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RuvA and RuvB Proteins Facilitate the Bypass of Heterologous DNA Insertions during RecA Protein-mediated DNA Strand Exchange*

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RecA protein-mediated DNA strand exchange between circular single-stranded DNA and linear duplex DNA readily bypasses short (up to 100 base pairs) heterologous inserts in one of the DNA substrates. Larger heterologous inserts are bypassed with decreasing efficiency, and inserts larger than 200 base pairs substantially block RecA-mediated DNA strand exchange. The RuvA and RuvB proteins dramatically facilitate the bypass of larger heterologous inserts. When the RuvA and RuvB proteins are added to an ongoing RecA protein-mediated strand exchange reaction, interior heterologous inserts of 1 kilobase pair are bypassed at significant frequencies. The RuvA, RuvB, and RecA proteins are all required for this activity. Bypass occurs only when homologous sequences are present on both sides of the insert. When the heterologous insert is positioned at either end of the linear duplex substrate, the RuvA and RuvB proteins do not significantly increase product formation in RecA protein-mediated DNA strand exchange reactions. The results suggest an important role for RuvA and RuvB in the bypass of DNA structural barriers during recombinational DNA repair.

The RecA protein of *Escherichia coli* is a central component of the system that promotes homologous genetic recombination and recombinational DNA repair *in vivo* (Cox, 1991; Roca and Cox, 1990). RecA protein promotes DNA strand exchange reactions *in vitro* which mimic the principal steps of recombinational DNA repair (Cox and Lehman, 1987; Radding, 1989; Roca and Cox, 1990). The three-strand exchange reaction, shown in Fig. 1*A*, is one of the best characterized *in vitro* reactions. The first step, which is facilitated by the *E. coli* SSB protein (Morrical and Cox, 1990), is the formation of a RecA protein filament on the ssDNA.[1] The second step is the pairing of a homologous linear duplex DNA with the nucleoprotein filament. Finally, a facilitated unidirectional branch migration, 5′ to 3′ relative to the ssDNA, leads to formation of nicked circular duplex and displaced single-stranded DNA products. (Cox and Lehman, 1981a, 1981b; Jain *et al.*, 1994; Kahn *et al.*, 1981; West *et al.*, 1981). The RecA protein is a DNA-dependent ATPase and hydrolyzes ATP throughout the DNA strand exchange reaction (Cox, 1994; Schutte and Cox, 1987).

The RuvA and RuvB proteins of *E. coli* are also important in DNA repair and homologous genetic recombination *in vivo* (Taylor, 1992; West, 1992, 1994). Biochemical characterization of these proteins has revealed a number of interesting properties. RuvA and RuvB proteins cannot by themselves initiate DNA strand exchange but are able to promote bidirectional branch migration in the absence of RecA if presented with appropriate substrates such as synthetic Holliday junctions (Iwasaki *et al.*, 1992; Parsons *et al.*, 1992) or deproteinized recombination intermediates generated from a RecA protein-mediated four-strand exchange reaction (Müller *et al.*, 1993a, 1993b; Tsaneva *et al.*, 1992b). The RuvB protein exhibits an ATPase activity that is stimulated in the presence of the RuvA protein and DNA (Iwasaki *et al.*, 1989a; Parsons and West, 1993; Shiba *et al.*, 1991; Shinagawa *et al.*, 1991). Recently, it was shown that the RuvA and RuvB proteins have a 5′ to 3′ helicase activity *in vitro* (Tsaneva *et al.*, 1993). The overall picture emerging from these studies indicates that the RuvA and RuvB proteins function largely in the processing of recombination intermediates (West, 1994). RuvA targets RuvB to a Holliday junction. Branch migration mediated by RuvB ensues. The Holliday junction is ultimately cleaved by the RuvC resolvase (Bennett *et al.*, 1993; Dunderdale *et al.*, 1991), in a reaction that might be facilitated in some manner by RuvA and RuvB.

Mutations in the *ruv*A and *ruv*B genes, however, have a negligible effect on homologous genetic recombination *in vivo* (Lloyd *et al.*, 1984). Defects in recombination are evident only when the mutations are in a *rec*BC *sbc*A, *rec*BC *sbc*B *sbc*C, or *rec*G genetic background. Evidence from studies both *in vivo* and *in vitro* indicates that RecG protein and perhaps other proteins have functions that overlap those of the RuvA and RuvB proteins in genetic recombination (Lloyd, 1991; Lloyd and Sharples, 1993). In contrast, *ruv*A or *ruv*B mutations individually produce a dramatic increase in the sensitivity of bacterial cells to DNA damaging agents (Iwasaki *et al.*, 1989b; Otsuji *et al.*, 1974). Genetic studies have provided evidence that the RuvA and RuvB proteins have important and possibly unique roles in recombinational DNA repair (Lloyd *et al.*, 1984; Shurvinton and Lloyd, 1982).

Current models for recombination are closely related to models for recombinational DNA repair, and similar steps involving DNA-DNA pairing, DNA strand exchange, and processing of branched recombination intermediates are believed to occur in both processes (Cox, 1993). One result is that few molecular distinctions are generally drawn for the recombination process envisioned in the generation of genetic diversity as opposed to DNA repair. However, the molecular requirements for recombinational DNA repair are substantially more stringent than those for recombination *per se*, and an argument can be made that the evolution of the RecA protein was impelled by the exigencies of DNA damage (Cox, 1993). The disparity seen in the effects of *ruv*AB mutations on recombination and repair

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

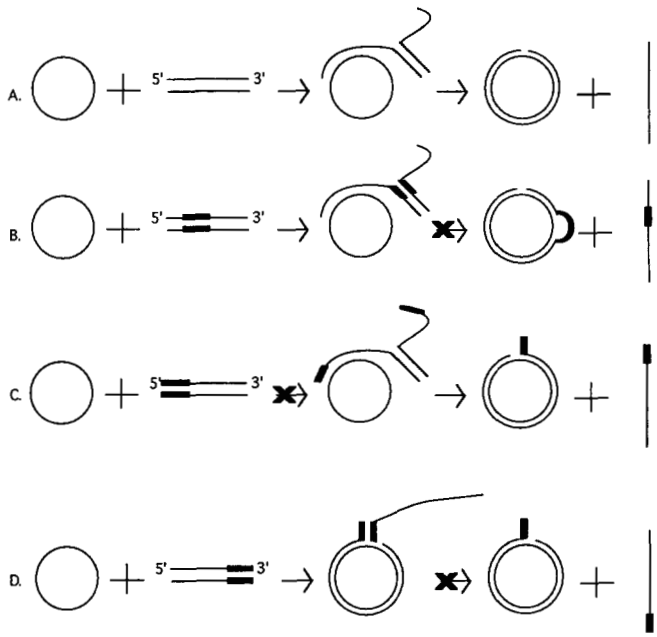


FIG. 1. **RecA protein-promoted three-strand exchange reactions.** An x indicates steps where a normal RecA protein-mediated DNA strand exchange reaction is blocked. A three-strand exchange reaction between completely homologous circular ssDNA (+) and linear duplex DNA produces a nicked circular duplex and a displaced linear (+) single strand. Reactions *B*, *C*, and *D* depict the observed results of reactions in which a long (>200 bp) heterologous sequence is placed in the interior, at the 5′ end, or at the 3′ end, respectively, of the linear duplex substrate. Heterologous sequences are indicated by *thickened lines*.

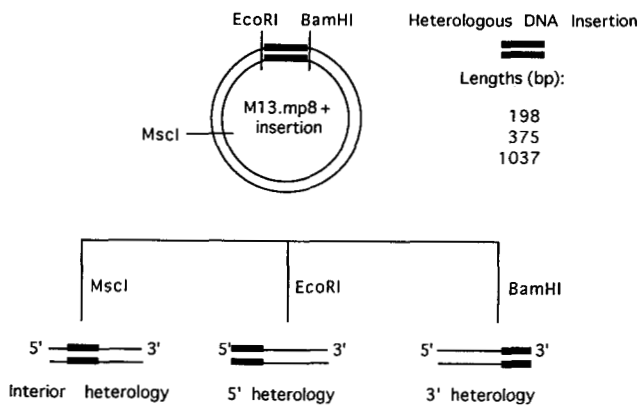


FIG. 2. **DNA substrates used in these experiments.**

suggests that a major function of the corresponding proteins involves some facet of the recombination process that is unique to recombinational DNA repair.

The recombination process in recombinational DNA repair is complicated by a requirement for the bypass of structural barriers in the DNA such as DNA lesions. Furthermore, homologous recombination can be accompanied by gene conversion events involving addition or deletion of DNA sequences (Lichten and Fox, 1984), an outcome that is likely to require the bypass of substantial barriers to DNA strand exchange in the form of differing sequences in one or both DNA substrates. In order to investigate the consequences of heterologous sequences in DNA molecules undergoing DNA strand exchange *in vitro*, RecA protein-mediated three-strand exchange reactions with substrates containing such barriers have been carried out. RecA protein is able to promote efficient DNA strand exchange with DNA substrates containing lesions, mismatches, and short insertions of heterologous DNA sequence (50–100 bp) (Bianchi and Radding, 1983; DasGupta and Radding, 1982; Hahn *et al.*, 1988; Kim *et al.*, 1992; Livneh and Lehman, 1982). The bypass of short heterologous inserts in one of the DNA substrates has recently been identified as one function of RecA-mediated ATP hydrolysis (Kim *et al.*, 1992; Müller *et al.*, 1990). Jwang and Radding (1992) demonstrated that torsional stress generated by the RecA protein was required to separate DNA strands in interior heterologous DNA insertions during strand exchange. On a molecular level, ATP hydrolysis may be coupled in some manner to DNA rotation to generate the torsional stress required for insertion bypass (Cox, 1994). The location of the heterologous DNA insertion also appears to have a significant effect on the strand exchange reaction. Interior heterologous DNA insertions are traversed more easily than heterologous DNA insertions located at the 5′ or 3′ end of the linear duplex DNA (Bedale *et al.*, 1991, 1993; Jwang and Radding, 1992).

