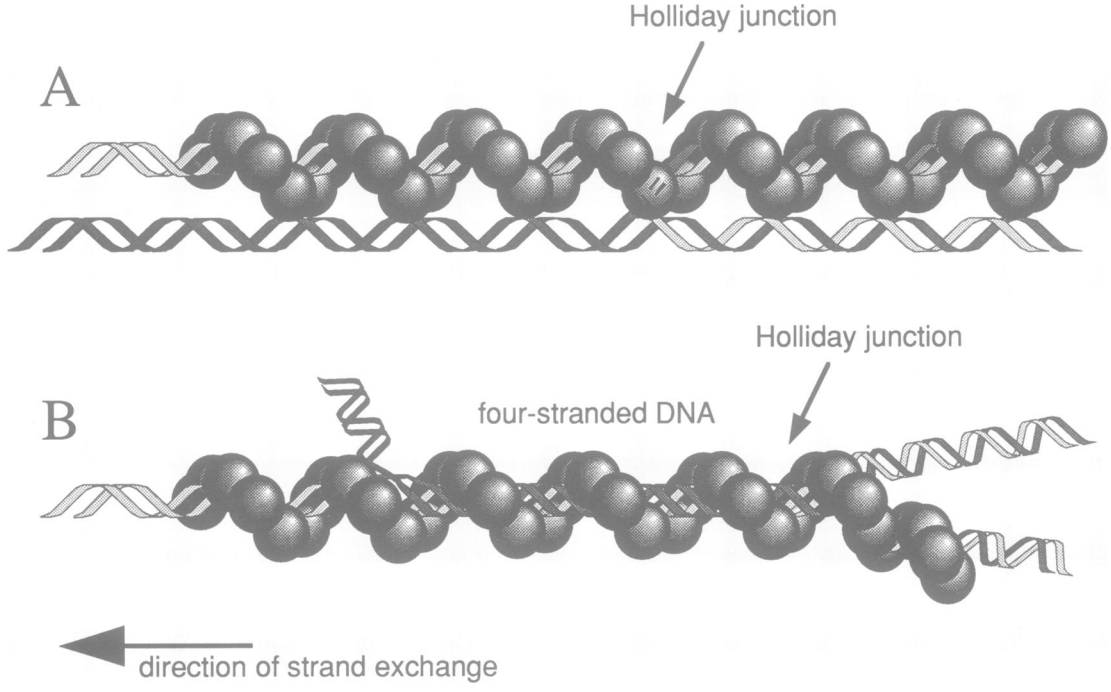

Fig. 6. Alternative possible configurations of RecA-mediated joints involving four strands of DNA. (**A**) A helical nucleoprotein filament formed between RecA protein and duplex DNA is connected to a homologous duplex by a Holliday junction. Additional homologous contacts occur at every turn of the helix. Rotation of the external DNA molecule around the nucleoprotein filament leads to heteroduplex formation by migration of the Holliday junction (Cox and Lehman, 1987). (**B**) Homologous duplex DNA is integrated into the helical nucleoprotein filament formed between RecA protein and duplex DNA, leading to the formation of a four-stranded recombination intermediate (Howard-Flanders *et al.*, 1984). Heteroduplex DNA formation could occur by rotation of the nucleoprotein filament along its longitudinal axis. In the drawing, RecA monomers are shown as shaded circles and only a few monomers are represented. In reality, the RecA nucleoprotein filament may extend over the entire DNA molecule. Similarly, the diameter of the nucleoprotein filament (100 Å) is underrepresented for diagrammatic purposes. Deproteinization of either structure (A) or (B) could lead to a classical Holliday junction.

occurred too rapidly to be measured. Since branch migration would be expected to progress by a random walk mechanism, they used the normal density function (Feller, 1968) to determine that the probability of a junction migrating at least 2450 bp within 10 min was 0.32 at 30°C (Warner *et al.*, 1978).

