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Blocked RecA Protein-mediated DNA Strand Exchange Reactions Are Reversed by the RuvA and RuvB Proteins*

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RecA protein is unable to complete a DNA strand exchange reaction between a circular single-stranded DNA and a linear duplex DNA substrate with heterologous sequences of 375 base pairs at the distal end. Instead, it generates a branched intermediate in which strand exchange has proceeded up to the homology/heterology junction. Addition of the RuvA and RuvB proteins to these stalled intermediates leads to the rapid conversion of intermediates back to the original substrates. The reversal reaction is initiated at the branch, and the hybrid DNA is unwound in the direction opposite to that of the RecA reaction that created it. Under optimal conditions the rate of the reaction exhibits only a modest dependence on the length of hybrid DNA that must be unwound. Products of the reversal reaction are detected within minutes after addition of RuvAB, and appear with an apparent first order progress curve exhibiting a $t_{1/2}$ in the range of 6–12 min under optimal conditions. Few molecules that have undergone only partial reversal are detected. This suggests that the assembly or activation of RuvAB on the branched substrate is rate-limiting, while any migration of RuvAB on the DNA to effect unwinding of the hybrid DNA (and reformation of substrate DNA) is very fast. The results are discussed in the context of the role of RuvA and RuvB proteins in recombinational DNA repair. We suggest that one function of the RuvAB proteins is to act as an antirecombinase, to eliminate intragenomic crossovers between homologous segments of the bacterial chromosome that might otherwise lead to deleterious inversions or deletions.

The order of genes in the chromosomes of *Salmonella typhimurium* and *Escherichia coli* has been evolutionarily conserved during the millions of years since these groups diverged (Ochman and Wilson, 1987). This is despite the fact that powerful recombination systems exist in these cells, along with repeated genomic sequences (*e.g.* the seven rRNA operons) that might facilitate genomic deletions or inversions via intramolecular recombination. Evidence has appeared supporting the existence of mechanisms to prevent deleterious intragenomic recombination events in bacteria (Segall *et al.*, 1988).

A central component of the bacterial recombination system is the RecA protein (Roca and Cox, 1990; West, 1992; Clark and Sandler, 1994; Cox, 1994; Kowalczykowski *et al.*, 1994; Stasiak and Egelman, 1994). RecA promotes a DNA strand exchange reaction *in vitro* that is believed to mimic key aspects of RecA function *in vivo* (Fig. 1*A*). This reaction occurs in at least 4 distinct phases. The first phase, which is facilitated by the *E. coli* SSB[1] protein, is the formation of a RecA protein filament on the ssDNA. The second phase is the alignment of a homologous linear duplex DNA with the ssDNA in the nucleoprotein filament, a process that might involve the formation of a triple-stranded DNA pairing intermediate (Rao *et al.*, 1991; Stasiak, 1992; Camerini-Otero and Hsieh, 1993). In the third phase, the complementary strand of the duplex DNA is transferred to the ssDNA to form a short (1–2 kbp) region of hybrid dsDNA in a process that does not require ATP hydrolysis (Menetski *et al.*, 1990; Rehrauer and Kowalczykowski, 1993; Rosselli and Stasiak, 1990). This nascent hybrid DNA is extended in the fourth phase, a facilitated unidirectional branch migration (5′ to 3′ relative to the ssDNA) that is coupled to ATP hydrolysis (Jain *et al.*, 1994).[2]

The RuvA and RuvB proteins of *E. coli* are also important in DNA repair and homologous genetic recombination *in vivo* (Lloyd, 1991; Lloyd *et al.*, 1984, 1987; Luisi-DeLuca *et al.*, 1989; Otsuji *et al.*, 1974), functioning largely in the processing of branched recombination intermediates (Muller and West, 1994; West *et al.*, 1993). The RuvA and RuvB proteins are able to promote bidirectional branch migration of strand exchange intermediates (Tsaneva *et al.*, 1992b). Complexes of the RuvA and RuvB proteins exhibit a 5′ to 3′ helicase activity which may be integral to the mechanism by which these proteins promote branch migration (Tsaneva *et al.*, 1993). The RuvA protein binds to Holliday junctions and is believed to be a molecular matchmaker (Sancar and Hearst, 1993) for the RuvB protein (Müller *et al.*, 1993). The RuvB protein exhibits significant ATPase activity in the presence of RuvA and DNA and is believed to be the motor of the RuvAB protein complex (West, 1994). Electron microscopic studies have shown that the RuvB protein forms a double hexameric ring structure which encircles dsDNA (Stasiak *et al.*, 1994). More recent results indicate that the active functional unit of RuvB is a hexameric structure (Mitchell and West, 1994). An elementary picture of RuvAB action is that RuvA binds to a Holliday junction, RuvB binds to create a RuvAB complex, and then branch migration mediated by the RuvA and RuvB proteins occurs. Ultimately, the Holliday junction may be cleaved by the RuvC resolvase in a reaction facilitated by RuvA and/or B (Dunderdale and West, 1994; West, 1994).

The function of the RuvA and B proteins partially overlaps that of the RecG protein (Lloyd, 1991; Luisi-Deluca *et al.*, 1989). When added to a RecA protein-promoted DNA strand

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[1] The abbreviations used are: SSB, single-stranded DNA binding protein; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; AMT, 4′-amino-4,5′,8-trimethylpsoralen; bp, base pair(s); kbp, kilobase pairs.

[2] W. A. Bedale and M. M. Cox, manuscript submitted for publication.

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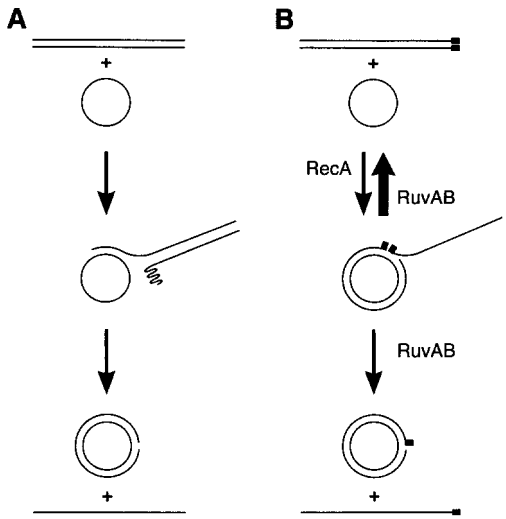


