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RecA Protein-facilitated DNA Strand Breaks

A MECHANISM FOR BYPASSING DNA STRUCTURAL BARRIERS DURING STRAND EXCHANGE*

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Wendy A. Bedale‡, Ross B. Inman, and Michael M. Cox§

From the Department of Biochemistry, College of Agriculture and Life Sciences, University of Wisconsin, Madison, Wisconsin 53706

RecA protein promotes an unexpectedly efficient DNA strand exchange between circular single-stranded DNA and duplex DNAs containing short (50–400-base pair) heterologous sequences at the 5′ (initiating) end. The major mechanism by which this topological barrier is bypassed involves DNA strand breakage. Breakage is both strand and position specific, occurring almost exclusively in the displaced (+) strand of the duplex within a 15-base pair region of the heterology/homology junction. Breakage also requires recA protein, ATP hydrolysis, and homologous sequences 3′ to the heterology. Although the location of the breaks and the observed requirements clearly indicate a major role for recA protein in this phenomenon, the molecular mechanism is not yet clear. The breakage may reflect a DNA structure and/or some form of structural stress within the DNA during recA protein-mediated DNA pairing which either exposes the DNA at this precise position to the action of a contaminating nuclease or induces a direct mechanical break. We also find that when heterology is located at the 3′ end of the linear duplex, strand exchange is halted (without DNA breakage) about 500 base pairs from the homology/heterology junction.

The recA protein of *Escherichia coli* is a DNA-dependent ATPase, and it promotes a number of *in vitro* DNA strand exchange reactions that mimic key steps in homologous genetic recombination (Cox and Lehman, 1987; Radding, 1989; Roca and Cox, 1990). One of the most useful and best studied of these reactions is the three-strand exchange shown in Fig. 1A. This reaction is promoted by a nucleoprotein filament that coats the ssDNA[1] as the first step in the reaction. Strand exchange proceeds unidirectionally 5′ to 3′ relative to the ssDNA (Cox and Lehman, 1981a; Kahn *et al.*, 1981; West *et al.*, 1981). In a reaction between completely homologous DNA substrates the products are equivalent to substrates in terms of total number of base pairs, and the equilibrium constant for this reaction should therefore be 1.0. No covalent bonds in the DNA are broken during the reaction, and little energy should be required to drive this reaction. This idea is reinforced by the observations that 1) substantial recA protein-mediated strand exchange can occur in the presence of ATPγS, which is minimally hydrolyzed by recA protein (Menetski *et al.*, 1990), and 2) several eukaryotic proteins have been isolated which promote strand exchange with no ATP requirement (Hsieh *et al.*, 1986; Kolodner *et al.*, 1987).

Not all strand exchange reactions in the cell, however, involve completely homologous DNA substrates. Genetic recombination is an important avenue for DNA repair, and strand exchange during recombinational repair must bypass many types of DNA lesions. In addition, homologous recombination is often accompanied by gene conversion events that can involve addition or deletion of DNA sequences (Lichten and Fox, 1984). *In vitro* recA protein-promoted DNA strand exchange with ATP proceeds efficiently past DNA lesions, mismatches, or short heterologous sequences in either DNA substrate in three-strand reactions (Bianchi and Radding, 1983; Das Gupta and Radding, 1982; Livneh and Lehman, 1982), and (less efficiently) in four-strand exchange reactions between gapped circular and linear duplex DNA (Hahn *et al.*, 1988).

Heterologous insertions in one of the DNAs represent a particularly imposing structural and thermodynamic barrier to strand exchange. Bianchi and Radding (1983) demonstrated that recA protein promotes some strand exchange past heterologous insertions in one of the DNA substrates even when the insertions are several hundred base pairs in length. This observation represents one of the most difficult phenomenons to accommodate within the current models for recA protein-mediated DNA strand exchange. Since recA protein lacks an intrinsic nuclease activity, strand exchange past a heterologous insert in the duplex substrate was presumed to involve an unwinding of the DNA within the insert to effect the bypass.

We have begun to examine the effects of heterologous DNA sequences in one substrate on recA protein-mediated strand exchange in an effort to extend the results of Bianchi and Radding. Our focus to date has been the effects of heterologies at the ends of the linear duplex DNA substrate. Known properties of the system led us to expect that heterology on the 3′ end of the duplex DNA (relative to the (+) strand) would permit strand exchange to proceed up to the boundary of the heterology (Fig. 1*B*), but heterology on the 5′ (proximal or initiating) end would effectively block a productive initiation event (Fig. 1*C*). We found instead that more strand exchange products (defined as DNA species that comigrate on agarose gels with the nicked circular duplex product shown in Fig. 1*A*) are formed when the heterology is at the 5′ end

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§ To whom correspondence should be sent. Tel.: 608-262-1181.

[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; FI DNA, supercoiled closed circular form of DNA as isolated from *E. coli* cells; FII DNA, the nicked circular form of the same molecule; FIII DNA, the linear form of the same molecule; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; AMT, 4′-amino-4,5′,8-trimethylpsoralen; bp, base pair(s).

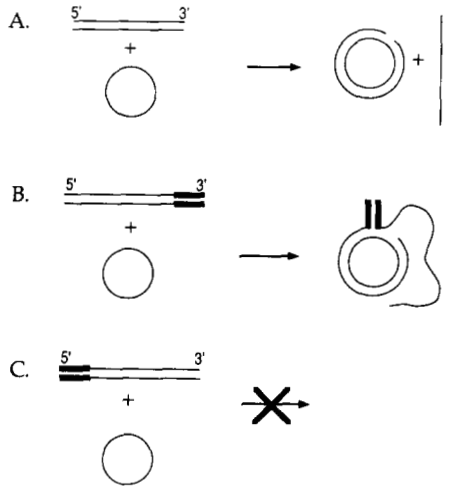


FIG. 1. **RecA protein-promoted three-strand exchange reactions.** *A*, three-strand exchange reaction between completely homologous circular ssDNA and linear duplex DNA, leading to the production of a nicked circular duplex and the displaced linear (+) strand. *B*, heterology at the 3′ end of the (+) strand of the linear duplex substrate would be predicted to prevent complete displacement of the (+) strand of the incoming duplex. This would result in the formation of a circular duplex molecule with a short double-stranded tail and a long single-stranded tail as shown. *C*, heterology at the 5′ end (relative to the (+) strand) of the linear duplex would be expected to prevent any productive strand exchange from occurring.