During homologous recombination, RecA protein polymerizes on DNA to form a right-handed helical nucleoprotein filament (for reviews see Howard-Flanders *et al.*, 1984; Stasiak and Egelman, 1988; West, 1992). The DNA lies along the longitudinal axis of the filament and is 1.5× longer than a B-form DNA helix. The primary function of the nucleoprotein filament is to bring two DNA molecules into homologous register, ready for strand exchange and the formation of heteroduplex DNA. Two models for the mechanism of strand exchange by RecA protein are shown in Figure 6. The first model (panel A) proposes that one DNA molecule lies within the nucleoprotein filament while the other is externally bound and linked to the first via a Holliday junction (Cox and Lehman, 1987; Roca and Cox, 1990). Deproteinization of this structure would be expected to lead to the classical representation of a Holliday junction in which two DNA molecules are linked by single-stranded cross-overs. However, recent studies of RecA-mediated strand exchange reactions between single-stranded and linear duplex DNA indicate that intermediates contain regions of triple-helical DNA (Hsieh *et al.*, 1990; Menetski *et al.*, 1990; Rao *et al.*, 1990, 1991). Surprisingly, triple helices made by RecA protein were found to be stable following deproteinization suggesting the presence of hydrogen bonds between the single-strand and the duplex DNA (Hsieh *et al.*, 1990; Rao *et al.*, 1990, 1991). The stability of the three-stranded structures led us to ask whether reactions involving four DNA strands could take place via a four-stranded DNA intermediate in which homologous DNA helices are interwound within the nucleoprotein filament (Figure 6B), as suggested by Howard-Flanders and coworkers (Howard-Flanders *et al.*, 1984; West *et al.*, 1981; West and Howard-Flanders, 1984). This structure may also be stabilized by additional non-Watson–Crick hydrogen bonds (Howard-Flanders *et al.*, 1984; McGavin, 1971, 1977; Wilson, 1979). In this model, strand exchange is proposed to occur by rotation of the nucleoprotein filament about its longitudinal axis (Radding, 1991). Deproteinization of the structure might be expected to lead either to a classical Holliday junction, or to a structure in which a region of four-stranded DNA would remain.

In the work described in this paper, we used RecA protein to prepare recombination intermediates (from gapped circular and linear duplex DNA) and these were deproteinized by treatment with proteinase K, EDTA and SDS, and the DNA purified by gel filtration chromatography. Double labelling experiments and electron microscopy showed that the recombination intermediates consisted of linear DNA molecules connected to circular DNA molecules. Interestingly, a number of different DNA forms were observed. We saw (i) DNA molecules that contained structures that resembled Holliday junctions (Figure 2B), (ii) structures in which there appeared to be a region at the junction point at which the two DNA molecules appeared to be aligned over a significant length (Figure 2C and D) and (iii) more complex structures which were uninterpretable. The region of DNA alignment at the junction point may indicate a stretch of four-stranded DNA in which the two duplexes are paired. Alternatively, it could simply be a consequence of the interwinding of the DNA at the site of the Holliday junction. The electron microscopic studies did not allow us to distinguish between these possibilities. The presence of dimer DNA molecules connected by a region of DNA alignment was also observed in preparations of G4 DNA isolated from *E.coli* cells (Fishel and Warner, 1986). Interestingly, upon linearization, these dimers were significantly more stable then the figure-of-eight DNA molecules used to determine the rates of branch migration (Thompson *et al.*, 1976; Warner *et al.*, 1978).

The thermal stability of the deproteinized recombination intermediates made by RecA protein was substantially greater than expected (Figure 3). Based on the treatment of branch migration described by Warner *et al.* (Thompson *et al.*, 1976; Warner *et al.*, 1978), assuming a step rate of 10 000 bp/s and using the same correlation between p and σ as these authors (i.e. $p^2/\sigma^2 = 1$ for a probability of 0.32 that a junction has migrated at least $2p$ bp), we calculated that the probability of a junction point migrating 5400 bp within 48.6 min at 30°C was 0.32. Therefore, after 48.6 min of incubation at 30°C, at least one-third of our recombination intermediates should have dissociated (assuming an extreme case in which branch migration through 5386 bp is required). At 37°C, the rate should be even faster. However, this was not the case and little or no dissociation was detected after 6 h at 37°C. Indeed, intermediates could still be detected following incubation at temperatures up to 72°C. Two explanations for these findings are possible: (i) spontaneous branch migration may be much slower then previously assumed or (ii) the recombination intermediates were connected by a structure that differs from a classical Holliday junction and therefore shows different thermal stability properties.