The investigation described in this report was initiated to determine if the RuvA and/or RuvB proteins affected the bypass of DNA structural barriers during recombinational DNA repair. The focus was on the bypass of long (>100 bp) heterologous DNA inserts during DNA strand exchange. The capacity of RecA protein to mediate three strand exchange reactions involving substrates with heterologous inserts falls off dramatically for inserts in this size range (Fig. 1, reactions *B–D*). We demonstrate here that the RuvA and RuvB proteins facilitate an efficient RecA protein-mediated strand exchange between circular single-stranded DNA and linear duplex DNA containing interior heterologous DNA insertions of 198–1037 bp and discuss how this result might affect our current understanding of RuvA and RuvB function.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—E. coli RecA protein was purified as described (Cox *et al.*, 1981). *E. coli* 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 RecA protein and SSB protein concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\epsilon_{280} = 0.58\ A_{280}$ mg^{-1} ml (Craig and Roberts, 1981), and $\epsilon_{280} = 1.5\ A_{280}$ mg^{-1} ml (Lohman and Overman, 1985), respectively. *E. coli* RuvA and RuvB proteins were purified as described below. The concentrations of RuvA and RuvB proteins were determined by the method of Bradford (Bradford, 1976) with bovine serum albumin as the protein standard (Bio-Rad assay kit). RecA K72R protein (Rehrauer and Kowalczykowski, 1993) was a gift from Qun Shan (this laboratory). It was purified to homogeneity using a procedure to be described elsewhere and was free of detectable nuclease activities under the conditions used in these experiments; its concentration was determined using the extinction coefficient reported above for wild type RecA protein.

Restriction endonucleases were purchased from New England Biolabs. Tris buffer was from Fisher Scientific. Proteinase K, creatine phosphokinase, phosphocreatine, ATP, lactic dehydrogenase, pyruvate kinase, bovine serum albumin, phosphoenolpyruvate, and nicotinamide adenine dinucleotide (reduced form) were purchased from Sigma. Amino-4,5′,8-trimethylpsoralen (AMT) was from Calbiochem. Oligonucleotides were synthesized by the University of Wisconsin Biochemistry Department Synthesis Facility. Plasmid pET21d was purchased from Novagen. The Sequenase version 2.0 sequencing kit was from U. S. Biochemical Corp. Isopropyl-1-thio-β-D-galactopyranoside was from Bachem. Bio-Rex-70 cation exchange resin was from Bio-Rad. The FPLC Mono Q column was purchased from Pharmacia Biotech Inc.

DNA—Duplex and ssDNA substrates were derived from bacteriophage M13mp8 (Fig. 2) (Messing and Vieira, 1982). Bacteriophage M13mp8.198 is bacteriophage M13mp8 with 198 bp (*Rsa*I-*Rsa*I fragment from the *E. coli gal*T gene) inserted into the *Sma*I site (Lindsley and Cox, 1990a). Bacteriophage M13mp8.375 is bacteriophage M13mp8 with a 375-bp fragment (*Eco*RI-*Bam*HI fragment of pBR322) replacing the 10-bp *Eco*RI-*Bam*HI fragment of bacteriophage M13mp8 (Bedale *et al.*, 1991). Bacteriophage M13mp8.1037 is bacteriophage M13mp8 with 1037 bp (*Eco*RV-*Eco*RV fragment from the *E. coli gal*T gene) inserted into the *Sma*I site (Lindsley and Cox, 1990b). Supercoiled circular duplex DNA and circular single-stranded DNA from bacteriophage

M13mp8 and its derivatives 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 μg ml^{-1} A_{260}^{-1}, respectively as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Complete digestion of supercoiled M13mp8 derivatives with appropriate restriction enzymes resulted in full-length linear duplex DNA substrates that contain heterology located internally or at the 5′ or 3′ end of the duplex DNA, relative to the viral (+) strand (Fig. 2). 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.

Polymerase Chain Reaction Cloning of the E. coli ruvA and ruvB Genes—The *ruvA* and *ruvB* genes were isolated individually from the *E. coli* genome (strain MG1655 (Guyer *et al.*, 1981), a gift from George Weinstock) by polymerase chain reaction using a Perkin-Elmer DNA Thermal Cycler. In brief, a pair of DNA primers was designed which were complementary to the 5′ DNA sequence and 3′ DNA sequence of the gene. For the *ruvA* gene, the 5′ DNA primer corresponded to bases 361–377 of the published DNA sequence (Shinagawa *et al.*, 1988), with the sequence 5′-CGTCA added to the 5′ end to introduce a *Bsp*HI restriction site. The 3′ DNA primer corresponded to bases 996–1010, with the sequence 5′-CCCAAGCTT added to the 5′ end to create a *Hin*dIII restriction site. For the *ruvB* gene, the 5′ primer corresponded to bases 980–996 of the published DNA sequence (Shinagawa *et al.*, 1988), with the sequence 5′-CTAGACTC added to the 5′ end to create a *Bsp*HI site. The 3′ primer corresponded to bases 1979–1993, with a *Hin*dIII site created at the 5′ end by adding the sequence 5′-CCCAATCTT. After polymerase chain reaction amplification of the predicted size fragment, complete digestion of the fragment with *Bsp*HI and *Hin*dIII endonucleases was carried out. The resultant fragment was ligated to the cloning vector pET21d (Novagen) with DNA ligase. The plasmid with the wild type *ruvA* gene was designated pEAW106, whereas the plasmid with the wild type *ruvB* gene was designated pEAW112. The integrity of the entire *ruvA* and *ruvB* genes in these constructs was verified by direct sequencing (Sequenase kit and protocol, U. S. Biochemical Corp.).[2] In order to express the RuvA and RuvB proteins, the strain BL21(DE3) (Studier and Moffatt, 1986) was transformed with pEAW106 and pEAW112, respectively (Sambrook *et al.*, 1989).

Purification of the E. coli RuvA Protein—A 30-liter culture of *E. coli* strain, BL21(DE3), with the plasmid pEAW106 was grown with aeration in a New Brunswick MPPF 30 liter fermentor at 37 °C in Luria broth (supplemented with 100 μg/ml ampicillin) for 10 h. Cells were collected with a Sharples centrifuge. The cell pellet (870 g) was washed with lysis buffer (100 mM Tris-HCl, 50% cation, pH 8, 2 mM EDTA, 5% glycerol) and then centrifuged again. The cell pellet was then resuspended in lysis buffer at 3 ml/g wet cells, fast-frozen in liquid nitrogen, and stored at −20 °C.

RuvA protein was purified as described (Tsaneva *et al.*, 1992a) with a few modifications. In brief, the DEAE-Bio-Gel step was identical to the published procedure except that the buffer contained 0.1% Triton X-100. The pooled peak fractions from the DEAE-Bio-Gel column were then dialyzed to remove the Triton X-100 before application to the ssDNA cellulose column. Another modification was that a Bio-Rex 70 cation exchange column (33-ml bed volume, 1.6 × 16.5 cm) was inserted into the purification scheme after the ssDNA cellulose column. A 170-ml 0.15–1 M NaCl linear gradient was applied to the column. Peak fractions (0.31–0.35 M NaCl) were pooled and then dialyzed against the appropriate buffer before being applied to a 1-ml Mono Q FPLC column (Pharmacia). The Mono Q column was used in place of the hydroxylapatite column, as suggested in the published protocol (Tsaneva *et al.*, 1992a). The final yield of RuvA protein from 55 g of cells was 2.2 mg. The RuvA protein was at least 95% pure as estimated by SDS-PAGE (Fig. 3) and was free of detectable endo- or exonuclease activities. The 22-kDa RuvA protein migrates in the position expected for a 27-kDa protein in SDS-PAGE (Tsaneva *et al.*, 1992a).

Purification of the E. coli RuvB Protein—A 30-liter culture of *E. coli* strain, BL21(DE3), with the plasmid pEAW112 was grown with aeration in a New Brunswick MPPF 30 liter fermentor at 37 °C in Luria broth (supplemented with 100 μg/ml ampicillin) to an OD_{650} = 1.2. Expression of RuvB protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.4 mM. The culture was then incubated at 30 °C for an additional 8 h. Cells were collected by centrifugation using a Sharples centrifuge. The cell pellet (240 g) was frozen in liquid nitrogen and stored at −20 °C.

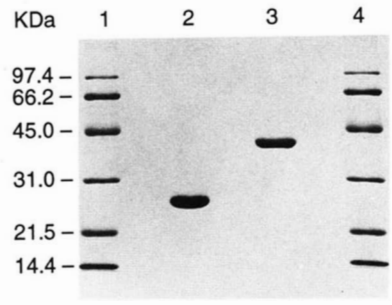


FIG. 3. **SDS-PAGE of purified RuvA and RuvB proteins.** The molecular masses of the RuvA and RuvB proteins are 22 and 37 KDa, respectively. *Lane 1*, molecular mass standards; *lane 2*, RuvA protein (5 μg); *lane 3*, RuvB protein (5 μg); *lane 4*, molecular mass standards.