FIG. 1. **DNA strand exchange reactions promoted by RecA protein.** *A*, a standard 3-strand exchange reaction yielding a nicked circular hybrid dsDNA product and a displaced linear ssDNA. *B*, DNA strand exchange with a linear dsDNA substrate containing heterologous sequences (thick lines) at the distal end. The RecA-mediated reaction produces the branched intermediate shown, with further branch migration blocked by the heterology. Two pathways for processing of this intermediate by the RuvA and B proteins are shown: reversal of the RecA reaction to re-form the original substrates, or unwinding of the heterologous DNA to generate a circular dsDNA with a short single-stranded tail.

exchange reaction with homologous DNA substrates, RuvA and B presumably promote branch migration predominantly in the same direction as RecA protein does, enhancing the formation of completed products of DNA strand exchange (Whitby *et al.*, 1993). In contrast, the addition of RecG protein to a similar RecA protein-mediated DNA strand exchange reaction leads to its reversal. A model for the productive application of this seemingly antirecombinogenic activity of RecG protein in post-replication repair and recombination has been proposed by Lloyd and colleagues (Whitby *et al.*, 1993).

Genetic studies have shown that mutations in the *ruv*A or *ruv*B genes exhibit only modest effects on recombination unless they are in either a *recBC sbc* or a *recG* genetic background (Lloyd, 1991; Luisi-Deluca *et al.*, 1989). However, the same mutations in a wild type background produce dramatic increases in the sensitivity of bacterial cells to DNA damaging agents (Sharples *et al.*, 1990). These studies directly implicate the RuvA and RuvB proteins in the process of recombinational DNA repair. Recombinational DNA repair is complicated by a requirement for the bypass of structural barriers in the DNA. RecA protein alone promotes efficient bypass of lesions, mismatches, and short insertions of heterologous DNA sequence (up to 50–100 bp) during strand exchange (Cox, 1994). Addition of the RuvA and RuvB proteins greatly enhances the heterology bypass capability of RecA, permitting the bypass of medial heterologous inserts up to 1000 bp in length (Iype *et al.*, 1994).

In RecA-mediated DNA strand exchange involving duplex DNA substrates with heterologous sequences on the distal[3] (3′ relative to the ssDNA substrate) end, hybrid DNA is formed up to the heterology/homology junction (Jain *et al.*, 1994; Iype *et al.*, 1994). We investigated the fate of blocked RecA-mediated strand exchange reactions (where barrier bypass was precluded) when RuvA and B were added (Fig. 1*B*). We have found that the strand exchange reaction is efficiently reversed, much as all strand exchange reactions are reported to be in the presence of RecG protein. The results suggest a role for RuvA and B as an antirecombinase system, capable of reversing blocked recombination events that are potentially deleterious to the bacterial genome.

[3] In a normal RecA protein-mediated DNA strand exchange reaction, hybrid DNA is formed progressively in the 5′ to 3′ direction relative to the single-stranded DNA on which the RecA filament forms. The ends of the linear duplex DNA substrate are often defined as proximal and distal, referring to the ends where a productive strand exchange is generally initiated and terminated, respectively.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—*E. coli* RecA protein was purified and stored as described previously (Cox *et al.*, 1981). The RecA protein concentration was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 0.58\ A_{280}\ mg^{-1}$ ml (Craig and Roberts, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described (Lohman *et al.*, 1986) with the minor modification that a DEAE-Sepharose column was added to ensure removal of single-strand exonucleases. The concentration of SSB protein was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}$ mg^{-1} ml (Lohman and Overman, 1985). *E. coli* RuvA protein and RuvB proteins were purified as described (Iype *et al.*, 1994; Tsaneva *et al.*, 1992a). The concentrations of RuvA and RuvB proteins were determined by the method of Bradford (1976) with bovine serum albumin as the protein standard (Bio-Rad assay kit). For assays, the RuvA and/or RuvB proteins were diluted as needed into a RuvAB dilution buffer containing 20 mM Tris chloride buffer (75% cation), 1 mM EDTA, 10% glycerol, 0.5 mM dithiothreitol, 150 mM NaCl, and 100 μg/ml bovine serum albumin. The final pH of this dilution buffer after addition of all components was 7.9.

Restriction endonucleases, calf intestinal phosphatase, β-agarose, and T4 polynucleotide kinase were purchased from New England Biolabs. Tris buffer was purchased from Fisher Scientific. Proteinase K, creatine phosphokinase, phosphocreatine, ATP, and bovine serum albumin were purchased from Sigma. Amino-4,5′,8-trimethylpsoralen (AMT) was purchased from Calbiochem.

DNA—Duplex and ssDNA substrates were derived from bacteriophage M13 mp8 (7229 bp; Messing and Vieira (1982)). Bacteriophage M13 mp8.375 (7594 bp) is bacteriophage M13 mp8 with a 375-bp fragment (*Eco*RI-*Bam*HI fragment of pBR322) replacing the 10-bp *Eco*RI-*Bam*HI fragment of bacteriophage M13 mp8 (Bedale *et al.*, 1991). Supercoiled circular duplex DNA and circular single-stranded DNA from bacteriophage M13 mp8 and M13 mp8.375 were prepared using methods described previously (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). The concentration of dsDNA and ssDNA stock solutions were determined by absorbance at 260 nm, using 50 and 36 $\mu g\ ml^{-1}\ A_{260}^{-1}$, respectively as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Complete digestion of FI M13 mp8.375 with *Bam*HI restriction enzyme resulted in full-length linear duplex (FIII) DNA substrates that contain heterology located at the 3′ end of the duplex DNA, relative to the viral (+) strand. After digestion, residual protein was removed by 1:1 extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) followed by ethanol precipitation. Further digestion of the full-length duplex DNA with *Msc*I or *Alw*NI restriction enzymes resulted in 1526- or 4414-bp linear duplex (FIII) DNA substrates, respectively. They each include 375 bp heterology at the distal end of the duplex DNA, and either 1151 or 4039 bp of sequences homologous to M13 mp8. These fragments are referred to in the text as the 1.5- and 4.4-kbp substrates. Both linear duplex DNA fragments were isolated from preparative low melting agarose gel using β-agarose according to the manufacturer's directions. Phosphatasing of linear duplex DNA with calf intestinal phosphatase and 5′-^{32}P-end labeling of linear duplex DNA with T4 polynucleotide kinase were performed as described (Sambrook *et al.*, 1989).