Since branch migration of junctions made by RecA protein is substantially slower than expected, we believe that the extension of heteroduplex DNA must be an actively driven process that requires the continued action of proteins. We found that the addition of stoichiometric amounts of RecA protein led to the re-initiation of branch migration, which progressed at normal rates in the presence of ATP and formed the expected products (Figure 4). The ability to study RecA-mediated branch migration without need for homologous pairing will now allow a direct study of the requirements for this reaction. Moreover, we found that recombination intermediates made by RecA protein could serve as substrates for two alternative types of processing events (Figure 5). Firstly, we found that the SOS-inducible recombination/repair proteins RuvA and RuvB could act together to promote branch migration. A detailed characterization of this ATP-dependent reaction is described elsewhere (Tsaneva *et al.*, 1992b). Secondly, we observed that purified RuvC protein (Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991), which is known to resolve recombination intermediates in the presence of RecA protein (Connolly *et al.*, 1991; Connolly and West, 1990; Dunderdale *et al.*, 1991), was able to act upon deproteinized intermediates and promote their resolution into recombinant DNA products. These results indicate that the role of RecA protein is to promote homologous pairing and strand exchange leading to the formation of recombination intermediates. These structures then serve as the substrate for other proteins which promote extensive heteroduplex formation and resolution into recombinant DNA products.

Materials and methods

Enzymes

The RecA (Müller *et al.*, 1990), RuvA (Tsaneva *et al.*, 1992a), RuvB (Tsaneva *et al.*, 1992a) and RuvC (Dunderdale *et al.*, 1991) proteins were purified as described; all concentrations refer to moles of monomeric protein.

DNA

^{3}H-labelled single-stranded DNA was prepared by phenol extraction of purified ϕX174 *am3* bacteriophage grown in the presence of [^{3}H]thymidine. Gapped circular duplex DNA (gDNA) and *Pst*I-linearized duplex DNA were prepared as described previously (Müller *et al.*, 1990). The linear duplex was 3′-^{32}P-labelled using terminal transferase and [α-^{32}P]ddATP. All DNA concentrations refer to moles of nucleotide residues.

Reaction conditions

RecA-mediated strand exchange reactions were performed in a buffer containing 20 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 2 mM dithiothreitol, 2 mM ATP, 20 mM phosphocreatine, 6 U/ml creatine phosphokinase and 100 μg/ml bovine serum albumin (BSA). RecA binding was facilitated by preincubation of gDNA (26 μM) with RecA protein (10 μM) for 5 min at 37°C. Recombination was then initiated by addition of 19 μM ^{32}P-end-labelled linear duplex DNA. Reactions were incubated at 37°C, stopped by addition of SDS (0.5%), EDTA (40 mM) and proteinase K (2 mg/ml) and incubated for 10–15 min at 37°C.

Deproteinization of recombination intermediates

After 20 min of incubation, large scale RecA-mediated strand exchange reactions (100–150 μl) were stopped and deproteinized with SDS, EDTA and proteinase K as described above. The mixture was then applied at room temperature to a 3.5 ml Sepharose CL-2B column (10 cm×0.7 cm) equilibrated with elution buffer [unless stated otherwise, this contained 20 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$, 2 mM dithiothreitol and 100 μg/ml BSA]. DNA was collected in fractions of two drops (~100 μl) and located by Cerenkov counting and gel electrophoresis. DNA concentrations were determined by quantification of ^{32}P-labelled DNA. Up to 14% of the recombination intermediates dissociated during this procedure.