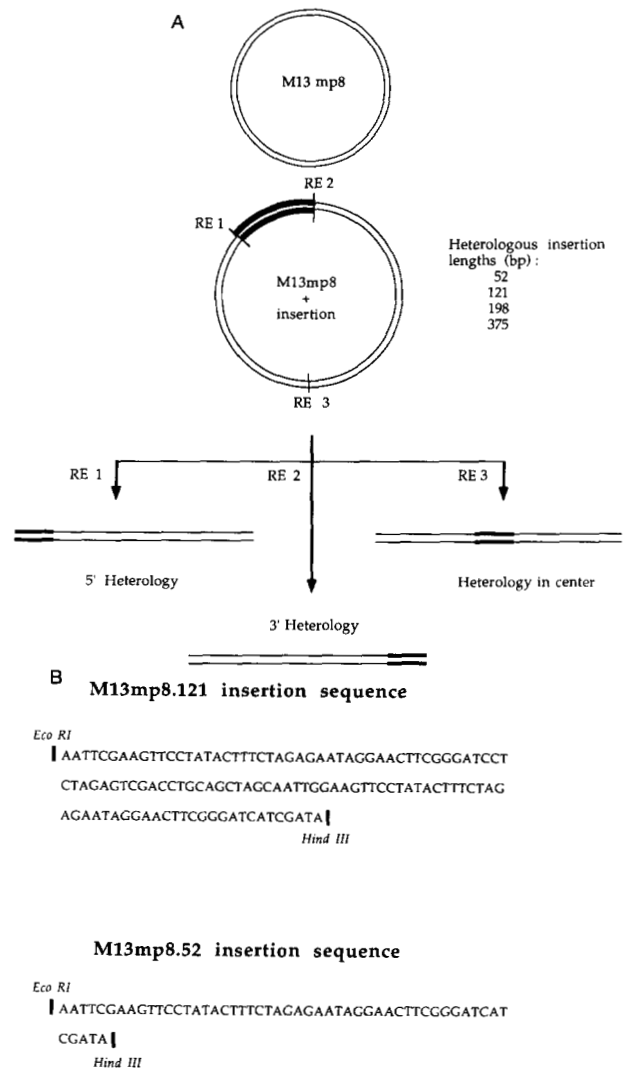


FIG. 2. **DNA substrates used for experiments.** *A*, heterologous insertions of various lengths were cloned into M13mp8 at a single site within the polylinker (M13mp8.198) or between two sites of the polylinker (M13mp8.52, M13mp8.121, and M13mp8.375) as described under "Experimental Procedures." Digestion of the supercoiled DNA with the appropriate restriction enzyme yielded linear duplex DNA substrates with the heterology located internally or at one of the two ends. *B*, DNA sequences of the heterologous insertions in M13mp8.121 and M13 mp8.52.

than when it is at the 3′ end of the duplex substrate. The mechanism by which most of these products are formed has proven to involve DNA strand breakage rather than unwinding of the heterologous DNA. In light of the ease in which large amounts of recA protein are purified in nearly homogeneous form and the lack of any reported nuclease activity by this protein, this result is unexpected. The strand breakage phenomenon is characterized in this report. We have also characterized the product formed when heterology is present at the 3′ end of the duplex DNA. Heterology at the 3′ end would be expected to permit strand exchange to proceed up to the boundary of the heterology (Fig. 1*B*), but our results indicate that strand exchange stops about 500 bp before reaching the heterology.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—E. coli recA protein was purified and stored as described previously (Cox *et al.*, 1981). Additional recA protein purified by the method of Griffith and Shores (1985) was the generous gift of the laboratory of Dr. Stephen Brenner. 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* SSB protein was purified as described (Lohman *et al.*, 1986). Additional SSB purified by the same method was the generous gift of the laboratory of Dr. Timothy Lohman. The concentration of the SSB protein stock solutions was determined by absorbance at 280 nm, with an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}$ mg^{-1} ml (Lohman and Overman, 1985). Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Calf intestinal alkaline phosphatase and Tris buffer were purchased from Boehringer Mannheim. Reverse transcriptase was purchased from Life Sciences. Proteinase K, pyruvate kinase, formamide, phosphoenolpyruvate, creatine kinase, phosphocreatine, and ATP were purchased from Sigma. The DNA sequences of M13mp8.52, M13mp8.121, and M13mp8.198 were verified using the Sequenase version 2.0 sequencing kit from U. S. Biochemical Corp. Radionucleotides were purchased from Amersham Corp., and 4′-amino-4,5′,8-trimethylpsoralen (AMT) was purchased from Calbiochem.

DNA—Duplex and ssDNA substrates were derived from bacteriophage M13mp8 (Fig. 2*A*) (Messing and Vieira, 1982). The bacteriophages M13mp8.52 and M13mp8.121 were constructed by replacing the 30-bp *Eco*RI-*Hind*III fragment of bacteriophage M13mp8 with short heterologous sequences (52 and 121 bp, respectively) originally derived from the plasmid pJFS36 (Senecoff *et al.*, 1985). The sequences of these insertions are shown in Fig. 2*B*. The bacteriophage M13mp8.375 was constructed similarly by replacing the 10-bp *Eco*RI-*Bam*HI fragment of bacteriophage M13mp8 with the 375-bp *Eco*RI-*Bam*HI fragment of pBR322. The bacteriophage M13mp8.198 is bacteriophage M13mp8 with a 198-bp (*Rsa*I-*Rsa*I fragment from the *E. coli gal*T gene) inserted at the *Sma*I site (Lindsley and Cox, 1990). Circular duplex and single-stranded DNA from M13mp8 and its derivatives described above were prepared using methods described previously (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). Single-stranded ϕX174 DNA was purchased from New England Biolabs. The concentration of ssDNA and dsDNA stock solutions were determined by absorbance at 260 nm, using 36 and 50 μg ml^{-1} A_{260}^{-1}, respectively, as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Complete digestion of FI M13mp8 and its derivatives with appropriate restriction enzymes yielded 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*A*). 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. Phosphatizing and radiolabeling of duplex DNA were performed as described (Sambrook *et al.*, 1989). The specific activity of the end-labeled DNA was typically about 1 μCi/μg, and the labeled DNA was generally used within 10 days of its preparation.

Reaction Conditions—Unless stated elsewhere, all reactions were performed at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol, and an ATP-regenerating system (5 units/ml of pyruvate kinase, 2.3 mM phosphoenolpyruvate, or 10 units/ml creatine kinase, 12 mM phosphocreatine). Duplex DNA and ssDNA, both at 20 μM, were preincubated with 6.7 μM recA protein for 10 min before ATP (3 mM) and SSB (2 μM) were added to start the reactions.