Strand Exchange Reaction Conditions—Reactions were performed at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation), 10 mM magnesium acetate, 3 mM potassium glutamate, 2 mM dithiothreitol, 5% glycerol, 100 μg/ml bovine serum albumin, and an ATP regenerating system (12.5 units ml^{-1} creatine phosphokinase, 15 mM phosphocreatine). The final pH after addition of all reaction components was 7.45. Duplex DNA and ssDNA, both at 21 μM, were preincubated at 37 °C with 7 μM RecA protein for 10 min before ATP (6 mM) and SSB (2 μM) were added to initiate the reaction. RuvA and RuvB proteins were mixed and left on ice for at least 30 min and then added to reactions as appropriate. Unless otherwise noted, the addition of RuvA and RuvB occurred 60 min after the addition of ATP and SSB.

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The final concentrations of RuvA protein and RuvB protein were 0.3 and 1 μM, respectively, unless noted otherwise in the text. If RecA protein converts all of the linear duplex substrate to the expected branched DNA intermediate, these RuvAB concentrations represent a 50-fold excess of RuvB hexamers relative to available DNA branch points.

In one series of reactions, the 1.5-kbp linear duplex DNA substrate was utilized. The strand exchange reaction conditions described above were followed for these reactions with one exception. The duplex DNA final concentration in these reactions was 4.4 μM while the ssDNA final concentration was 21 μM. The reduced duplex DNA concentration maintained a 2-fold excess of circular ssDNA molecules to linear duplex DNA molecules.

Agarose Gel Assays—Aliquots (15 μl) of strand exchange reactions described above were removed at each time point, and the reactions were stopped by the addition of 5 μl of gel loading SDS buffer (30% glycerol, 0.03% bromphenol blue, 30 mM EDTA, 4% SDS). Samples were electrophoresed in a 0.8% agarose gel.

Cross-linking DNA during the Strand Exchange Assay—In the experiments presented in Figs. 6 and 7, strand exchange reactions were performed essentially as described with the important addition of a DNA cross-linking step prior to RuvAB addition. At 60 min after the addition of ATP and SSB, the reaction mixture was mixed with AMT (0.5 or 1.0 μg ml^{-1}, final concentration, as indicated), incubated at 25 °C for 3 min, and irradiated with long-wave UV light for 4 min at 25 °C. The reaction was then transferred to 37 °C and incubated in the dark during subsequent steps of the reaction. The RuvAB proteins were added and samples of the strand exchange reaction were removed at appropriate time points and electrophoresed as described above. Samples of the strand exchange reaction were also prepared for electron microscopy as described below.

The AMT concentration used in these experiments was determined empirically, with the intention of introducing about one cross-link per DNA molecule. A titration was carried out in which reversal reactions were done after cross-linking with various concentrations of AMT. The concentration chosen was the one that resulted in about a 50% reduction in the amount of intermediate converted back to substrate. As noted under "Results," the cross-linking density in the experiments was somewhat greater than one cross-link per DNA molecule.

Electron Microscopy—Samples for electron microscopy were obtained by spreading the entire strand exchange reaction mixture. Reaction mixtures were cross-linked with AMT prior to examination by electron microscopy to prevent spontaneous branch migration, if such could occur, during sample preparation. For these reactions, aliquots (15 μl) of the strand exchange reaction were mixed with AMT (30 μg ml^{-1}, final concentration), incubated at 25 °C for 3 min, and irradiated with long-wave UV light for 4 min at 25 °C (Jain *et al.*, 1992). Cross-linked DNA samples were incubated with proteinase K (1 mg ml^{-1} final) and SDS (0.9% final) for 60 min at 37 °C. The samples were dialyzed into 20 mM NaCl and 5 mM EDTA overnight at 25 °C on Millipore type VM (0.05 μm) filters and were then spread as described previously (Inman and Schnös, 1970). Photography and measurements of the DNA molecules were performed as described previously (Littlewood and Inman, 1982).

The length of DNA exchanged in intermediates from strand exchange reactions was estimated for a representative sample of intermediates. Because of the large number of samples, obtaining accurate measurements of significant numbers of intermediates in all of the samples was impractical. The ratio of the exchanged DNA region (length of the ds segment within the circle) to the unexchanged DNA region (length of ds tail) was visually judged (Jain *et al.*, 1994). This ratio is converted to the length of DNA exchanged by using the total number of base pairs in the linear duplex DNA. For convenience, the linear duplex DNA was divided into 8 segments which each represent 1000 bp (the first segment at the proximal end is only 500 bp). The estimates of DNA exchanged were then sorted by 1000-bp segments. Data was plotted as a percentage of total intermediates per 1000 bp of DNA exchanged (see Figs. 3 and 5). For the data presented in Fig. 5, the grids from this reaction were assigned an undescriptive identification code (by L. E. I.) and provided in a random sequence (to R. B. I.) without further identification. The length of DNA exchanged in the intermediates was then estimated (by R. B. I.).

Kinetics Assays—Strand exchange reactions with the 1.5-kbp linear duplex substrate were performed as described above except that radiolabeled linear duplex (FIII) substrate was utilized. After samples were electrophoresed in 0.8% agarose gels, the gels were dried and the bands were visualized by autoradiography and by PhosphorImager (Molecular Dynamics). For each time point the quantity of radiolabeled DNA in the substrate (S) and intermediate (I) bands were quantitated using ImageQuant software (Molecular Dynamics). In order to correct for any variability in sample loading onto the agarose gel, a ratio of the I and S bands was determined. The zero time point for the RuvAB reaction was the time point 60 min after the initiation of the RecA reaction. At this zero time point the sample contained a small amount of unreacted linear duplex DNA substrate which was designated S_o. The fraction of reaction intermediate remaining was calculated as $I/[I + (S\text{-}S_o)]$. Data was plotted as $I/[I + (S\text{-}S_o)]$ *versus* time after RuvAB addition.

Assays with the 7.5-kbp linear duplex substrate were quantified differently. The DNA was not radiolabeled. Samples were electrophoresed on a 0.8% agarose gel. The gel was stained with ethidium bromide and photographed over a ultraviolet transilluminator. The intensities of DNA bands were quantified by scanning the photographic negatives using a Molecular Dynamics Personal Densitometer SI and analyzing the scanned image with ImageQuant software.