Thermal stability studies

Recombination intermediates (200 μl at 8.5 μM) were deproteinized as described above except that the column buffer was 20 mM Tris–HCl (pH 7.5), 15 mM $MgCl_2$. Aliquots (25 μl) were incubated at various temperatures and times, chilled on ice and analysed by agarose gel electrophoresis.

Agarose gel electrophoresis

DNA samples were analysed on 0.8% agarose gels using 40 mM Tris–acetate (pH 8.0), 1 mM EDTA as the buffer system. Electrophoresis was performed at room temperature at 6 V/cm with buffer recirculation. Gels were dried and the DNA visualized by autoradiography on Fuji RX or Kodak XAR films. To measure the amount of DNA in each band, agarose gel electrophoresis was performed using low melting point agarose (FMC Bioproducts). The DNA was detected by staining with ethidium bromide and each band was excised. The agarose was melted at 75°C in 1 ml water, the mixture supplemented with 9 ml Aquasol scintillation fluid (DuPont) and the amounts of ^{32}P-labelled and ^{3}H-labelled DNA determined by scintillation counting.

Electron microscopy

Recombination intermediates were deproteinized using a Sepharose CL-2B column as described above, except that the elution buffer was 20 mM Tris–acetate (pH 7.5), 5 mM magnesium acetate. Prior to adsorption onto glow-discharged carbon supports, intermediates were diluted to a concentration of 3 μM in 5 mM magnesium acetate (Arcidiacono *et al.*, 1980). Samples were then dried in absolute ethanol, rotary shadowed with tungsten at an angle of 5° in a modified Leybold–Heraeus EPA-100 vacuum coating unit equipped with electron beam evaporators and a quartz-crystal film thickness monitor. Specimens were visualized in a JEOL JEM-1200 EX electron microscope operated at 80 kV.

To visualize nucleoprotein filaments formed between RecA protein and the intermediates, deproteinized intermediates (240 μl at 6.6 μM) were eluted from the Sepharose CL-2B column in 20 mM triethanolamine acetate (pH 7.5), 2 mM magnesium acetate, 2 mM dithiothreitol and 100 μg/ml BSA, adjusted to 2 mM ATP and supplemented with RecA protein (12 μM). Following incubation for 7 min at 37°C, complexes were fixed by addition of glutaraldehyde (to 0.25%) and incubated for further 45 min 37°C. Fixed complexes were then separated from free protein using a 1 ml Sepharose CL-2B column equilibrated with 5 mM magnesium acetate. Complexes were adsorbed onto carbon supports, shadowed and visualized as described above.

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References

Arcidiacono,A., Stasiak,A. and Koller,T. (1980) In Brederoo,P. and de Priester,W. (eds), *Proceedings of the 7th European Congress on Electron Microscopy*. Seventh European Congress on Electron Microscopy Foundation, Leiden, Vol. 2, pp. 516–523.

Benson,F.E., Illing,G.T., Sharples,G.J. and Lloyd,R.G. (1988) *Nucleic Acids Res.*, **16**, 1541–1550.

Chase,J.W. and Richardson,C.C. (1974) *J. Biol. Chem.*, **249**, 4545–4552.

Connolly,B. and West,S.C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8476–8480.

Connolly,B., Parsons,C.A., Benson,F.E., Dunderdale,H.J., Sharples,G.J., Lloyd,R.G. and West,S.C. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 6063–6067.

Cox,M.M. and Lehman,I.R. (1987) *Annu. Rev. Biochem.*, **56**, 229–262.

DasGupta,C., Wu,A.M., Kahn,R., Cunningham,R.P. and Radding,C.M. (1981) *Cell*, **25**, 507–516.

Dressler,D. and Potter,H. (1982) *Annu. Rev. Biochem.*, **51**, 727–762.

Dunderdale,H.J., Benson,F.E., Parsons,C.A., Sharples,G.J., Lloyd,R.G. and West,S.C. (1991) *Nature*, **354**, 506–510.

Feller,W. (1968) *An Introduction to Probability Theory and its Applications*, 3rd edition. Wiley, New York.