RuvB protein was purified by a published procedure (Tsaneva *et al.*, 1992a) with modifications. In brief, a Bio-Rex 70 cation exchange column (33-ml bed volume, 1.6 × 16.5 cm) was included in the purification scheme after the hydroxylapatite column. Flow-through fractions were pooled and then applied to a 1-ml Mono Q FPLC column. The final yield of RuvB protein from 13 g of cells was 5.5 mg. The RuvB protein was at least 95% pure as estimated by SDS-PAGE (Fig. 3) and was free of detectable endo- or exonuclease activities.

For assays, the RuvA and/or RuvB proteins were diluted as needed into a RuvAB dilution buffer containing 20 mM Tris chloride buffer (65% 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.

Strand Exchange Reaction Conditions—All 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 (10 units ml^{-1} creatine phosphokinase, 12 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 (to 6 mM final) and SSB (to 2 μM final) 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/or RuvB occurred immediately (10–20 s) after the addition of ATP and SSB. The final concentrations of RuvA protein and RuvB protein were 0.3 μM and 1 μM, respectively, unless noted otherwise in the text.

Agarose Gel Assays—Aliquots (15 μl) of reactions described above were removed at each time point, and the reactions were stopped by the addition of SDS (0.9% final) and proteinase K (1 mg ml^{-1} final). The aliquots were incubated at 37 °C for 30 min followed by addition of 5 μl of gel loading buffer (50% glycerol, 0.05% bromphenol blue, 50 mM EDTA). Samples were electrophoresed overnight in a 0.8% agarose gel at 2–2.5 V cm^{-1}.

Electron Microscopy—Samples for electron microscopy were obtained by spreading the entire strand exchange reaction mixture. Some reaction mixtures were cross-linked with AMT prior to examination by electron microscopy to prevent spontaneous branch migration 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 and noncross-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 (10–20 μl) 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).

In order to identify and quantify the different DNA species in DNA strand exchange reactions, noncross-linked samples (90-min time points) were prepared for electron microscopy. The categories of molecules observed are shown in Tables I and IV.

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 measure-

[2] We found one discrepancy in the ruvA sequence as reported by the Shinagawa (Shinagawa *et al.*, 1988) and Lloyd (Benson *et al.*, 1988) groups. At codon 166, the Shinagawa sequence specifies an alanine residue, whereas the Benson *et al.* (1988) sequence specifies Arg. Our sequence agrees with Benson *et al.* (1988).

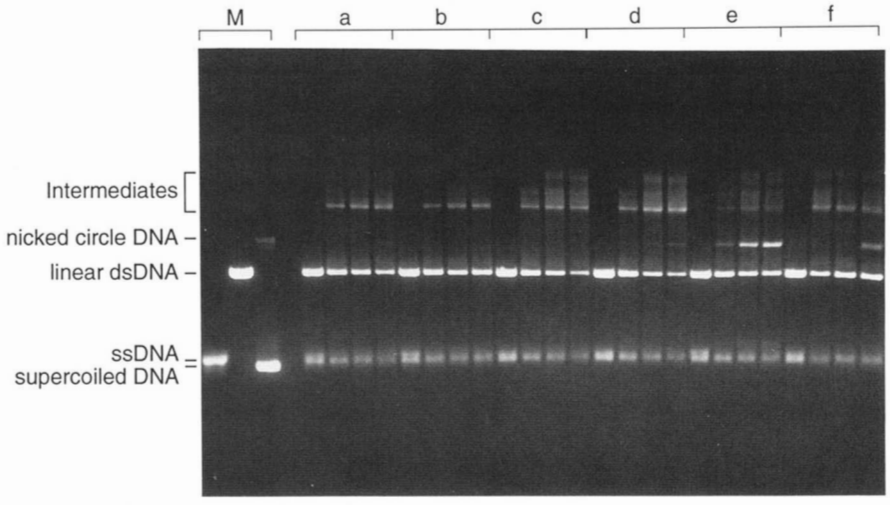


FIG. 4. **Effect of RuvA and RuvB proteins on RecA protein-mediated DNA strand exchange when the duplex DNA substrate contains an internal 198-bp heterologous insertion.** Reactions were carried out under standard reaction conditions as described under "Experimental Procedures," and included (in 60 μl of total volume) 21 μM M13mp8 circular ssDNA, 21 μM M13mp8.198 dsDNA, and 2 μM SSB. RecA (7 μM final), RuvA (0.3 μM final), and RuvB (1 μM final) proteins were included as indicated below. Markers (*M*) are (from *left* to *right*) circular M13mp8 (+) ssDNA, M13mp8.198 linear duplex DNA (linearized with *Msc*I), and M13mp8.198 circular duplex DNA (supercoiled and a minor nicked circular component). In reactions *A–E*, the time points correspond to 0, 30, 60, and 90 min of reaction. The reactions included are: *A*, RecA protein; *B*, RecA protein and 2.75 μl of RuvA/B protein dilution buffer; *C*, RecA protein and RuvA protein; *D*, RecA protein and RuvB protein; and *E*, RecA protein, RuvA protein, and RuvB protein. In reaction *F*, a RecA protein-mediated DNA strand exchange reaction was initiated. After 60 min, at 37 °C, two aliquots (14 μl each) of the reaction mix were placed in separate tubes. To one tube the RuvA and RuvB proteins (0.3 and 1 μM final, respectively) were added, and to the other tube no additional components were added. Both tubes were incubated at 37 °C for an additional 30 min to give a total reaction time of 90 min. Time points are (from *left* to *right*): 0, 60, 90 min (–RuvA and RuvB proteins), 90 min (+RuvA and RuvB proteins).

ments 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. 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 eight segments which each represent 1.0 kbp. The estimates of DNA exchanged were then sorted by 1.0-kbp segments. Data were plotted as a percentage of total intermediates per 1.0 kbp of DNA exchanged (see Fig. 8). As a check on these visual estimates of DNA exchanged, careful measurements of the length of DNA exchanged were carried out as described (Littlewood and Inman, 1982) for the intermediates generated from the strand exchange reaction with a 1037-bp heterologous DNA insertion. An estimated and measured data set were compared by the χ^2 two-way contingency test at the 95% confidence level and found to be in excellent agreement ($p = 0.93$).

DNA-dependent ATPase Assay—This assay was used as an indirect measure of RecA protein binding to DNA (Morrical *et al.*, 1986; Lindsley and Cox, 1990a). A Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two thermojacketed cuvette holders, each capable of holding six cuvettes, was used for absorbance measurements. The cell path length and the band pass were 0.5 cm and 2 nm, respectively. The coupled assay used to measure ATP hydrolysis was described previously (Morrical *et al.*, 1986). The regeneration of ATP from ADP and phosphoenolpyruvate with the oxidation of NADH can be followed by the decrease in absorbance at 340 or 380 nm. Absorbances were measured at 380 nm, instead of 340 nm (the absorbance maximum for NADH), to remain within the linear range of the spectrophotometer. High concentrations of NADH (2 mM) were necessary to ensure that a reproducible steady state (end point) was reached under all conditions. No component of the coupling system limited the observed rate of ATP hydrolysis. Doubling the concentrations of the phosphoenolpyruvate and/or the enzymes in the regenerating system had no effect on results. ATP is constantly regenerated in this system so no product accumulation occurs. An NADH extinction coefficient of $\epsilon_{380} = 1.21\ mM^{-1}\ cm^{-1}$ was used to calculate the rate of ATP hydrolysis. All data were fit by linear regression. For reactions containing the RuvA and RuvB proteins, the two proteins were mixed and left on ice and then added to the assay at the appropriate time.

ATP hydrolysis was measured as a means to measure RecA protein binding to ssDNA. In brief, 10 μM M13mp8 circular single-stranded DNA was preincubated with the indicated proteins and all other reaction components except ATP and SSB at 37 °C for 10 min. The assay was initiated by the addition of SSB (1 μM final) and ATP (6 mM final). The decrease in A_{380} was then followed for approximately 20 min. In order to measure ATP hydrolysis for 40 min, the concentrations of M13mp8 circular single-stranded DNA and all proteins was decreased by 50%. Under these conditions, rates of ATP hydrolysis are essentially a linear function of RecA protein concentration until stoichiometric (1 RecA monomer per 3 nucleotides of ssDNA) concentrations are reached, at which point the rate levels off (data not shown).

ATP hydrolysis during DNA strand exchange was measured as described previously (Schutte and Cox, 1987). In brief, 5 μM M13mp8 circular ssDNA and 5 μM M13mp8 linear dsDNA were incubated with RecA protein at concentrations indicated in the text, along with all other reaction components (except ATP and SSB) at 37 °C for 10 min. The assay was initiated by the addition of SSB (0.5 μM final) and ATP (6 mM final). The decrease in A_{380} was then followed for approximately 40 min. RuvA and RuvB proteins were added as appropriate at times indicated in the text.