Agarose Gel Assays—Aliquots (10 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 buffer (0.125% bromphenol blue, 0.125% xylene cyanol FF, 15% glycerol, 5% SDS). The aliquots were stored on ice until after the last time point was taken. Samples were electrophoresed overnight in a 0.8% agarose gel at 2–2.5 V cm^{-1}. The percentage of DNA that had completed strand exchange to form FII DNA product was determined by scanning photographic negatives of the gels stained in ethidium bromide. The 100% value was defined as the amount of FIII DNA in the zero time point lane. The densitometric scans were performed on a Zeineh Soft Laser scanning densitometer, SL-504-XL, from Biomed Instruments, Inc.

Denaturing Gel Assays—Aliquots (5 μl) of strand exchange reactions performed with ^{32}P end-labeled dsDNA were removed at each time point, and the reactions were stopped by the addition of 0.5 μl of 1% SDS. Each aliquot was stored on ice until the last time point was taken, at which time 0.5 μl of 10 mg/ml proteinase K was added. After incubation at 65 °C for 10 min, 6 μl of formamide loading buffer (Sambrook *et al.*, 1989) was added to each sample. The samples were boiled for 3 min and immediately loaded onto a denaturing 6% polyacrylamide gel that had been prerun at 35 watts for 45 min. After electrophoresis at 35 watts for 90 min, the gel was dried, and bands were visualized by autoradiography.

Electron Microscopy—Samples for electron microscopy were obtained by either elution of DNA product bands from agarose gels or by spreading whole reaction mixtures. Elution of DNA from agarose gels was performed using a unidirectional electroeluter (International Biotechnologies, Inc.) according to the manufacturer's instructions or by electroelution into dialysis membranes (Sambrook *et al.*, 1989). Some reaction mixtures to be examined by electron microscopy were treated with the psoralen derivative AMT. For these reactions, aliquots (30 μl) of strand exchange reactions were immediately mixed with AMT (60 μg/ml, final concentration), incubated at room temperature for 3 min, and irradiated with UV light for 5 min on ice (Umlauf *et al.*, 1990). This AMT-cross-linking treatment resulted in at least one cross-link/220 bp (data not shown). Cross-linked and noncross-linked DNA samples were prepared for electron microscopy similarly. Proteinase K (0.5 mg/ml final) and SDS (0.5% final) were added, and the samples were then incubated at 37 °C for 30 min. The samples were loaded onto small spun columns (Umlauf *et al.*, 1990), and the column flow-through was dialyzed extensively against 20 mM NaCl and 5 mM EDTA. The dialyzed samples and the electroeluted samples were spread as described previously (Inman and Schnos, 1970). Photography and measurements of the DNA molecules were performed as described previously (Littlewood and Inman, 1982).

RESULTS

Experimental Design—The overall purpose of the experiments described in this paper was to examine the effects of heterologous sequences at either end of the linear duplex DNA on recA protein-promoted strand exchange. Several complementary methods of investigation were employed to examine the products of these reactions. First, agarose gel assays were used to provide a simple and rapid means of monitoring the strand exchange reactions (Cox and Lehman, 1981b). To identify and characterize the products formed during these reactions more completely, they were examined by electron microscopy. In the case of 5′ heterology in the duplex DNA, electron microscopy revealed some unusual products that seemed to be the result of DNA strand breakage. This DNA strand breakage was characterized further by denaturing polyacrylamide gel assays. In the descriptions below, 5′ and 3′ refer to the (+) strand of the linear duplex substrate, unless otherwise specified. The 5′ end is therefore the end at which strand exchange would normally be initiated if the substrates were completely homologous.

Form II-like Products Are Formed More Efficiently with 5′ Heterology Than with 3′ Heterology in the Duplex DNA—In the case of strand exchange between completely homologous circular ssDNA and linear duplex DNA, strand exchange is efficient and leads to the formation of a nicked circular duplex DNA (FII) product (Cox and Lehman, 1981b). This FII product can be easily distinguished from the linear duplex substrate on agarose gels and can be used to assess the extent and efficiency of the strand exchange reaction (Fig. 3*A*, reaction *a*; Fig. 3*B*, reaction *a*). We predicted that strand exchange with duplex DNA substrates possessing 3′ or 5′ heterology would yield the results depicted in Fig. 1, *B* and *C*. These predictions were based on the observed polarity of recA protein-promoted strand exchange and the presumed requirement for homology at the 5′ end of the (+) strand of the dsDNA substrate (Bianchi *et al.*, 1983; Cox and Lehman, 1981a; Kahn *et al.*, 1981; West *et al.*, , 1981). In either case, FII production would be expected to be minimal. In the case

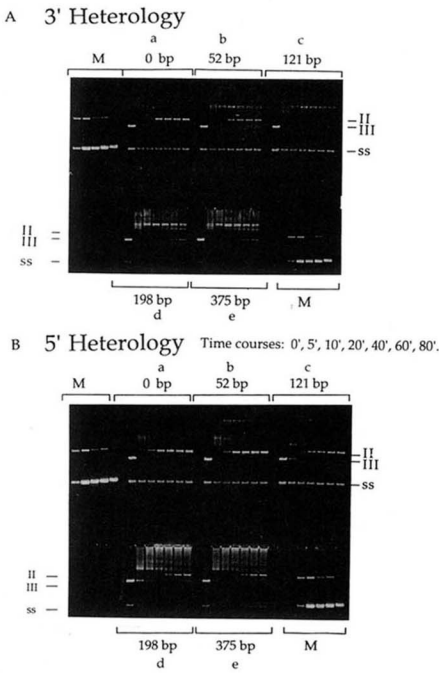


FIG. 3. **Effects of 5′ or 3′ heterologous ends in the duplex DNA on recA protein-promoted strand exchange.** Strand exchange reactions were performed under standard conditions with 6.7 μM recA protein, 20 μM ssDNA, 20 μM dsDNA, and 2.3 mM phosphoenolpyruvate as described under "Experimental Procedures." For each reaction, the time points shown correspond to 0, 10, 20, 40, 60, and 80 min of reaction. In each reaction, the single-stranded DNA is M13mp8 (+). *A*, effect of 121 bp of heterology in the duplex DNA on strand exchange. Markers are (from *left* to *right*) M13mp8 (FI and FII) and M13mp8.121 (FI and FII). (Single-stranded and FI M13mp8 DNA migrate very similar under these electrophoretic conditions.) The linear duplex DNA in each reaction is *a*, M13mp8 (*Eco*RI) (completely homologous reaction); *b*, M13mp8.121 (*Eco*RI) (5′ heterology in dsDNA); *c*, M13mp8.121 (*Alw*NI) (heterology in center of dsDNA); *d*, M13mp8.121 (*Hin*dIII) (3′ heterology in dsDNA). *B*, effect of 198 bp of heterology in dsDNA on strand exchange. Markers are (from *left* to *right*) M13mp8 (FI and FII DNA) and M13mp8.198 (FI and FII DNA). The linear duplex DNA in each reaction is *a*, M13mp8 (*Eco*RI) (completely homologous reaction); *b*, M13mp8.198 (*Eco*RI) (5′ heterology in dsDNA); c, M13mp8.198 (*Bam*HI) (3′ heterology in dsDNA).