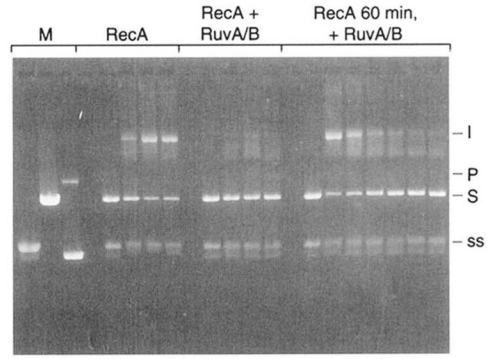


FIG. 2. **Reversal of a blocked DNA strand exchange reaction by RuvA and RuvB proteins.** Reactions were carried out as described under "Experimental Procedures." The substrates were circular M13 mp8 ssDNA and linear duplex M13 mp8.375 DNA, the latter cleaved to place the 375-bp heterologous sequence at the distal end. Symbols are: *I*, branched reaction intermediates resulting from a stalled strand exchange reaction; *P*, the product (a circular dsDNA with a short single-stranded tail) that would be generated if the heterologous duplex tail in I were unwound; *S*, linear duplex substrate DNA; *ss*, circular ssDNA substrate. The M13-derived circular ssDNA substrate migrated as a doublet in this gel system, even though greater than 90% of the circles were intact. The marker lanes (*M*) contain, from left to right, circular ssDNA substrate, linear duplex substrate (cleaved M13 mp8.375), and supercoiled M13 mp8. A minor band of nicked circular M13 mp8 is also present in the third marker lane, migrating at the position labeled P. The first reaction is one with RecA alone, with time points at 0, 30, 60, and 90 min, left to right. The second reaction and time course is identical to the first, except that RuvA and RuvB proteins were added simultaneously (to final concentrations of 0.3 and 1 μM, respectively) immediately after initiating strand exchange by addition of SSB and ATP. In the final reaction, the first two lanes represent a RecA-mediated strand exchange at 0 and 60 min. RuvA and B were then added at the concentrations described above, and the remaining 5 lanes show the progress of the reaction 5, 10, 15, 20, and 25 min later, respectively.

RESULTS

RuvA and RuvB Proteins Promote a Reversal of Blocked RecA Protein-mediated DNA Strand Exchange Reactions—A strand exchange reaction between circular ssDNA and linear duplex DNA containing a 375-bp heterologous DNA insertion at the distal end is shown in Fig. 2. The expected product of this reaction, if it went to completion, is a nicked circular duplex with a 375-nucleotide single-stranded tail. This DNA species, designated P, is not generated to any appreciable extent in the reaction with RecA and SSB alone. However, a prominent DNA band indicative of branched strand exchange intermediates is present (Fig. 2, reaction A, species designated *I*). When the RuvA and RuvB proteins were added immediately after the initiation of RecA-mediated DNA strand exchange with these substrates, there was a very slight increase in the amount of P observed in the gel (Fig. 2, reaction B). The more significant effect was the reduction in the amount of I coupled with an increase in substrates when compared with the reaction without Ruv proteins. This result contrasts with the negligible

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effect of RuvA and B on RecA-mediated strand exchange reactions with completely homologous DNA substrates (Iype *et al.*, 1994). The RuvA and RuvB proteins promoted this reduction in intermediates and increase in substrates even when added quite late in the reaction. A strand exchange reaction was initiated with RecA and SSB and allowed to progress for 60 min (Fig. 2, reaction C). At this point, there is a prominent DNA band corresponding to I. Continued incubation of the intermediates generated from the RecA reaction over an additional 60-min period showed that the intermediates were stable and did not dissociate to form either substrates or the nicked circular product with a ssDNA tail (data not shown). After the 60-min RecA reaction, RuvA and RuvB proteins were added and time points were taken over a 25-min incubation period. The quantity of the intermediate diminished and the quantity of substrate increased substantially after RuvA and RuvB protein addition. This result indicates that the RuvA and RuvB proteins are converting intermediates to substrates and thereby reversing RecA protein-mediated DNA strand exchange. Intermediates from RecA protein-mediated strand exchange reactions with duplex substrates containing different distal heterologous DNA insertions (198 bp or 1037 bp) were also converted to substrates by the RuvAB proteins (data not shown).

At relatively high concentrations, the RuvB protein promotes branch migration on deproteinized strand exchange intermediates independently of RuvA (Tsaneva *et al.*, 1992b). However, addition of RuvB protein concentrations ranging from 0.2 to 2 μM RuvB protein (in a RecA-mediated strand exchange reaction with 21 μM DNA substrates) did not reverse strand exchange, indicating that the requirement for RuvA protein cannot be circumvented under the conditions of the present experiments (data not shown).

Strong RuvAB-mediated reversal of DNA strand exchange occurs only when the 375-bp heterologous insert is located on the distal end of the linear duplex DNA substrate. When the insert was 673 bp to over 6000 bp from the distal end, the major reaction upon RuvAB addition was insertion bypass (Iype *et al.*, 1994). When the insertion was 169 bp from the distal end, bypass was reduced but reversal did not substantially increase (data not shown). We do not know if larger heterologous insertions would alter this result.

Analysis of Reverse Strand Exchange Reactions by Electron Microscopy—A complementary analysis of the strand exchange reaction described above was carried out by electron microscopy. A strand exchange reaction was initiated with RecA and SSB and allowed to progress for 60 min. RuvA and RuvB proteins were then added to the reaction and time points were taken over a 20-min incubation period. Molecules typical of those observed are shown in Fig. 3, *panels A-F*. The prominent intermediate species is shown in *panels A* and *B*. These intermediates characteristically have a short double-stranded tail and a long single-stranded tail with lengths consistent with a strand exchange reaction that has proceeded up to the distal heterologous sequences as shown schematically in Fig. 1*B*. These intermediates represented 60% of the duplex DNA-containing species 60 min after the RecA reaction had begun. *Panels C-F* show species generated by processing the intermediates shown in *panels A* and *B*. *Panels C* and *D* show duplex circles with a short ssDNA tail, a species created when the 375-bp heterologous sequence (the duplex DNA tail in *panels A* and *B*) was unwound. *Panels E* and *F* illustrate the linear duplex and circular ssDNA substrates that are generated by a reversal of DNA strand exchange. The species in *panel G* is described below.