RESULTS

Experimental Design—The purpose of this investigation was to determine if the RuvA and RuvB proteins had any effect on RecA protein-mediated DNA strand exchange through a particular DNA structural barrier, an insertion of heterologous DNA sequence. Three methods were utilized: agarose gel electrophoresis to monitor strand exchange reactions, electron microscopy to characterize DNA species formed during strand exchange reactions, and a spectrophotometric assay to measure ATP hydrolysis and DNA binding of the RecA protein. In these experiments, the ends of the duplex DNA substrate are identified as 5′ or 3′ with respect to the strand that is identical to the single-stranded circular DNA substrate.

RuvA and RuvB Proteins Facilitate the Bypass of Large Heterologous DNA Insertions during RecA Protein-mediated DNA Strand Exchange—When one of the DNA substrates contains an interior heterologous DNA insertion (greater than 150 bp), the insertion represents a barrier that will halt a RecA protein-mediated DNA strand exchange reaction (Bedale *et al.*, 1991, 1993; Bianchi and Radding, 1983; Kim *et al.*, 1992). A strand exchange reaction between circular ssDNA and linear duplex DNA containing an interior 198-bp heterologous DNA insertion is shown in Fig. 4. The expected product of this reaction is a

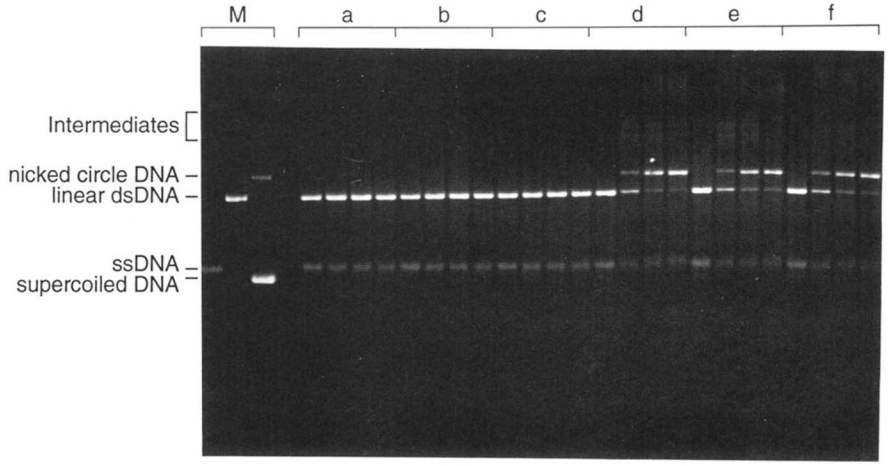


FIG. 5. **Effects of RuvA and RuvB proteins on RecA protein-mediated DNA strand exchange with homologous DNA substrates.** Reaction conditions were identical to those in Fig. 4, except that M13mp8.198 circular ssDNA (21 μM) replaced the M13mp8 circular ssDNA. For each reaction, the time points are 0, 30, 60, and 90 min. Markers (*M*) are (from *left* to *right*) circular M13mp8.198 (+) ssDNA, M13mp8.198 linear duplex DNA, M13mp8.198 circular duplex DNA. Reactions are: *A*, RuvA protein alone; *B*, RuvB protein alone; *C*, RuvA and RuvB proteins; *D*, RecA protein; *E*, RecA protein and 2.75 μl of RuvA/RuvB protein dilution buffer; *F*, RecA protein, RuvA protein, and RuvB protein.

nicked circular duplex with a 198-nucleotide single-stranded loop. The loop, as shown below, does not have a significant effect on the mobility of this DNA species in the agarose gels employed in this study. Very little of this DNA species is generated in the reaction with RecA and SSB alone, with a prominent new DNA band appearing at a position typical of branched strand exchange intermediates (Fig. 4, reaction *a*). However, when RuvA and RuvB proteins were added to this reaction mixture, a substantial amount of substrate was converted into product (Fig. 4, reaction *e*). Neither RuvA or RuvB individually had this effect on the reaction (Fig. 4, reactions *b* and *c*). Adding RuvA/RuvB dilution buffer in place of the RuvA and RuvB proteins (2.75 μl addition to a 60-μl reaction mix) also had no effect on the reaction (Fig. 4, reaction *b*).

Several results indicated that the bypass of long heterologous inserts was a result of the RecA, RuvA, and RuvB proteins acting together. First, the RuvA and RuvB proteins facilitated insertion bypass 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. 4, reaction *f*). At this point, two aliquots of the reaction mixture were withdrawn. One reaction aliquot was supplemented with the RuvA and RuvB proteins, whereas the other aliquot was not supplemented. After an additional 30-min incubation, reaction products were evident only in the aliquot with the RuvA and RuvB proteins (Fig. 4, reaction *f*). Second, the RuvA and RuvB proteins do not mediate DNA strand exchange on their own. A strand exchange reaction with completely homologous DNA substrates yielded no products or intermediates when RuvA, RuvB, or both proteins were used in the absence of RecA (Fig. 5, reactions *a–c*). This is consistent with previous results showing that the RuvA and RuvB proteins are unable to initiate DNA strand exchange (Iwasaki *et al.*, 1992). Finally, the RuvA and RuvB proteins do not inhibit RecA-mediated DNA strand exchange with homologous substrates. A similar yield of strand exchange product was observed with or without RuvA and B (Fig. 5, reactions *d* and *f*). A control in which the RuvA/RuvB protein dilution buffer was added in place of the RuvA and RuvB proteins also showed no effect (Fig. 5, reaction *e*).

Effects of Heterologous Insertion Size and the Concentration of RuvA and RuvB—Strand exchange reactions between circular ssDNA and linear duplex DNA containing either a 375-bp (Fig. 6, reactions *a–c*) or a 1037-bp (Fig. 6, reactions *d–f*) interior heterologous DNA insertion were examined. In these reactions, the expected product would contain a single-stranded loop corresponding in size to the substrate insert, and these products would have slightly reduced mobility as compared with a M13mp8 nicked duplex DNA marker. In the strand exchange reaction with RecA protein only (Fig. 6, reactions *a* and *d*), intermediates were present but the product was absent. When the same strand exchange reaction was carried out in the presence of the RecA, RuvA, and RuvB proteins (Fig. 6, reactions *b* and *e*), the intermediates were diminished, and there was significant product formation. Results were again similar even when the RuvA and RuvB were added 30 min after initiation of strand exchange with RecA alone. Product was formed only in the reaction aliquots supplemented with the RuvA and RuvB proteins (Fig. 6, reactions *c* and *f*).

A broad range of RuvA and RuvB protein concentrations were effective in facilitating the formation of product from a DNA strand exchange reaction using linear duplex DNA with a 375-bp heterologous DNA insertion. Under standard reaction conditions (7 μM RecA protein, 21 μM of each DNA), similar levels of product formation were seen in reactions where the RuvA/RuvB protein concentrations ranged from 36 nM/250 nM to 286 nM/1200 nM (data not shown). The concentrations chosen for the experiments described above are at the high end of this range.

At relatively high concentrations, the RuvB protein promotes branch migration independently of RuvA. Stoichiometric concentrations of RecA protein combined with high RuvB protein concentrations (ranging from 1 to 10 μM RuvB protein in a strand exchange reaction with 21 μM DNA substrates) were unable to promote a strand exchange reaction past a 375-bp insert, indicating that the requirement for RuvA protein cannot be circumvented (data not shown).

Additional requirements for bypass of a heterologous insertion during a strand exchange reaction were determined (data not shown). First, omission of SSB protein dramatically reduced formation of strand exchange products. This reduction in product is probably due to the documented instability of RecA nucleoprotein filaments in the absence of SSB (Cox *et al.*, 1987; Kowalczykowski, 1991; Radding, 1989). Second, the order of addition of the DNA and protein components in the DNA strand exchange reaction affected the results. When the RuvA and B proteins were added before or concurrently with RecA protein, product formation was reduced about 30–50% relative to experiments in which RuvA and B were added after a reaction had been initiated with RecA protein. Third, a change in the location of the 375 heterologous DNA insertion (4039 bp instead of 1150 bp from the 5′ end) had no discernible effect on the results.

Analysis of Strand Exchange Reactions by Electron Microscopy—A complementary analysis of each of the strand exchange reactions described above was carried out by electron microscopy. First, the different DNA species were identified. Standard reactions with duplex substrates containing each heterologous insert (198, 375, or 1037 bp) were carried out with or