of 3′ heterology, recA protein-promoted strand exchange might begin and continue through the homologous region. Upon reaching the heterology, strand exchange must stop since there is no complementary sequence in the circular ssDNA to which the (−) strand of the duplex can be transferred. Such a strand exchange reaction would result in the formation of a double-stranded circular molecule with the normally displaced (+) strand still attached via base pairing at the heterologous tail (Fig. 1*B*). If the recA protein possessed a DNA helicase or unwinding activity, as has been suggested to account for recA protein-promoted strand exchange through heterologous insertions in the center of the duplex DNA (Bianchi *et al.*, 1985; Bianchi and Radding, 1983), this heterologous tail may be unwound. Unwinding of this heterology would result in complete displacement of the (+) strand of the original duplex, leading to the formation of a double-stranded circular DNA molecule with a short single-stranded tail. For 5′ heterology, one might predict that no reaction at all could occur since the 5′ end of the (+) strand of the duplex cannot react with the ssDNA; in effect, this short 5′ heterologous sequence would be expected to serve as a topological barrier to strand exchange.

Strand exchange was examined with a series of duplex substrates containing short lengths of 5′ or 3′ heterology (52, 121, 198, or 375 bp). Unexpectedly, a significant amount of the substrate DNA was converted to a product that migrated at the position of the FII DNA marker on agarose gels when a short 5′ heterologous sequence was present in the duplex substrate (Fig. 3*A*, reaction *b*; Fig. 3*B*, reaction *b*). When this heterologous sequence was present near the center of the duplex substrate, some of the substrate DNA was converted to a product migrating at the position of FII, in agreement with the results of Bianchi and Radding (1983) although the conversion was less efficient than when the heterology was positioned at the 5′ end of the molecule. For 3′ heterology, much less product migrating at the position of FII was observed; instead, much of the substrate DNA was converted to a product migrating substantially slower than the FII marker (Fig. 3*A*, reaction *d*; Fig. 3*B*, reaction *c*). The striking difference in efficiency and extent of reactions between ssDNA and dsDNA containing 3′ *versus* 5′ heterology is not the result of an inherent difference in reactivity of the two DNA substrates; with completely homologous ssDNA both duplex substrates react identically (data not shown). An apparently more efficient reaction for 5′ heterology in the dsDNA was observed for all lengths of heterology tested (Fig. 4). Essentially no products migrating at the position of FII were observed for 3′ heterologous sequences longer than 198 bp whereas substantial FII-like product formation was obtained even with 1,037 bp of 5′ heterology (data for the 1,037-bp heterology is not shown). In Fig. 5, the percent of FIII substrate DNA which has been converted to a band migrating at the position of FII DNA is plotted *versus* the length of 5′ or 3′ heterology. Reactions with 5′ and 3′ heterology were performed on different days so the efficiencies of product formation between completely homologous substrates were used to normalize other data from the 2 days. For both 3′ and 5′ heterology in the dsDNA, as the length of heterology increases the amount of linear dsDNA converted to a product that migrates at the position of FII decreases. However, in every case, 5′ heterology resulted in greater amounts of this product than 3′ heterology (Fig. 5). Similar experiments were performed 22 times over a period of 13 months with this same general result.

Strand Exchange Reactions with 3′ or 5′ Heterology Result in Characteristically Branched Products—To learn more about

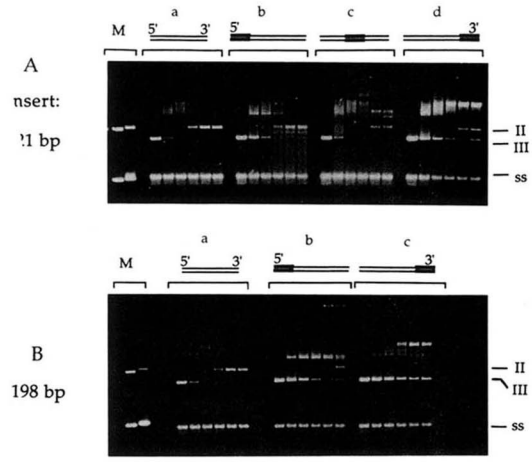


FIG. 4. **Effect of increasing lengths of 3′ or 5′ heterologous ends in the duplex DNA on recA protein-promoted strand exchange.** For both *A* and *B*, markers (*M*) are FI and FII DNA from (*left* to *right*) M13mp8, M13mp8.52, M13mp8.121, M13mp8.198, and M13mp8.375. Strand exchange reactions were performed as described under "Experimental Procedures" and the legend to Fig. 3. For each reaction, the time points shown correspond to 0, 5, 10, 20, 40, 60, and 80 min of reaction. In each reaction, the single-stranded DNA is M13mp8 (+). *A*, effect of increasing lengths of 3′ heterology on strand exchange. The linear duplex DNA in each reaction is *a*, M13mp8 (*Eco*RI); b, M13mp8.52 (*Hin*dIII); *c*, M13mp8.121 (*Hin*dIII); *d*, M13mp8.187 (*Bam*HI), and M13mp8.375 (*Bam*HI). *B*, effect of increasing lengths of 5′ heterology on strand exchange. The linear duplex DNA in each reaction is *a* M13mp8 (*Eco*RI); *b*, M13mp8.52 (*Eco*RI); *c*, M13mp8.121 (*Eco*RI); *d*, M13mp8.198 (*Eco*RI); and M13mp8.375 (*Eco*RI).