The different DNA species present at several time points, beginning with the 60-min RecA reaction just prior to RuvAB addition (defined as time 0), were counted and the percentage of the duplex DNA molecules present in various forms (DNA substrates or intermediates) in each of the samples was calculated (Fig. 4). In addition to the intermediates present at time 0 (60% of the total), 18% of the duplex-containing molecules were unreacted linear substrates. Another 13% of the linear duplexes were interacting at a single internal point with circular ssDNA, with no obvious strand exchange (point contacts). The remaining 9% of the molecules were either complex (*e.g.* one circular ssDNA interacting with two linear duplexes) or represented broken molecules. The fraction of each sample present as complex or broken molecules was similar in subsequent time points. The tabulation in Fig. 4 of the fraction of duplex DNA present as identifiable intermediates or substrates shows that the intermediates are converted back to substrates with an apparent $t_{1/2}$ of 4–5 min. Since the reactions are not stopped abruptly at the time indicated (the cross-linking procedure used for each sample, incubation with AMT plus UV irradiation, takes 7 min) the actual $t_{1/2}$ is probably somewhat longer. DNA species in which the 375-bp tail was unwound (Fig. 3, *C* and *D*), were also observed after RuvAB addition, but at very low levels (data not shown). Approximately 90% of the intermediates had been converted to substrate in a sample taken 20 min after RuvA and RuvB protein addition, indicating that the RuvA and RuvB proteins can rapidly reverse strand exchange.

A control was carried out in which RuvAB dilution buffer was added to the RecA reaction at 60 min instead of the RuvAB proteins. After further incubation for 60 min, virtually all the substrate DNA was still present as intermediates, and the branch point in all of the intermediates was at the homology/heterology junction (data not shown). This indicates that the reversal of DNA strand exchange is completely RuvAB-dependent.

In order to characterize the action of the RuvA and RuvB proteins on intermediates generated during RecA protein-mediated strand exchange, we decided to examine the length of DNA exchanged in the intermediates that still remained at each time point (Fig. 4). The majority of the intermediates present before RuvAB addition are halted at the homology/heterology junction. However, there is a minor population with a variable length of hybrid DNA among the intermediates. Surprisingly, the distribution of the lengths of hybrid DNA in the intermediates did not change significantly after RuvAB addition, even as the total number of intermediates decreased. The histograms show that the majority of the intermediates remaining at each time point still contained hybrid DNA up to the homology/heterology junction, even after 20 min of reaction. Intermediates which were in the process of being converted to substrate (which should have shorter lengths of hybrid DNA) were rare at all time points. We either saw the initial stalled intermediate or the final substrate but little in between.

We considered the possibility that an activity in the RuvA and RuvB protein preparations might cleave the intermediate produced in the strand exchange reaction to regenerate a linear duplex DNA with the appearance and length of a substrate molecule. A strand exchange reaction between 7.2 kb circular M13 mp8 ssDNA and a 4.4-kbp linear duplex DNA (with a 375-bp heterologous sequence on the distal end) was carried out, subjected to RuvAB reversal, and examined by electron microscopy (data not shown). If the intermediate in the reaction were cleaved by the RuvA and RuvB proteins, cleavage of the intermediate generated with the 4.4-kbp substrate would generate linear dsDNA molecules which had 4.4 kbp of dsDNA

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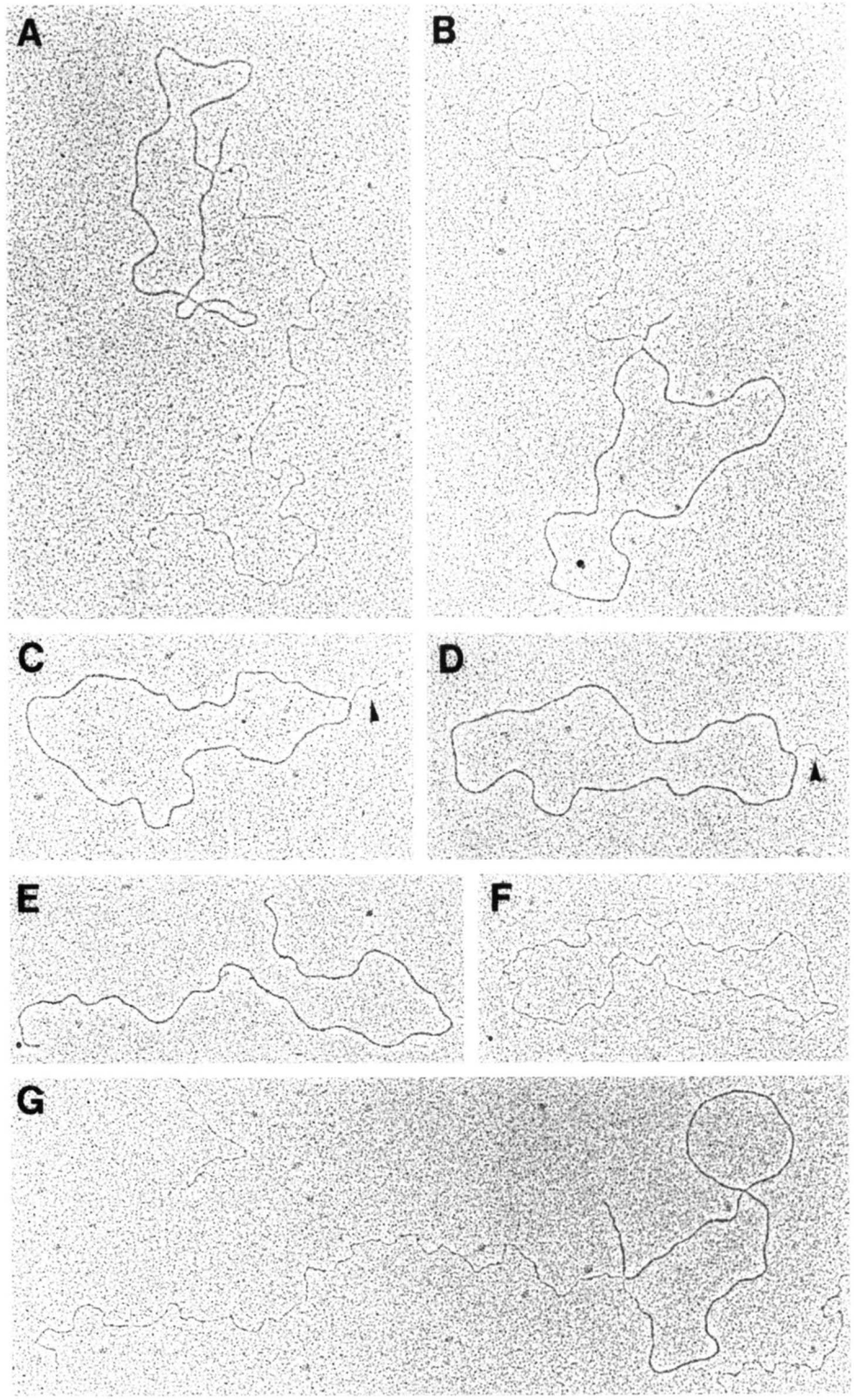