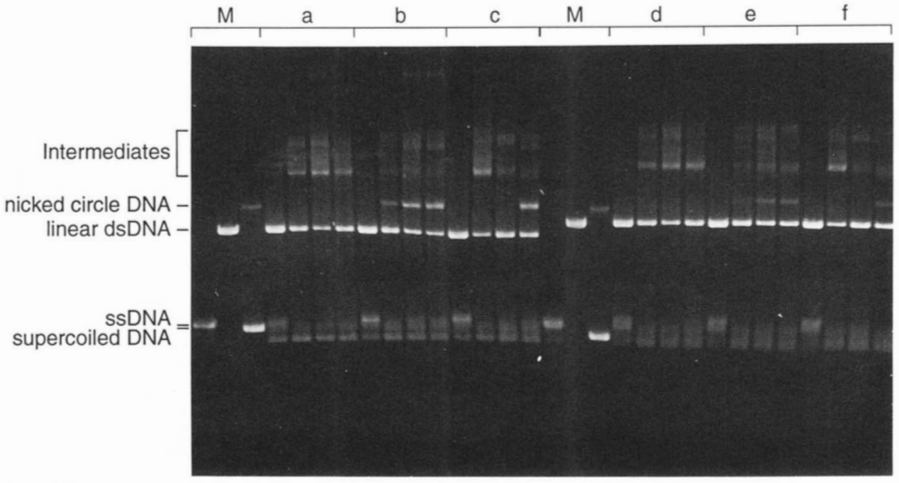


FIG. 6. **Effect of RuvA and RuvB proteins on RecA protein-mediated DNA strand exchange when the linear duplex DNA substrate contains internal 375- or 1037-bp heterologous insertions.** Reactions contained 21 μM M13mp8.375 dsDNA (reactions *A–C*) or 21 μM M13mp8.1037 (reaction *D–F*); reaction conditions were otherwise identical to those in Fig. 4. In both cases, the duplex substrates were linearized with *Msc*I. For reactions *A, B, D,* and *E*, the time points correspond to 0, 30, 60, and 90 min. Markers (*M*) for reactions *A–C* are (from *left* to *right*): circular M13mp8 (+) ssDNA, M13mp8.375 linear duplex DNA, and M13mp8 circular duplex DNA (supercoiled and a minor nicked circular component); for reactions *D–F*: circular M13mp8 (+) ssDNA, M13mp8.1037 linear duplex DNA, and M13mp8 circular duplex DNA. The ssDNA migrated as a doublet in this gel system, although greater than 95% of the ssDNA was circular (data not shown). The reactions included: *A* and *D*, RecA protein; *B* and *E*, RecA protein, RuvA protein, and RuvB protein; *C* and *F*, a RecA protein-mediated DNA strand exchange reaction was initiated. After a 30-min incubation at 37 °C, two aliquots (14 μl each) of the reaction mix were placed in separate tubes. The RuvA and RuvB proteins (0.3 and 1 μM final, respectively) were added to one of the tubes; nothing was added to the other. Both tubes were incubated at 37 °C for an additional 60 min to give a total reaction time of 90 min. Time points (from *left* to *right*): 0, 30, 90 min (–RuvA and RuvB proteins), 90 min (+RuvA and RuvB proteins).

without the addition of the RuvA/B proteins. The results are summarized in Table I. Molecules identified as intermediates all have the branched structure shown in Fig. 1. In the case of the 198-bp heterologous DNA insertion, the addition of the RuvA and RuvB proteins brought about a 5-fold increase in the production of nicked circular products, each with a small single strand loop as shown below. For the 375-bp heterologous DNA insertion, the increase effected by RuvA and RuvB was about 8-fold. For the 1037-bp insertion, the increase was >14-fold. The data in Table I are consistent with results from the agarose gel assays.

There are two categories in Table I which reflect DNA species that are not normal substrates, intermediates, or products and together represented a notable proportion of the sample. Broken molecules included those which may have arisen from DNA strand exchange between circular ssDNA and nicked or short double-stranded linear molecules. Nicked or shortened DNA molecules comprise a small percentage of every DNA substrate preparation. Miscellaneous molecules consisted of DNA species presumed to result from side reactions. Two examples of such side reactions are interactions of a single-stranded circle with more than two linear duplex molecules or the interaction of a circular duplex product with a circular ssDNA molecule. Molecules in the miscellaneous category were generally reduced whenever RuvA and RuvB were present.

Typical reaction products are shown in Fig. 7. The single-stranded loop was clearly visible for reactions involving substrates with the 1037 or 375 bp of heterologous DNA (Fig. 7, *A* and *B*). The 198-bp loops are more difficult to see and are identified by *arrows* in the photographs (Fig. 7*C*). To verify that the looped-out regions in the product had the expected length, the size of the loops were measured for products arising from strand exchange reactions with substrates containing the 375 bp (six molecules measured) or 1037-bp (17 molecules measured) heterologous insertion. The loops were found to be 380 ± 30 bp and 1030 ± 120 bp in length, respectively.

We considered the possibility that the RuvA and RuvB proteins might nick the intermediate produced in the strand exchange reactions to generate the observed product. If no additional nicking had occurred during the reaction, there should be just one nick in the product located about 1 kbp from the loop. Reactions containing all protein components were cross-linked with AMT prior to deproteinization and then completely denatured in order to visualize the free ends at a strand break. Of the 42 molecules counted from reactions involving substrates with the 375-bp heterologous DNA insertion, 20 contained one nick, 7 had no visible nicks, and 15 had more than one nick. Of the 33 molecules counted with the 1037-nucleotide loop, 24 contained one nick, 3 had no nicks, and 6 had more than one nick. Molecules that did not appear to have nicks can be explained by the random nature of AMT cross-linking. There are regions within molecules which are cross-linked to a sufficiently high degree that upon denaturation, they will appear to be native. In the present experiments, about 5–10% of each molecule appeared to be native duplex DNA, and any nick that occurred in such a region would not be visible. This also leads to a slight underestimation of molecules with one or more nicks. Molecules having more than one nick may arise from the UV light treatment required during the AMT cross-linking process. Nicks were not localized to a precise position in the DNA molecule relative to the loops because of the uncertainty about where a loop begins and ends when the DNA is denatured as in this protocol. The results are consistent with a mechanism for bypass of heterologous insertions that does not involve DNA strand breaks.

We next changed our focus from products to reaction intermediates. Estimates of the length of DNA exchanged in intermediates from the DNA strand exchange reactions were made as described under "Experimental Procedures." First, intermediates from strand exchange reactions in the presence or absence of RuvA and RuvB using linear duplex substrates with the 198-bp heterologous DNA insertion were examined (Fig. 8*A*). Although there appeared to be more intermediates stalled at the heterologous insertion after 90 min of reaction without RuvA and RuvB, the histograms in *A* showed little difference when subjected to a χ^2 two-way contingency test ($p = 0.1$). Therefore, in the absence as well as the presence of the RuvA and RuvB proteins, there appears to be a distribution in the

TABLE I

Percent of species generated during a strand exchange reaction involving a linear duplex DNA with a heterologous insertion

Strand exchange reactions between M13mp8 circular ssDNA and M13mp8.198 or M13mp8.375 or M13mp8.1037 linear duplex DNA were carried out as described under "Experimental Procedures." For each set of reactions, aliquots from 90-min time points were spread for electron microscopy as described under "Experimental Procedures." Values in the table are given as percentage of molecules in the counted DNA sample. Standard deviations of these values ranged from ± 2 to ± 4 (these errors are statistical counting errors rather than variations between different experiments). *n* represents the total number of molecules counted for each reaction. A description of the broken species and miscellaneous species categories are given in the text. Reactions – and + are results obtained in the absence or presence of RuvA/B, respectively.

Category	Length of heterologous DNA insertion					
	198 bp		375 bp		1037 bp	
	–	+	–	+	–	+
Linear duplex DNA	49	39	40	33	61	49
Intermediates	23	12	29	12	26	21
Nicked circular products	7	36	5	43	0	14
Broken molecules	8	7	6	7	3	9
Miscellaneous molecules	13	6	20	5	10	7
n	234	251	166	316	143	222

length of DNA exchanged in the intermediates. Note that the electron micrographs do not reveal which end of the linear duplex is undergoing reaction. Given the lack of products seen in the absence of RuvA and RuvB, the intermediates that have longer regions of exchanged DNA are probably not molecules that have bypassed the insert, but are instead derived from transient reactions at the 3′ or distal end of the duplex. There may also be some spontaneous branch migration that occurs during the processing of the samples for electron microscopy.

In reactions using linear duplexes with 375- or 1037-bp inserts, the results were more clear-cut (Fig. 8, *B* and *C*). The majority of intermediates generated in the absence of RuvA and RuvB had exchanged DNA up to the region of the heterologous DNA insertion. A smaller percentage again had exchanged regions of DNA that were longer. These probably arose from strand exchange at the 3′ end (Jain *et al.*, 1994),[3] since none of them had the expected single-strand loop that would be present if exchange had bypassed the insert (data not shown).

Intermediates from the strand exchange reaction with RuvA and RuvB proteins exhibited a much wider spectrum in the lengths of DNA exchanged (Fig. 8, *B* and *C*). Surprisingly, none of the intermediates (among over 100 examined) contained the single-strand loop that would identify a molecule that had undergone exchange beyond the insertion. As indicated in Table I, these same samples contained large numbers of products which clearly contained the loops. This result is addressed under "Discussion."

Analysis of RecA Protein Binding to DNA—One mechanism by which the RuvA and RuvB proteins could facilitate insertion bypass would be by promoting dissociation of the RecA protein filament and then completing the reaction without RecA protein. To determine if the RecA protein dissociated from DNA in the presence of the RuvA and RuvB proteins, the rate of RecA protein-mediated ATP hydrolysis was monitored. This assay is indirect, but it is rapid and reproducible. It also has the considerable advantage of minimizing the ambiguity that would result in more direct assays for DNA binding when more than one DNA-binding protein is present (*e.g.* RecA, SSB, RuvA, and RuvB).

Under the conditions used in this study, levels of ATP hydrolysis correlate well to RecA protein binding measurements obtained by more direct assays (Morrical *et al.*, 1986). The contribution of the RuvB ATPase is modest and was evaluated independently. If the presence of the RuvA and RuvB proteins caused a significant decrease in the observed ATP hydrolysis rate, this would suggest a reduction in RecA protein binding to the ssDNA that would merit further study.

The ssDNA-dependent ATP hydrolysis rates were measured for RecA protein only; for RuvA and RuvB proteins only; for RecA, RuvA, and RuvB proteins incubated simultaneously; and for RecA protein with the RuvA and RuvB proteins added 10 min after the reaction was initiated (Table II). As before, SSB was present in all experiments. The protein concentrations used in this assay maintained the ratios of protein to ssDNA present in the strand exchange reactions described earlier.