Fishel,R.A. and Warner,R.C. (1986) *Virology*, **148**, 198–209.

Holliday,R. (1964) *Genet. Res.*, **5**, 282–304.

Howard-Flanders,P., West,S.C. and Stasiak,A.J. (1984) *Nature*, **309**, 215–220.

Hsieh,P., Camerini-Otero,C.S. and Camerini-Otero,R.D. (1990) *Genes Dev.*, **4**, 1951–1963.

Iwasaki,H., Takahagi,M., Shiba,T., Nakata,A. and Shinagawa,H. (1991) *EMBO J.*, **10**, 4381–4389.

McGavin,S. (1971) *J. Mol. Biol.*, **55**, 293–298.

McGavin,S. (1977) *Heredity*, **39**, 15–25.

Menetski,J.P., Bear,D.G. and Kowalczykowski,S.C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 21–25.

Meselson,M. (1972) *J. Mol. Biol.*, **71**, 795–798.

Meselson,M.M. and Radding,C.M. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 358–361.

Müller,B., Jones,C., Kemper,B. and West,S.C. (1990) *Cell*, **60**, 329–336.

Parsons,C.A., Tsaneva,I.R., Lloyd,R.G. and West,S.C. (1992) *Proc. Natl. Acad. Sci. USA*, in press.

Radding,C.M. (1982) *Annu. Rev. Genet.*, **16**, 405–437.

Radding,C.M. (1991) *J. Biol. Chem.*, **266**, 5355–5358.

Rao,B.J., Jwang,B. and Radding,C.M. (1990) *J. Mol. Biol.*, **213**, 789–809.

Rao,B.J., Dutreix,M. and Radding,C.M. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 2984–2988.

Roca,A.I. and Cox,M.M. (1990) *Crit. Rev. Biochem. Mol. Biol.*, **25**, 415–456.

Rusche,J.R., Konigsberg,W. and Howard-Flanders,P. (1985) *J. Biol. Chem.*, **260**, 949–955.

Shinagawa,H., Makino,K., Amemura,M., Kimura,S., Iwasaki,H. and Nakata,A. (1988) *J. Bacteriol.*, **170**, 4322–4329.

Shurvinton,C.E. and Lloyd,R.G. (1982) *Mol. Gen. Genet.*, **185**, 352–355.

Stasiak,A. and Egelman,E.H. (1988) In Kucherlapati,R. and Smith,G.R. (eds), *Genetic Recombination*. American Society for Microbiology, Washington, DC, pp. 265–308.

Szostak,J.W., Orr-Weaver,T.L., Rothstein,R.J. and Stahl,F.W. (1983) *Cell*, **33**, 25–35.

Thompson,B.J., Camien,M.N. and Warner,R.C. (1976) *Proc. Natl. Acad. Sci. U.S.A*, **73**, 2299–2303.

Tsaneva,I.R., Illing,G.T., Lloyd,R.G. and West,S.C. (1992a) *Mol. Gen. Genet.*, in press.

Tsaneva,I.R., Müller,B. and West,S.C. (1992b) *Cell*, in press.
Tsang,S.S., Chow,S.A. and Radding,C.M. (1985) *Biochemistry*, **24**, 3226–3232.
Umlauf,S.W., Cox,M.M. and Inman,R.B. (1990) *J. Biol. Chem.*, **265**, 16898–16912.
Warner,R.C., Fishel,R.A. and Wheeler,F.C. (1978) *Cold Spring Harbor Symp. Quant. Biol.*, **43**, 957–968.
West,S.C. (1992) *Annu. Rev. Biochem.*, **61**, 603–640.
West,S.C. and Howard-Flanders,P. (1984) *Cell*, **37**, 683–691.
West,S.C., Cassuto,E. and Howard-Flanders,P. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 2100–2104.
West,S.C., Countryman,J.K. and Howard-Flanders,P. (1983) *Cell*, **32**, 817–829.
Wilson,J.H. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 3641–3645.

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