FIG. 3. **DNA species observed in these reactions by electron microscopy.** *Panels A* and *B* show typical blocked strand exchange intermediates. *Panels C* and *D* show strand exchange products generated by unwinding the short double-stranded tail seen in *panels A* and *B*. The short single-stranded tails in the circular duplex product are indicated by the *arrows*. *Panels E* and *F* show the linear duplex and circular ssDNA substrates, respectively. *Panel G* shows an intermediate in the process of a reversal reaction, with reversal blocked by an AMT cross-link. Note the longer double-stranded tail.

and a 2.8-kb ssDNA tail. However, the only linear dsDNA molecules observed were full-length 4.4-kbp linear duplex DNA. We did not observe any molecules that could be interpreted as arising from a DNA cleavage reaction.

Determining the Direction in Which Intermediates Are Unwound to Produce Substrates—In principle, the intermediates generated by RecA protein could be converted to substrates by unwinding the hybrid DNA from either end. The results described above indicated that the intermediates were rapidly converted to substrates. In order to "catch" the intermediates in the process of being reversed to substrate by the RuvA and RuvB proteins, we decided to cross-link the hybrid DNA in the intermediates at the end of the strand exchange reaction but before the addition of RuvA and B. A low concentration of AMT, selected empirically (see "Experimental Procedures"), was added to a RecA protein-mediated strand exchange reaction which had progressed for 60 min and then the reaction was UV cross-linked. These randomly placed cross-links would be expected to block a complete reversal reaction mediated by RuvA and B. Reversal would proceed up to the cross-link, creating a population of intermediates with more varied lengths of hybrid DNA. If this procedure added one cross-link per DNA molecule

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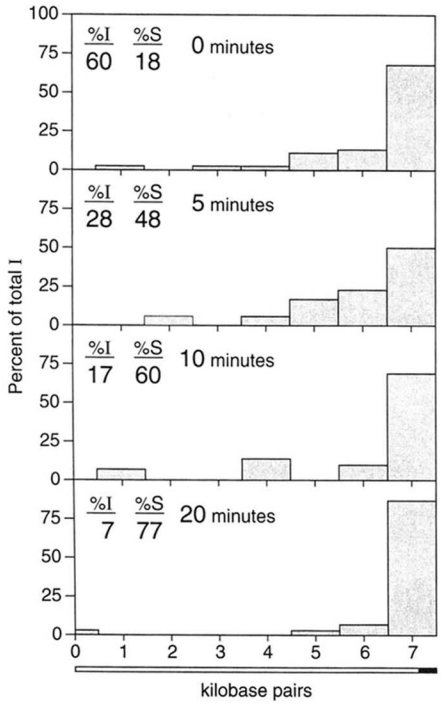


FIG. 4. **Analysis of a reversal reaction by electron microscopy.** A RecA-mediated DNA strand exchange reaction was carried out as described under "Experimental Procedures" for 60 min, followed by addition of RuvA and B proteins to 0.3 and 1 μM, respectively. Panels from top to bottom show successive time points after addition of RuvA and B. The fraction of the duplex DNA present as I or S is noted in each panel. Only the intermediates were measured and included in the histograms. The lengths of the hybrid DNA in individual intermediates were judged as described under "Experimental Procedures," and plotted relative to the DNA scale for the substrate duplex DNA shown at bottom. In the scale drawing, the solid segment at the right end indicates the 375-bp heterologous sequence. At least 30 intermediates were judged to generate each histogram.

on average, there would be a distribution of molecules in which 37% had one cross-link, 26% had more than one cross-link, and 37% had no cross-link. The results described below indicate that the degree of cross-linking was somewhat greater.

RuvA and RuvB proteins were added to the reaction and time points were taken during a 60-min incubation. Fig. 5 shows a comparison between a normal reversal reaction and a reaction with intermediates that had been cross-linked. A typical RuvA and RuvB reversal reaction shows reduction of the intermediate band and an increase in the substrate band (reaction A). In the cross-linked reversal reaction (reaction C), the intermediate band decreases slightly and the substrate band increases minimally. The large intermediate band indicated that the RuvA and RuvB proteins were unable to complete the reversal reaction. Surprisingly, there was a large increase in the nicked circle product with a short single-strand tail in the cross-linked reversal reaction (species labeled P). In order to ascertain if the AMT affected the RuvA and RuvB reversal reaction, AMT was added to a RecA strand exchange reaction after 60 min but the reaction was not irradiated to induce cross-linking (Fig. 5, reaction B). The RuvA and RuvB proteins were then added to the reaction. The results demonstrate that the RuvA and RuvB reversal reaction is not affected by AMT unless it is irradiated to produce cross-links. Another control was done in which RecA-bound, cross-linked intermediates were incubated further for 60 min without addition of RuvAB. This produced no change in the distribution of DNA between the intermediate and substrate bands (data not shown).

These reactions were examined by electron microscopy. The lengths of the hybrid DNA in the intermediates were judged

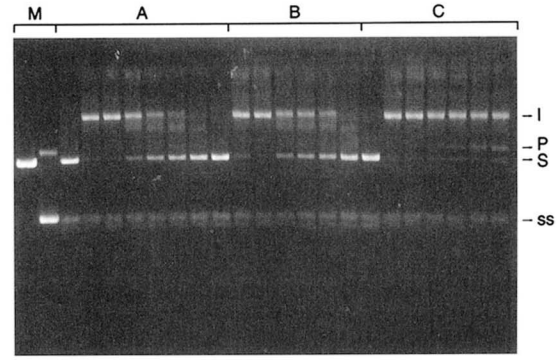


FIG. 5. **Effect of AMT cross-links on RuvAB-mediated reversal of DNA strand exchange.** Reactions were carried out as described under "Experimental Procedures," with the substrates used in Figs. 2 and 3. The symbols are as in Fig. 2. The first and second marker lanes (*M*) contain linear duplex substrate (cleaved M13 mp8.375), and supercoiled M13 mp8, respectively. A minor band of nicked circular M13 mp8 is also present in the second marker lane, migrating at the position labeled P. In each reaction, the first three time points are, from left to right, 0, 60, and 67 min after initiation of a RecA-mediated DNA strand exchange. RuvA (to 0.3 μM) and RuvB (to 1 μM) proteins were then added. The last four lanes show the reaction at 5, 10, 20, and 60 min after addition of RuvAB. In reaction A, no AMT was added. In reaction B, 1 μg ml^{-1} AMT was added at 60 min after the RecA reaction was initiated, but the samples were not irradiated to generate cross-links. Reaction C is the same as B, but the reaction was irradiated to generate cross-links after the 60-min time point, immediately prior to the addition of RuvA and B. In reaction C, the 67-min time point is the first sample containing cross-links.