The rate of RecA-mediated ATP hydrolysis in the absence of RuvA and B was 85.8 μM min^{-1}; if it is assumed that there is one bound monomer per 3 nucleotides of ssDNA present, this rate yields a k_{cat} for bound RecA of 26 min^{-1}. This compares reasonably well with published k_{cat} values for ssDNA-dependent ATP hydrolysis that approach 30 min^{-1}. The ATP hydrolytic activity measured with RuvA and RuvB proteins alone was very low in comparison (about 1 μM min^{-1}). Previous studies (Iwasaki *et al.*, 1989a; Parsons and West, 1993; Shiba *et al.*, 1991; Shinagawa *et al.*, 1991) have generally reported a "low" rate of ATP hydrolysis for RuvB. The rates for RuvB were at the detection limit for this spectrophotometric assay and therefore varied somewhat more than the rates obtained in the presence of RecA. We made no attempt to determine a turnover number for the RuvB ATPase in this study, but it was no greater than 1 min^{-1}.

When the RecA, RuvA, and RuvB proteins were added to the reaction mixture simultaneously, the observed rate of ATP hydrolysis was reduced about 60%. As noted above, there was also a reduction in the formation of strand exchange products when all of these proteins were added simultaneously. This suggests a competitive binding of RecA with RuvA and/or RuvB. Consistent with this idea, the observed rate of ATP hydrolysis decreased 4-fold when the concentration of RuvA and RuvB was doubled.[4] When RuvA and RuvB were added prior to RecA, much lower rates of ATP hydrolysis were observed, indicating that RuvA and RuvB could block RecA binding to ssDNA. In each case, once the reaction was initiated, there was no further change in the rate of ATP hydrolysis that might indicate a further binding or dissociation of RecA.[4]

When the RuvA and RuvB proteins were added to a RecA-mediated ATP hydrolysis assay 10 min after it was initiated, there was no significant change in rate, even if the reaction was followed for 40 min (data not shown). These results indicate that there is no substantial effect of RuvA and RuvB on preformed RecA nucleoprotein filaments.

Similar experiments were carried out to determine if the RuvA and RuvB proteins caused RecA protein to dissociate from DNA during DNA strand exchange. In this assay, circular ssDNA and linear duplex DNA containing a 375-bp heterologous DNA insertion were used. Rates of ATP hydrolysis were measured during RecA protein-mediated strand exchange

[3] S. K. Jain and M. Cox, unpublished results.

[4] L. Iype, unpublished data.

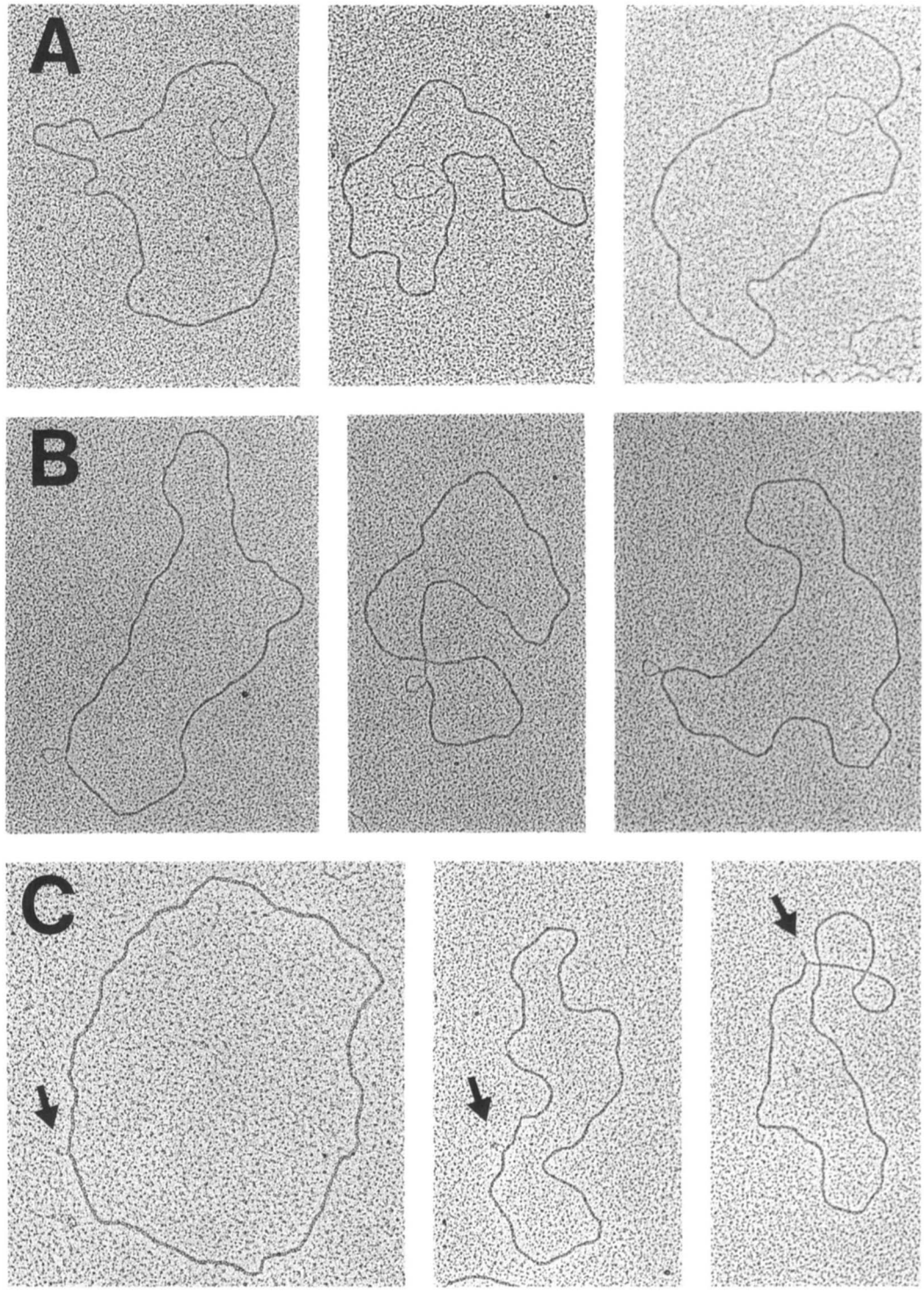


FIG. 7. **Electron microscopy of DNA strand exchange products.** RecA protein-mediated DNA strand exchange reactions (with RuvA and RuvB proteins) were carried out between M13mp8 circular ssDNA and M13mp8.1037 (*A*), M13mp8.375 (*B*), or M13mp8.198 (*C*) linear duplex DNA as described in the legends to Figs. 4 and 6. After 90 min, the reactions were stopped, and the DNA was prepared for electron microscopy as described under "Experimental Procedures." Samples were not cross-linked. The molecules shown are representative of the products observed; each contains the expected single-stranded loop derived from the heterologous insert. The *arrows* in *C* indicate the small 198-bp single-stranded loops.

(with SSB present), with either no Ruv proteins or with Ruv proteins added simultaneously, or 2 or 10 min after initiation of the reaction (Table III). The protein concentrations used in this assay were somewhat reduced relative to the previous set of experiments to permit examination of the rate of ATP hydrolysis over a longer period of time, but the ratios of each protein to DNA and to each other were identical to those in the strand exchange experiments of Figs. 4–8.

When homologous duplex DNA is paired with the ssDNA in a RecA nucleoprotein filament, the intrinsic rate of ATP hydrolysis or k_{cat} decreases abruptly by about 30%, then remains at the new level throughout the ensuing strand exchange reaction (Schutte and Cox, 1987). If it is again assumed that the RecA is bound at a level of 1 monomer/3 nucleotides of ssDNA, the observed rate of ATP hydrolysis in the present experiments yielded a k_{cat} for bound RecA during strand exchange of about 20 min^{-1}, consistent with published values (Schutte and Cox, 1987). The ATP hydrolysis in the reaction with the RuvA and

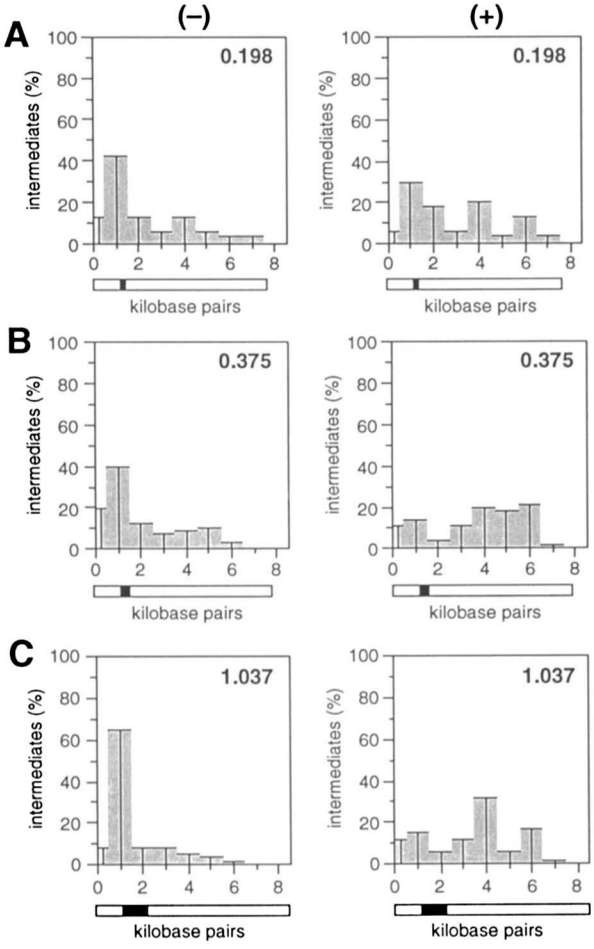


FIG. 8. **Estimated lengths of exchanged DNA in intermediates.** RecA protein-mediated DNA strand exchange reactions (with or without RuvA and RuvB proteins) were carried out between M13mp8 circular ssDNA and M13mp8.198 (*A*), M13mp8.375 (*B*), or M13mp8.1037 (*C*) linear duplex DNA as described in the legends to Figs. 4 and 6. After 90 min, the reactions were stopped, and the DNA was prepared for electron microscopy. Samples were not cross-linked. The length of DNA exchanged was then estimated and plotted as described under "Experimental Procedures." The linear duplex DNA used in each experiment is depicted as a *horizontal bar*. The heterologous DNA insertion is shown as a *solid black box*. The – and + symbols denote reactions without and with RuvA and RuvB proteins, respectively.