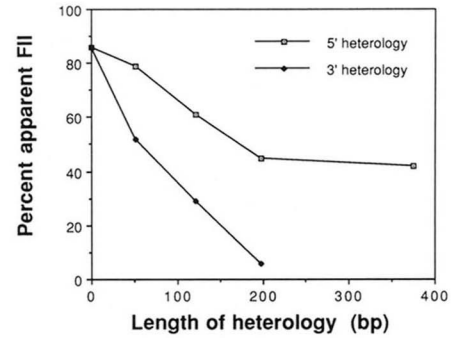


FIG. 5. **Effect of increasing lengths of 5′ or 3′ heterologous ends in the duplex substrate on recA protein-promoted strand exchange.** Strand exchange reactions were performed as described under "Experimental Procedures" and the legend to Fig. 3. The percent of original FIII substrate DNA that has been converted to a product migrating at the position of FII was determined by densitometry as described under "Experimental Procedures." This value was plotted *versus* the length of heterology present at the 5′ end of the duplex substrate (⊡——⊡) or the 3′ end of the duplex substrate (◆——◆).

the products formed during recA protein-promoted strand exchange reactions when 3′ or 5′ heterology is present on the linear duplex DNA substrate, the reactions were examined by electron microscopy. The DNA products of some of these reactions were cross-linked with AMT under strand exchange reaction conditions when aliquots were removed from the reaction mixture. The AMT cross-linking was designed to prevent spontaneous branch migration after the subsequent removal of recA protein.

After 40 min of reaction, the major product obtained when 198 bp of heterology was present at the 3′ end of the duplex

DNA appeared similar to what was predicted; extensive (near complete) heteroduplex formation between the homologous regions of the two substrates had occurred, and the (+) strand of the original duplex DNA was displaced but still attached to the rest of the product by the base pairing in the short heterologous tail (Figs. 6 and 7). As expected, this species migrates more slowly than FII in an agarose gel. In complete reaction mixtures spread after 40 min of reaction, this species represented 79 and 88% of all DNA species (excluding unreacted ssDNA) observed by electron microscopy in two independent samples of 52 and 150 total molecules, respectively. Only one molecule in either of the samples appeared to be a circular duplex with a short single-stranded tail, suggesting that unwinding of the 3′ heterologous tail does not occur to a significant extent. The remainder of the DNA molecules in these samples were not characterized extensively and consisted primarily of unbranched circular duplexes, some possessing single-stranded regions. These molecules probably account for the appearance of small amounts of product migrating at the position of FII DNA on agarose gels (Figs. 3 and 4). The lengths of the double-stranded tails in these products were measured for randomly chosen molecules taken from both uncross-linked and AMT-cross-linked samples. For the cross-linked molecules, the double-stranded tail lengths were fairly uniform. In a set of 21 molecules, the tail length averaged 757 bp, with 18 of these tail lengths falling in a narrow range between 579 and 984 bp (the exceptions measured 304, 383, and 1581 bp, respectively). Subtracting the known length of the heterologous tail from these tail measurements indicates that recA protein-mediated strand exchange halts 556 ± 248 bp from the heterologous tail. The length of single-stranded DNA in the circle (adjacent to the tail) measured 542 bp on average, consistent with the conclusions obtained from the tail measurements.

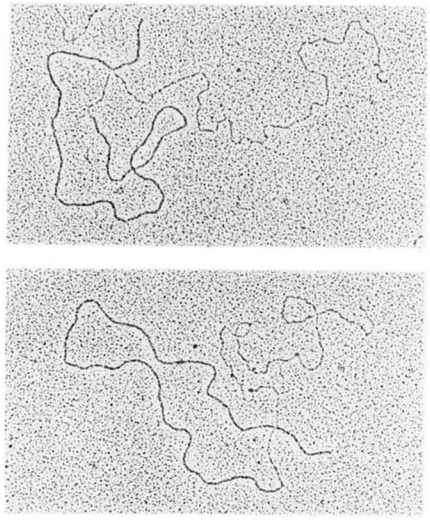


FIG. 6. **Electron microscopy of the major product formed when heterology is present at the 3′ end of the (+) strand of the duplex DNA.** Strand exchange reactions between single-stranded M13mp8 (+) and M13mp8.198 (*Bam*HI) were performed as described under "Experimental Procedures." After 40 min of reaction, the products were cross-linked with AMT, the recA protein was removed, and the DNA was spread for electron microscopy. The two molecules shown are representative of the major product observed for this reaction.

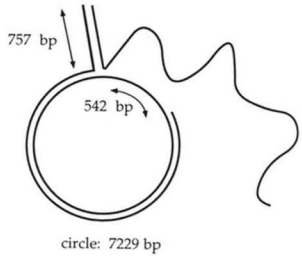


FIG. 7. **Schematic representation of the major product formed when 198 bp of 3′ heterology is present in the duplex DNA.** Computer-assisted length measurements were made from electron micrographs as described previously (Littlewood and Inman, 1982). All measured lengths were calculated in base pairs relative to the length of unreacted circular single-stranded M13mp8 (7,229 nucleotides) also observed in micrographs. A normalization factor of 0.95 was applied to all measurements of ssDNA or single-stranded regions to account of the extension of ssDNA relative to dsDNA. Additionally, correction factors were applied to the measurements of the double-stranded tail and the double-stranded part of the circle to account for the observed extension of AMT-cross-linked duplex DNA in the absence (0.963) or presence (0.941) of recA protein (W. A. Bedale, R. B. Inman, and M. M. Cox, unpublished results). These correction factors were determined by comparing measured lengths of M13mp8 FIII (naked and uncross-linked, after AMT cross-linking of naked DNA, and after AMT cross-linking of recA protein-bound DNA.

A problem encountered in the cross-linked samples was random breakage of the long single-stranded tail (displaced strand). These single strands were broken in a majority (71%) of the molecules examined. This is apparently an artifact of the AMT-cross-linking procedure. In uncross-linked samples, 83% of the single-stranded tails had the correct length. In the uncross-linked molecules, however, the range of lengths of the double-stranded tails was expanded (average 1,399 bp; range from about 300 to 4,800 bp). This indicates that substantial branch migration did occur in these molecules subsequent to protein removal; therefore, the cross-linked samples probably provide a better indication of the location of the branch when the reaction was stopped.