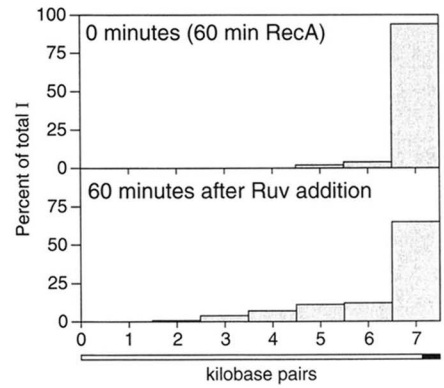


FIG. 6. **Lengths of hybrid DNA in intermediates undergoing reversal, with reversal blocked by AMT cross-linking.** Reactions were carried out as described in the legend to Fig. 5, reaction C, except that the AMT concentration was reduced to 0.5 μg ml^{-1}. Lengths of hybrid DNA were judged and plotted in a histogram as described under "Experimental Procedures" and in the legend to Fig. 4. The *top panel* shows the distribution of the lengths of hybrid DNA in intermediates generated by a RecA-mediated DNA strand exchange reaction immediately after cross-linking but before RuvAB addition. The *bottom panel* shows the same reaction 60 min after addition of RuvA and RuvB proteins (to 0.3 and 1 μM, respectively).

with the results shown in Fig. 6. The *top panel* shows the distribution of the length of hybrid DNA present in the cross-linked intermediates before RuvAB addition. The majority of the intermediates have halted at the homology/heterology junction. The *bottom panel* shows the distribution of the lengths of hybrid DNA in the cross-linked intermediates 60 min after RuvA and RuvB protein addition. The histogram shows that there is a greater distribution in the lengths of hybrid DNA in these intermediates, as expected for a reversal reaction blocked by random cross-links. A typical intermediate that has been partially reversed in this experiment is shown in Fig. 3*G*. The length of the dsDNA tail in the intermediate is longer than the ds tail in the stalled intermediates shown in Fig. 3, *panels A*

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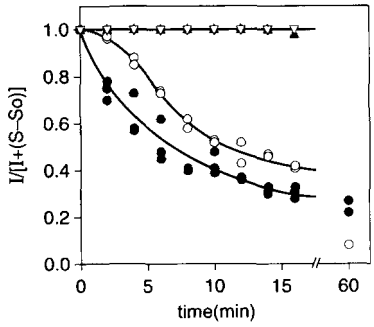


FIG. 7. **Kinetics of RuvAB-mediated reversal of DNA strand exchange.** Reactions were carried out under standard reaction conditions as described under "Experimental Procedures." RecA protein-mediated DNA strand exchange proceeded for 60 min, with the 7.6 (○) or 1.5 (●) kbp linear duplex DNA substrates. Reversal reactions were initiated by addition of RuvA and RuvB proteins, and data is plotted from that time point. In control reactions (*open* and *closed triangles* for reactions with the 7.6- and 1.5-kbp linear duplex, respectively), RuvAB dilution buffer was added in place of the RuvA and RuvB proteins.

and *B*. All of the intermediates examined had this structure, in which hybrid DNA extends to the proximal end. The data sets for the two histograms in Fig. 6 were compared by the χ^2 contingency test at the 95% confidence level and found to be significantly different ($p = 7.6 \times 10^{-7}$). The data therefore support the idea that the RuvA and RuvB proteins are reversing strand exchange by unwinding the hybrid DNA in the 3′ to 5′ direction, the direction opposite to that of RecA protein-mediated strand exchange.

Kinetics of the Reversal Reaction—To better characterize the kinetics of the reversal reaction, the process was monitored with the agarose gel assay and quantified as described under "Experimental Procedures." After the RuvA and RuvB proteins were added to a reaction, samples were removed at 2-min intervals for the first 16 min of the reaction. Results are presented in Fig. 7. In the first set of reactions, the intermediates contain 7.23 kbp of hybrid DNA, with the strand exchange blocked by the standard 375-bp heterologous sequence on the distal end of the substrate. In the two experiments with this DNA substrate plotted in Fig. 7, there is lag of about 2 min before the substrate band begins to increase; the RuvAB-mediated reversal then proceeds with a $t_{1/2}$ of approximately 10 min. After 60 min, approximately 90% of the intermediates have been converted back to substrate. A control reaction in which the RuvAB protein dilution buffer was added in place of the RuvA and RuvB proteins showed no conversion of intermediates back to substrates (Fig. 7). The 2-min lag (during which time the intermediate band sometimes intensified) was observed with many, but not all of the reactions using these substrates. In other cases, the conversion of intermediates to substrates was evident at the 2-min time point, and the progress curve approximated that of a first order reaction throughout its length (data not shown).

Mitchell and West (1994) have reported that incubation conditions affect the oligomerization state of RuvB protein. With 15 mM Mg^{2+}, the RuvB dodecamer predominates, while with 15 mM Mg^{2+} and ATP the RuvB hexamer predominates. We raised the Mg^{2+} concentration to 15 mM in a series of RecA-mediated strand exchange reactions, and observed no significant effects on RecA-mediated DNA strand exchange. Extended preincubation of RuvA and B in the higher Mg^{2+} conditions at 37 °C, with or without 1 mM ATP, also did not significantly affect the rate of reversal (data not shown).

In order to determine if the kinetics of the reversal was dependent on the length of the hybrid DNA in the intermediate, a shorter DNA fragment was used as a substrate. The reversal of intermediates generated from a strand exchange reaction between circular ssDNA and a 1.5-kbp linear duplex DNA containing a 375-bp heterologous DNA insertion at the distal end is also shown in Fig. 7. Approximately 6–8 min after RuvA and RuvB protein addition, 50% of the intermediates were converted to substrate (Fig. 7). After 60 min, nearly 80% of the intermediates were converted back to substrate. The results indicate that the kinetics of the reversal reaction are only minimally affected by a 5-fold change in the length of hybrid DNA to be unwound to regenerate substrate DNA.