RuvB proteins only was very low. When the RecA, RuvA, and RuvB proteins were added simultaneously, the rate of ATP hydrolysis was reduced significantly (about 52% on average). When the RuvA and RuvB proteins were added to the strand exchange reaction after either 2 or 10 min, there was no significant change in the rate of ATP hydrolysis. These results paralleled those obtained for the ssDNA-dependent ATPase activity. They indicate that RuvA and B compete with RecA for binding to DNA, but do not substantially displace RecA once it is bound.

It seemed possible that an interaction with RecA might increase the ATPase activity of RuvB and that this increase might mask a decrease in RecA-mediated ATP hydrolysis due to dissociation. As one approach to this question, the same assay was used to measure ATP hydrolysis during strand exchange, but a mutant RecA protein, RecAK72R, was employed in place of wild type RecA. RecAK72R is deficient in ATP hydrolysis, but is still able to bind ATP and catalyze limited strand exchange (Rehrauer and Kowalczykowski, 1993). In this assay RecAK72R had negligible ATP hydrolysis but ATP hydrolysis mediated by the RuvB protein was detectable (data not shown). The low levels of RuvB-mediated ATPase activity were increased no more than 2-fold in the presence of RecAK72R under strand exchange conditions. A similar increase in the presence of wild type RecA would still make the RuvB-mediated ATPase a minor background reaction that would be insufficient to mask a significant dissociation of RecA.

TABLE II
RecA protein-mediated ssDNA-dependent ATPase rates in the presence and absence of RuvA and RuvB proteins

RecA protein ATP hydrolysis rates were determined as described under "Experimental Procedures" and in the text. In this assay, the circular ssDNA concentration was 10 μM, and the final concentration of proteins were: RecA protein, 3.3 μM; RuvA protein, 0.15 μM; RuvB protein, 0.5 μM, and SSB protein, 1 μM. ATP hydrolysis was followed for 20 min. Rates presented are from three independent experiments (A, B, and C).

	Rates of ATP hydrolysis		
	A	B	C
	μM min^{-1}		
Proteins in reaction			
RecA	85.8	85.8	84.2
RuvA and RuvB	1.8	0.9	0.5
RecA, RuvA and RuvB added simultaneously	39.6	31.4	34.7
RecA reaction with RuvA and RuvB added after 10 min			
Rate before addition of RuvA/B	84.2	82.5	84.2
Rate after addition of RuvA/B	84.2	79.2	80.8

TABLE III
RecA protein-mediated ATPase rates during strand exchange in the presence and absence of RuvA and RuvB proteins

The circular ssDNA and linear duplex DNA concentrations were both 5 μM, and the final concentration of proteins were: RecA protein, 1.7 μM; RuvA protein, 0.075 μM; RuvB protein, 0.25 μM, and SSB protein, 0.5 μM. ATP hydrolysis was followed for 40 min. ATP hydrolysis rates presented are from three independent experiments (A, B, and C).

	Rates of ATP hydrolysis		
	A	B	C
	μM min^{-1}		
Proteins in reaction			
RecA	31.4	38	34.6
RuvA and RuvB	0.3	0.4	0.6
RecA, RuvA, and RuvB added simultaneously	13.2	14	23.1
RecA reaction with RuvA and RuvB added after 2 min			
Rate before and after addition of RuvA/B	36.3	38	39.6
RecA reaction with RuvA and RuvB added after 10 min			
Rate before addition of RuvA/B	35.5	36.3	39.6
Rate after addition of RuvA/B	34.3	38.0	39.6

Effects of RuvA and RuvB Proteins when the Heterologous DNA Insertion Is at One End of the Linear Duplex DNA—Previous studies have shown that the RecA protein-mediated three-strand exchange reaction is impeded when a substantial heterologous sequence is present at the 5′ end of the linear duplex (Fig. 1, reaction *C*), and little product formation occurs. When the insertion is at the 3′ end, strand exchange proceeds to the homology/heterology junction and is then blocked (Fig. 1, reactions *D*) (Bedale *et al.*, 1991, 1993; Jwang and Radding, 1992). The effect of the RuvA and RuvB proteins on a heterologous insertion located at the 5′ or 3′ end of the linear duplex DNA was examined.

Results of experiments using linear duplex substrates with a

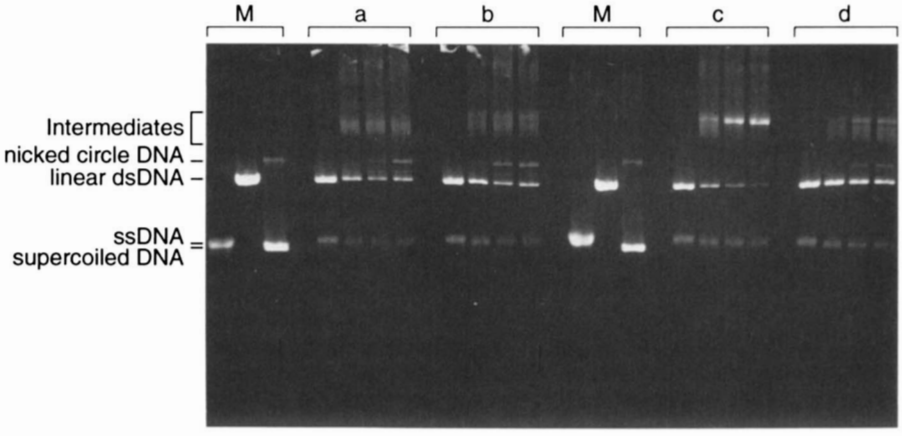


FIG. 9. **Effect of RuvA and RuvB proteins on RecA protein-mediated DNA strand exchange when the duplex DNA substrate contains a 198-bp heterologous insertion at the 5′ or 3′ end.** Reactions were carried out under standard reaction conditions as described under "Experimental Procedures" and included (in 80 μl of total volume) 21 μM M13mp8 circular ssDNA, 21 μM M13mp8.198 dsDNA, and 2 μM SSB. RecA (7 μM final), RuvA (0.3 μM final), and RuvB (1 μM final) proteins were included as indicated below. The M13mp8.198 dsDNA was linearized with *Eco*RI in reactions *A* and *B*, placing the heterology at the 5′ end; the same DNA was linearized with *Bam*HI in reactions *C* and *D*, placing the heterology at the 3′ end. For all reactions, the time points correspond to 0, 30, 60, and 90 min. Markers for reactions *A* and *B* are (from *left* to *right*): circular M13mp8 (+) ssDNA, M13mp8.198 linear duplex DNA (linearized with *Eco*RI), and M13mp8.198 circular duplex DNA (supercoiled and a minor nicked circular component). Reaction *A* contained RecA protein alone. Reaction *B* contained RecA protein along with RuvA and RuvB proteins. Markers for reactions *C* and *D* are (from *left* to *right*): circular M13mp8 (+) ssDNA, M13mp8.198 linear duplex DNA (linearized with *Bam*HI), and M13mp8.198 circular duplex DNA (supercoiled and a minor nicked circular component). Reaction *C*, RecA protein; reaction *D*, RecA protein, RuvA protein, and RuvB protein.

198-bp heterologous sequence at one end are shown in Fig. 9. When the heterologous sequence is on the 5′ end, there is a very limited production of nicked circular duplex product with RecA protein that is not significantly altered when RuvA and RuvB are added (Fig. 9, reactions *a* and *b*). The DNA species which do form are branched intermediates as shown below. This experiment was also carried out with a 375-bp heterologous insertion at the 5′ end of the linear duplex DNA. There was no product formation in this experiment, and no substantial change in the formation of products or intermediates when the RuvA and RuvB proteins were added (data not shown).