The products of reactions with duplex DNA containing 198 bp of 5′ heterology were also examined by electron microscopy. A major product of such reactions migrated at the same position as the M13mp8.198 FII marker on agarose gels (Figs. 3 and 4). We reasoned that complete displacement of the (+) strand of the original duplex would require unwinding of the heterologous sequences, and the result would be a double-stranded circular molecule with a short (198-base) single-stranded tail. This product would be expected to essentially comigrate with M13mp8.198 FII on an agarose gel, as the single-stranded tail would be small compared with the size of the circular duplex. After 1 h of reaction, the major product observed by electron microscopy of uncross-linked reactions was indeed a double-stranded circular molecule with a short branch or tail, but the tail was double stranded rather than single stranded (Fig. 8). This product represented 39 out of 65 total species electroeluted from the FII region of agarose gels after electrophoresis of the reaction mixture. (Each separate species viewed by electron microscopy, whether an isolated substrate molecule or a complex joint species composed of several molecules, was counted as one for statistical purposes.) Other products observed in the electroeluted samples included linear duplex DNA and circular duplexes with and without single-stranded regions. Circular duplexes with short single-stranded tails accounted for 5 of 65 electroeluted species, suggesting that unwinding of the 5′ heterology is a minor mechanism for generating products migrating at the position of FII on agarose gels. In complete reaction mixtures, the double-stranded tailed species represented 9% of all the DNA species (161 total) observed. The majority of DNA species observed in complete reaction mixtures consisted of ssDNA (52%, of which approximately 71% was circular and 29%

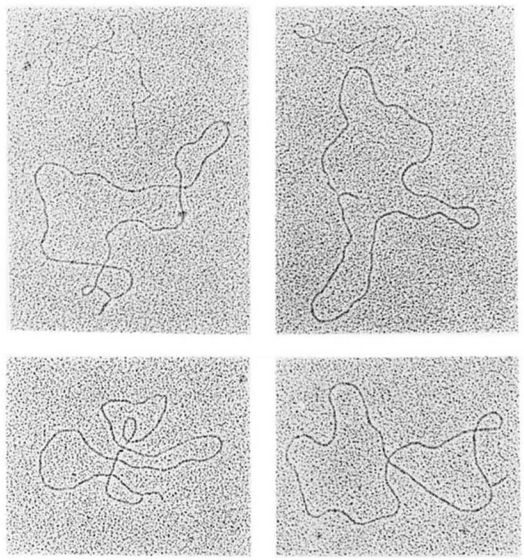


FIG. 8. **Electron microscopy of the major product formed when heterology is present at the 5′ end of the (+) strand of the duplex DNA.** Strand exchange reactions between single-stranded M13mp8 (+) and M13mp8.198 (*Eco*RI) were performed as described under "Experimental Procedures." The sample molecules shown are representative of the major product observed following electroelution of the band migrating at the position of FII on agarose gels.

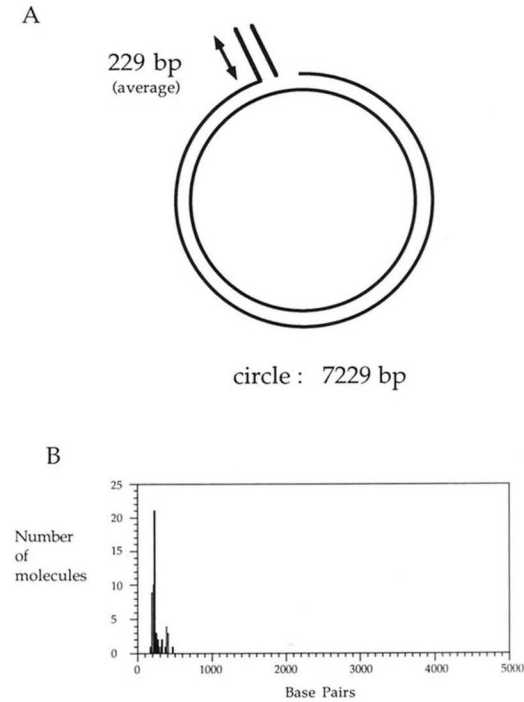


FIG. 9. **Products formed when 198 bp of heterologous DNA are present at the 5′ end of the (+) strand of the duplex DNA.** *A*, schematic representation of products formed when 198 bp of heterologous DNA are present at the 5′ end of the (+) strand of the duplex DNA. The measured length of the circular component of the product was normalized to 7,229 bp. This normalization factor was used to determine the length of the double-stranded tail in terms of base pairs. *B*, distribution of the measured lengths of the double-stranded tails for 58 randomly chosen molecules.

linear ssDNA). The large amount of single-stranded DNA observed reflects the fact that ssDNA is present in 2-fold excess relative to the duplex DNA. Complex aggregates (species consisting of ≥3 substrate molecules in undefined associations) constituted 22% of all species. To confirm the double-stranded nature of the tails, DNA products were AMT cross-linked and denatured before spreading. Denaturation bubbles in the circle and (rarely) in the short tail confirmed that the molecules were entirely duplex (data not shown). This result eliminates the possibility that the FII-like species is actually a triple-stranded circle with a duplex tail, as might be formed if the linear duplex simply wrapped itself around the ssDNA throughout the region of homology. Products with a duplex tail (Figs. 8 and 9) could arise only if the (+) strand of the original linear duplex DNA is broken, allowing the homologous portion of the (+) strand of the original duplex to be displaced via strand exchange but leaving the heterologous portion of the (+) strand still base paired to its complement in the (−) strand. From measurements of 58 molecules chosen at random, the average length of the tails was determined to be 229 ± 68 bp, with a range in lengths of 151–447 bp (Fig. 9). This measured length is very close to the length of the heterology present at the 5′ end of the duplex DNA (198 bp). As shown in Fig. 9*B*, most of the tail lengths fall between 200 and 220 bp although a small cluster of tail lengths near 400 bp is also apparent. These longer tails are described in more detail below.

Characterization of DNA Breakage by Denaturing Gel Electrophoresis—To investigate further the apparent DNA strand breakage that was occurring in the reactions described above a denaturing gel assay was employed. The duplex DNA for these reactions was 5′ end labeled with ^{32}P. Products from these reactions were deproteinized, denatured, and subjected to electrophoresis on a denaturing 6% polyacrylamide gel. Denaturation of the products observed by electron microscopy (Fig. 9*A*) would be expected to generate two labeled products: a very long, linear, single-stranded DNA, and a 200 base single-stranded fragment. The results for the duplex substrate containing 198 bp of 5′ heterology are shown in Fig. 10, *lanes 2–6*. The major product band appearing on the autoradiogram migrated between 201 and 217 bases, which correlates well with the length of the double-stranded tail observed by electron microscopy (229 bp) and with the length of the heterology present at the 5′ end of the FIII substrate (198 bp). In addition, this band appeared with approximately the same kinetics as the band on agarose gels which migrates at the position of FII (Fig. 4*B*, reaction *d*; Fig. 10, *lanes 2–6*). This observation suggests that strand breakage and production of the band migrating at the position of FII are temporally related; for example, breakage may allow subsequent strand exchange to proceed efficiently through the homologous regions shared by the two substrates. The slow rate of appearance of these products as observed on agarose and sequencing gels suggested that strand exchange itself is not the rate-limiting step in their production since unhindered strand exchange between completely homologous substrates occurs much faster (Figs. 3 and 4).