The concentrations of RuvA and RuvB proteins used in most of these experiments (0.3 and 1 μM, respectively) were found to be optimal for the reversal reaction. An increase in RuvB concentration to 4 μM did not increase the rate of reversal (data not shown). Substantial reversal reactions were observed for RuvA and RuvB concentrations down to 50 and 300 nM, respectively, although the rates of the appearance of substrate DNA generally slowed as RuvAB concentration decreased. When the RuvA or B concentration was decreased still further, the reversal reactions were much reduced and the kinetics became more variable (data not shown). The requirements for RuvA and RuvB were not changed substantially when the RecA and DNA substrate concentrations were reduced 8-fold (data not shown).

DISCUSSION

Our primary conclusion is that the RuvA and RuvB proteins reverse RecA protein-mediated strand exchange when a heterologous DNA insertion is present at the 3′ end of linear duplex DNA. RecA protein alone is able to promote branch migration up to the homology/heterology junction and a branched intermediate persists. However, addition of the RuvA and RuvB proteins produces a rapid reversal of strand exchange and hence, the conversion of these intermediates back to substrate.

We cannot offer a complete mechanism for the reversal of strand exchange, but our results suggest some general characteristics. Under optimal reaction conditions (with 1 μM RuvB and 0.3 μM RuvA protein), a 7.2-kbp length of hybrid DNA is unwound with almost the same kinetics as a 1.2-kbp length of hybrid DNA. Stalled intermediates or substrates are observed, but few intermediates that can be interpreted as being in the process of reversal are found. These results suggest that the unwinding of the hybrid DNA and re-formation of substrate DNA is very fast and does not limit the rate of the overall reaction. We estimate that reversal in an individual intermediate requires a few minutes or less, suggesting that it occurs at a rate of a few thousand base pairs per min. The rate-limiting process is evidently an initiation step, perhaps an assembly or activation of a RuvAB complex at the branch point.

Only after cross-links were introduced into the intermediate molecules were we able to observe intermediates in the process of RuvAB reverse strand exchange. These allowed us to confirm that the reversal began at the DNA branch, and proceeded in the direction opposite to that of RecA protein-mediated DNA strand exchange. Under the conditions of these experiments, both RuvA and RuvB proteins are required for reversal.

Although we document a prominent strand exchange reversal reaction in these experiments, there was much evidence for bidirectional branch migration mediated by RuvAB, especially with the intermediates containing over 7 kbp of hybrid DNA. Immediately after RuvA and RuvB addition there often appears to be a short lag in which the quantity of intermediates sometimes increases rather than decreases (Fig. 7). The results suggest that the RuvA and RuvB proteins facilitate a rapid branch migration up to the homology/heterology junction for those molecules in which the RecA reaction is not complete. In addition, when the reversal was blocked by cross-linking in the hybrid DNA, a significant number of the intermediates were

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processed by unwinding the short heterologous dsDNA tail (Fig. 3, *panels C* and *D*). In general, the RuvAB proteins seem to process branched intermediates predominantly by whatever path represents that of least resistance.

We do not know if the RecA protein is displaced by RuvAB protein in these reactions. West and colleagues have reported that RuvA and RuvB proteins will displace RecA protein from dsDNA (Adams *et al.*, 1994). Rates of ATP hydrolysis in these reactions, which we attribute primarily to RecA protein, were not substantially reduced in these reactions upon addition of RuvA and B (data not shown). However, we do not know to what extent the ATP hydrolysis reflects the RuvB ATPase activity. It is noteworthy that if disassembly of the RecA nucleoprotein filament begins at the branch point and proceeds in the direction that the hybrid DNA is being unwound, it would involve removal of RecA monomers from the filament end opposite to that from which disassembly normally occurs (Lindsley and Cox, 1990).

RecG protein is another protein which has emerged as an important component of DNA repair and homologous genetic recombination in *E. coli* (Lloyd and Sharples, 1993; West, 1994; Whitby *et al.*, 1994). Evidence from *in vivo* and *in vitro* studies indicate that RecG protein has functions that overlap those of the RuvA and B proteins (Lloyd, 1991; Luisi-Deluca *et al.*, 1989). When RecG protein acts on branched DNA intermediates formed in a RecA-mediated DNA strand exchange reaction involving homologous DNA substrates, it promotes a reversal of the reaction (Whitby, *et al.*, 1994). RecG protein therefore tends to promote branch movement in the direction opposite to that of RecA. Lloyd and colleagues (Whitby *et al.*, 1993) have proposed that the directionality of branch migration may be an important difference between the RuvAB proteins and the RecG protein *in vivo*. Our data supports the idea that with a branched molecule in which further strand exchange is blocked, the RuvA and RuvB proteins are also effective in promoting reversal of strand exchange.

The RuvA and RuvB proteins also have an important role in recombinational DNA repair. West and colleagues have shown that RuvAB-mediated branch migration efficiently processes DNA containing large numbers of pyrimidine dimers (Tsaneva *et al.*, 1992b). We have previously shown that RuvA and B greatly facilitate the bypass of heterologous insertions in DNA substrates during RecA protein-mediated DNA strand exchange (Iype *et al.*, 1994). The present work shows that the RuvA and B proteins can process a stalled branch point by reversing DNA strand exchange. Together, these results could indicate a role for RuvA and B in postreplication repair along the lines of that suggested for RecG protein by Lloyd and colleagues (Whitby *et al.*, 1993).

We suggest one other possible function for the RuvAB system. There are long repeated sequences in the bacterial genome, such as the multiple rRNA operons. Intragenomic recombination between these sequences could generate substantial chromosomal deletions and inversions. DNA damage is highly recombinogenic (Cox, 1993), and UV radiation and other agents which cause DNA damage may trigger the initiation of intragenomic recombination. Given the conservation of chromosomal gene order in divergent bacterial species, a mechanism may exist to reverse these recombination events or process them in an innocuous way. The activities of RuvA and B exhibited here recommend consideration of these proteins as potential components of such a system. The sensitivity of *ruv* mutant strains might then be explained, at least in part, by an inability to process deleterious crossovers within the genome that are induced by DNA damage. The RecG protein might also be part of such a system.

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