A similar experiment was carried out using a linear duplex DNA with a 198-bp heterologous DNA insertion located at the 3′ end (Fig. 9). The strand exchange reaction with RecA protein yields intermediates which are halted at the heterologous DNA insertion; products were absent (Fig. 9, reaction *c*). When the RuvA and RuvB proteins were added to this strand exchange reaction, there was a slight increase in product formation (Fig. 9, reaction *d*). However, the more significant effect appeared to be a reduction in the amount of intermediates and an increase in substrates when compared with the reaction without the Ruv proteins. The increased quantity of substrates could be explained by a poor strand exchange reaction with these substrates in the presence of the RuvA and RuvB proteins or it could be that the RuvA and RuvB proteins promote the conversion of intermediates to substrates. To test these possibilities, a reaction was carried out with these substrates in which the Ruv proteins were added 30 or 60 min after strand exchange was initiated (Fig. 10). Before the addition of the RuvA and RuvB proteins, there was an accumulation of the band corresponding to the stalled intermediate. However, after RuvA and RuvB protein addition, the quantity of intermediate diminished and the quantity of substrate increased substantially. West and colleagues (Tsaneva *et al.*, 1992b) have made similar observations.

These same reactions were analyzed by electron microscopy (Table IV), which served in this case to confirm the observations made with the agarose gel assays. When the heterologous sequence was at the 5′ end, the electron microscopy counts indicated that addition of RuvA and RuvB increased product formation slightly, but elicited no other significant changes.

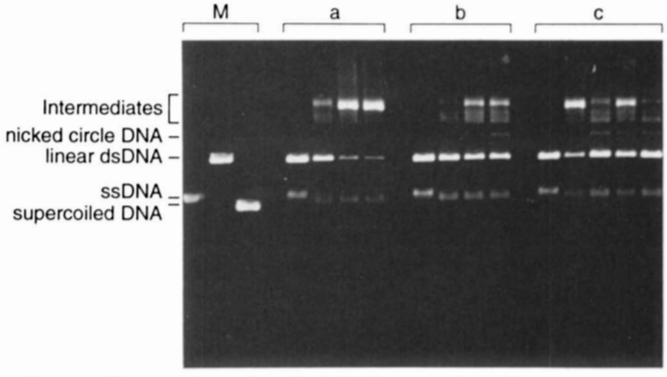


FIG. 10. **Conversion of stalled DNA strand exchange intermediates to substrates by RuvA and RuvB proteins.** Reactions were carried out under standard reaction conditions as described under "Experimental Procedures" and included (in 80 μl of total volume) 21 μM M13mp8 circular ssDNA, 21 μM M13mp8.198 dsDNA (linearized with *Bam*HI to place the heterology on the 3′ end), and 2 μM SSB. RecA (7 μM final), RuvA (0.3 μM final), and RuvB (1 μM final) proteins were included as indicated below. Markers are (from *left* to *right*): circular M13mp8 (+) ssDNA, M13mp8.198 linear duplex DNA (linearized with *Bam*HI), and M13mp8 circular duplex DNA (supercoiled and a minor nicked circular component). For reactions *A* and *B*, the time points correspond to 0, 30, 60, and 90 min. Reaction *A* contains RecA protein. Reaction *B* contains RecA protein along with the RuvA and RuvB proteins. In reaction *C*, a RecA protein-mediated DNA strand exchange reaction was initiated as in reaction *A* and allowed to proceed for 30 min at 37 °C. The reaction was then divided into four aliquots of 14 μl each. These aliquots were treated as follows: tube 1, no addition; tubes 2–4, RuvA and RuvB proteins were added (to 0.3 and 1.0 μM final concentrations, respectively), but at different times. In tube 2, the RuvA and RuvB were added immediately, whereas in tubes 3 and 4, the RuvA and RuvB were added after 30 additional min at 37 °C. Tubes 1 and 2 were then incubated at 37 °C for 60 min to give a total reaction time of 90 min. Tube 3 was incubated for 30 min after the RuvA and RuvB addition to give a total reaction time of 90 min. Tube 4 was incubated for 60 min after the RuvA and RuvB addition to give a total reaction time of 120 min. *Lane 1* in reaction *C* is a 0 time point. Succeeding lanes from *left* to *right* are the reactions in tubes 1–4.

When the heterology was located at the 3′ end, there was again a slight increase in product formation, but the more significant effect was the apparent reversal of strand exchange which served to convert stalled intermediates to substrates when RuvA and RuvB were added.

TABLE IV

Percent of species generated in a strand exchange reaction with linear duplex DNA substrates containing heterology located at the 5′ or 3′ end

Strand exchange reactions (90 min) between M13mp8 circular ssDNA and M13mp8.198 linear duplex DNA with 5′ heterologous insertion or 3′ heterologous insertion were carried out, and the reactions were spread for electron microscopy as described under "Experimental Procedures." Values in the table are given as percentage of molecules in the counted DNA sample. Standard deviations of these values ranged from ± 2 to ± 5 (these errors are statistical counting errors rather than variations between different experiments). *n* represents the total number of molecules counted for each reaction. A description of the broken molecule and miscellaneous molecule categories are given in the text. Reactions − and + are results obtained in the absence or presence of RuvA/B, respectively.

Category	5′ 198-bp heterology		3′ 198-bp heterology	
	−	+	−	+
Linear duplex DNA	49	41	30	73
Intermediates	18	25	59	14
Nicked circular products	10	19	4	4
Broken molecules	13	11	4	3
Miscellaneous molecules	10	4	3	6
n	213	217	104	178

DISCUSSION

Our primary conclusion is that the RuvA and RuvB proteins facilitate the bypass of heterologous DNA insertions during RecA protein-mediated DNA strand exchange. Whereas RecA protein alone can promote the bypass of short heterologous insertions (with reasonable efficiency up to about 100 bp), longer insertions are a barrier to strand exchange. Addition of the RuvA and RuvB proteins permits an effective bypass of insertions up to at least 1037 bp in length. Bypass of the insertions is brought about without introducing nicks in the DNA.

This activity represents a new function for the RuvA and RuvB proteins. Previously, these proteins had been implicated in the processing of Holliday junctions during homologous genetic recombination, with the promotion of branch migration thought to be their primary activity (West, 1994). The new activity fits in with one theme evident in earlier work, that these proteins are required at late stages of recombination processes and act on branched recombination intermediates. However, the present results provide a function more closely associated with recombinational DNA repair, suggesting that these proteins may be important in situations requiring the bypass of structural barriers that RecA alone cannot effect.

The data we have obtained so far suggest that the Ruv proteins act together with RecA protein to bypass heterologous inserts, but we do not know if the proteins act synergistically or sequentially. RuvA and RuvB effected insertion bypass even when added to a reaction 60 min after it had been initiated, but there was no evidence for significant dissociation of RecA bound to the branched intermediates when the Ruv proteins were added. The Ruv proteins cannot initiate a strand exchange reaction (Iwasaki *et al.*, 1992) and do not inhibit an ongoing RecA-mediated strand exchange reaction with homologous substrates.

While RuvA and RuvB do not appear to displace RecA in preformed filaments, they seem to compete with RecA for available DNA binding sites. This aspect of the reaction with all three proteins merits further investigation.

The bypass of heterologous insertions occurred only when the insertion was located internally within the linear duplex. This result suggests that RecA/RuvA/RuvB-mediated bypass requires homology on both sides of the insertion just as with RecA-mediated bypass of shorter insertions (Jwang and Radding, 1992).[5] When the heterologous sequences were present on the 5′ (proximal) end of the duplex, the addition of RuvA and RuvB had little discernible effect on the outcome of the reaction. When the heterologous sequences were present on the 3′ end of the duplex, RuvA and RuvB actually effected a partial reversal of the reaction, leading to conversion of a significant fraction of the stalled intermediates to substrates. We have not yet investigated the latter reaction in detail, although it suggests that at least one aspect of RuvAB action exhibits a polarity that is opposite to that of RecA protein-mediated DNA strand exchange.

We cannot offer a complete mechanism for this reaction, but our results address at least one obvious possibility. The simplest mechanism would be one in which the RecA protein promoted strand exchange until a barrier was reached that it could not bypass. The RuvA and RuvB proteins would then recognize the stalled intermediate, load onto the DNA, and unwind the duplex DNA in the insertion by means of their demonstrated helicase activity. The inability of RuvA and RuvB to unwind heterologous sequences located at the 3′ end of the duplex argues against this scheme in its simplest forms. The helicase activity of RuvAB appears to be ineffective in any case for unwinding duplex DNAs exceeding about 550 bp in length (Tsaneva *et al.*, 1993).

Another observation may have mechanistic significance. There was an increase in the length of exchanged DNA in observed strand exchange intermediates when RuvA and RuvB proteins were present. However, *none* of these intermediates had ssDNA loops to indicate a bypassed heterologous insertion, implying that the intermediates had initiated at the 3′ or distal end of the linear duplex DNA. This suggests that RuvA and RuvB facilitate a strand exchange reaction in the direction opposite to that normally mediated by RecA protein or at least stabilize intermediates formed at the 3′ end so they are not as readily eliminated by normal RecA action. The apparent absence of intermediates in which the insertion had been bypassed could mean that bypass is the final step in the pathway, preceded by strand exchange on both sides of the insertion.

Although the addition of RuvA and B does not result in substantial displacement of RecA, the ATPase assay we used to investigate the status of RecA binding is indirect and could easily miss changes in binding involving only a few percent of the monomers in the filaments. Therefore, we cannot eliminate the possibility that a small amount of RecA is displaced at the point at which RuvA and RuvB proteins are loaded onto the DNA.

Helicases and topoisomerases must play a significant role in genetic recombination and DNA repair *in vivo*. This study suggests some new avenues for examining the function of an unusual DNA helicase in recombinational DNA repair.

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