Essentially no short DNA fragments were observed on denaturing gels as the result of strand exchange reactions when the short heterologous sequence was present at the 3′ end of the (+) strand in the 5′ end-labeled duplex DNA (Fig. 10, *lanes 7–11*; Fig. 11, *lanes 5–7*). This observation is consistent with the electron microscopy and agarose gel results discussed above; extensive strand exchange between the substrates occurs, but little or no specific strand breakage is seen. In addition, no breakage was seen when the duplex DNA is 3′ end labeled regardless of whether 5′ or 3′ heterology was present (Fig. 11, *lanes 8–13*). These results indicate that breakage is occurring exclusively near the 5′ end of the (+) strand of the duplex DNA and only when heterology is present

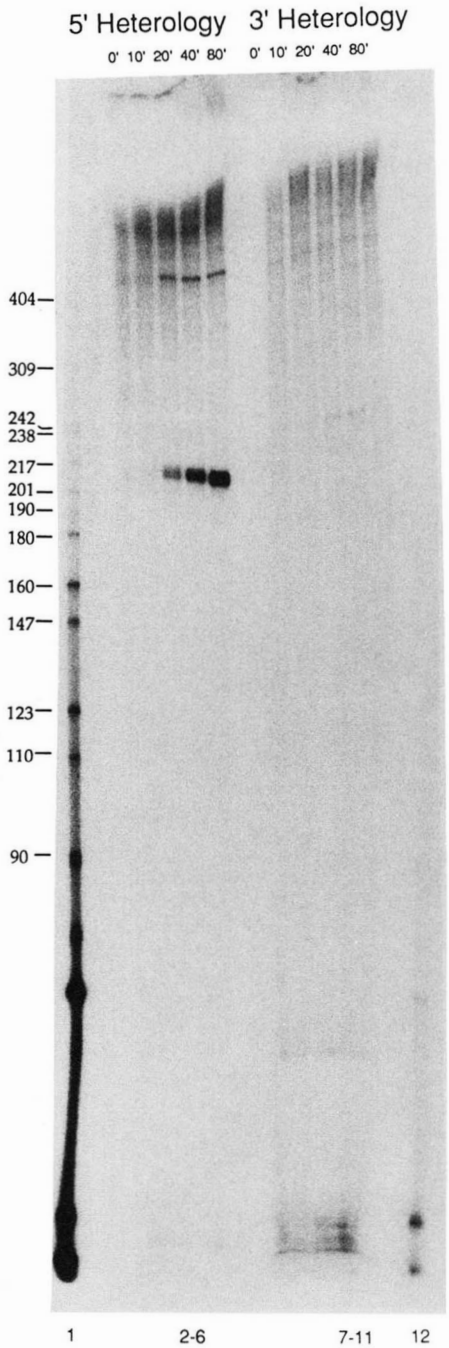


FIG. 10. **Denaturing acrylamide gel of strand exchange reactions with 198 bp 5′ or 3′ heterology in the duplex DNA.** Strand exchange reactions were performed with 5′ end-labeled DNA as described under "Experimental Procedures" and in the legend to Fig. 3. The markers (*Lanes 1* and *12*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. The time course for each reaction is 0, 10, 20, 40, and 80 min. *Lanes 2–6* correspond to the reaction between single-stranded M13mp8 (+) and M13mp8.198 (*Eco*RI) (5′ heterology in the (+) strand of the duplex DNA). *Lanes 7–11* correspond to the reaction between M13mp8 (+) and M13mp8.198 (*Bam*HI) (3′ heterology in the (+) strand of the duplex DNA).

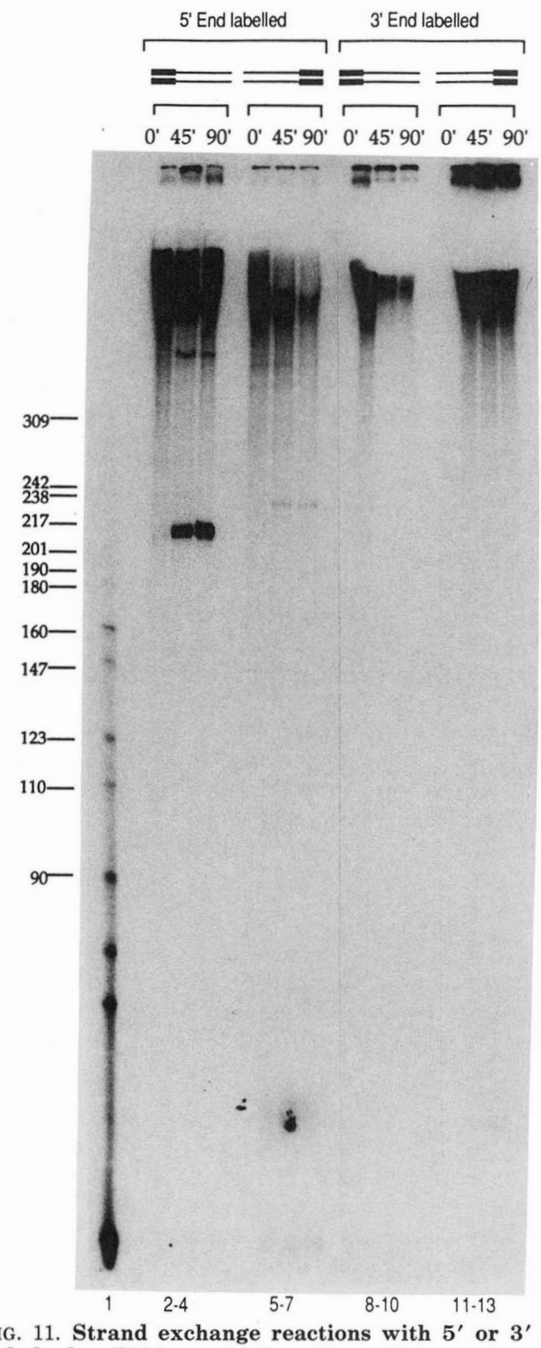


FIG. 11. **Strand exchange reactions with 5′ or 3′ end-labeled duplex DNA containing 5′ or 3′ heterology.** Strand exchange reactions were performed with 5′ (*lanes 2–7*) or 3′ end-labeled (*lanes 8–13*) DNA as described under "Experimental Procedures" and the legend to Fig. 3. The markers (*lane 1*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. The time course for each reaction is 0, 45, and 90 min. *Lanes 2–4* and *8–10* correspond to the reaction between single-stranded M13mp8 (+) and M13mp8.198 (*Eco*RI) (5′ heterologous end). *Lanes 5–7* and *11–13* correspond to the reactions between single-stranded M13mp8 (+) and M13mp8 (*Bam*HI) (3′ heterologous end).

at the 5′ end of the duplex. In agreement with the electron microscopy observations discussed above, the breaks are both position and strand specific.

Additional denaturing gel assays were performed to characterize further the requirements for DNA strand breakage (Figs. 11 and 12). Reactions with completely homologous ssDNA did not result in strand breakage (Fig. 12, *lanes 2–4*), indicating that some heterology in the dsDNA is essential for breakage. Homology is required, however, since breakage was also not observed when completely heterologous ssDNA (ϕX174) was used (Fig. 12, *lanes 8–10*). RecA protein purified by the method of Griffith and Shores (1985) also promoted breakage (Fig. 12, *lanes 11–13*), suggesting that breakage is not the result of a contaminating nuclease found exclusively in our recA protein preparation. Breakage requires recA protein and partially homologous ssDNA since omission of either

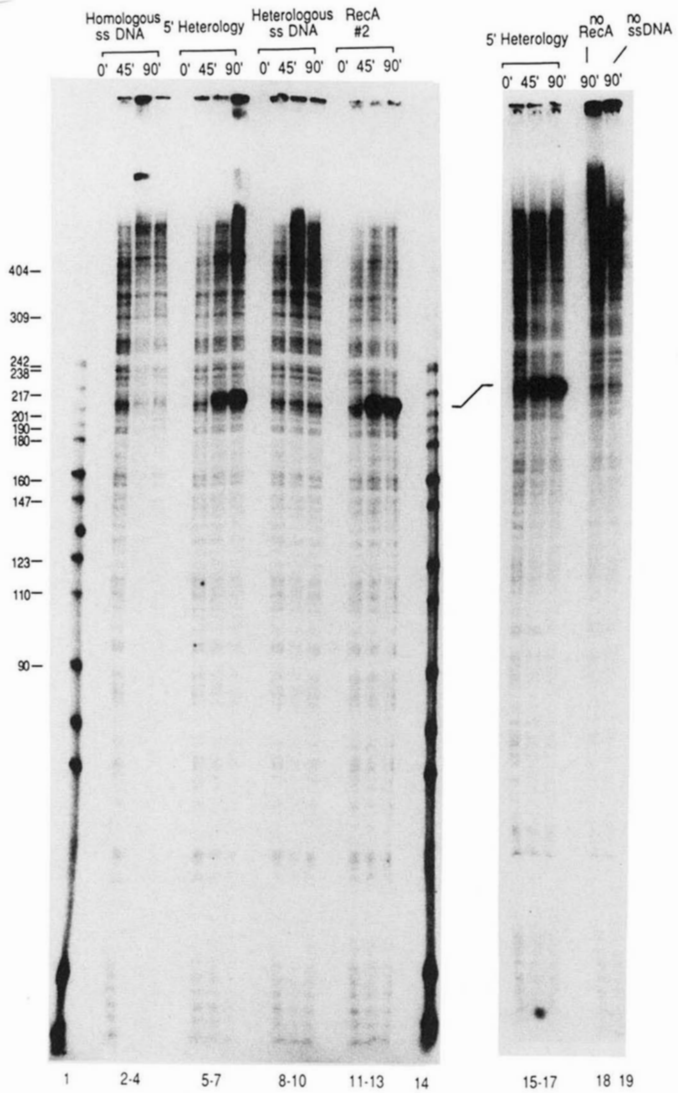


FIG. 12. **Requirements for DNA strand breakage.** Strand exchange reactions were performed with 5′ end-labeled M13mp8.198 (*Eco*RI) as described under "Experimental Procedures" and the legend of Fig. 3. The markers (*lanes 1* and *13*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. The time course for each reaction is 0, 45, and 90 min. *Lanes 2–4* correspond to the reaction with single-stranded M13mp8.198 (+) (complete homology). *Lanes 5–7* and *15–17* correspond to the reaction with single-stranded M13mp8 (+) (5′ heterologous end in the duplex DNA). *Lanes 8–10* correspond to the reaction with single-stranded ϕX174 (+) (complete heterology). *Lanes 11–13* correspond to the reaction with single-stranded M13mp8 (+) (5′ heterologous end in the duplex DNA) using recA protein obtained from the laboratory of Dr. Stephen Brenner. *Lanes 18* and *19* correspond to the 90-min time points of reactions lacking recA protein or ssDNA, respectively.

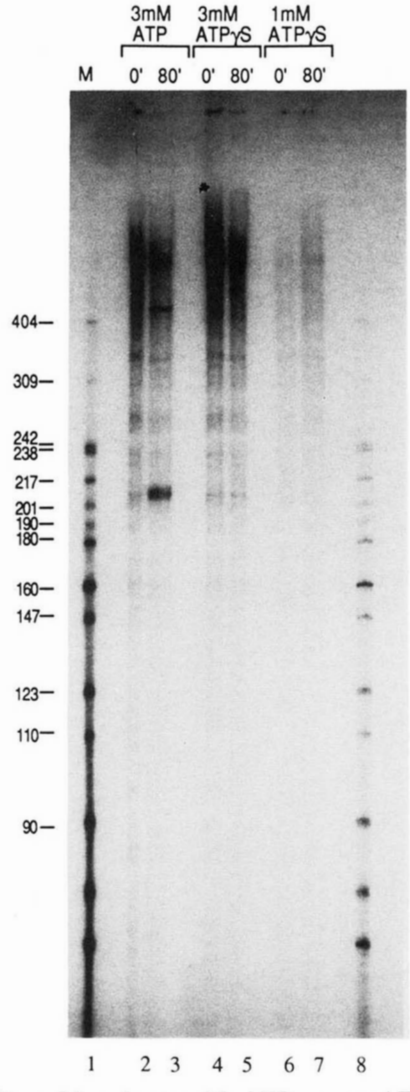


FIG. 13. **Strand breakage with ATP *versus* ATPγS.** Strand exchange reactions were performed with single-stranded M13mp8 (+) and 5′ end-labeled M13mp8.198 (*Eco*RI) as described under "Experimental Procedures" and the legend to Fig. 3. The markers (*lanes 1* and *8*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. The time points for each reaction are 0 and 80 min. *Lanes 2* and *3* correspond to the standard reaction (3 mM ATP). *Lanes 4* and *5* correspond to an identical reaction except that 3 mM ATPγS is substituted for the ATP, and the ATP-regenerating system is omitted. *Lanes 6* and *7* correspond to a reaction using the reaction conditions of Menetski *et al.* (1990) which permit stable heteroduplex formation with ATPγS.

from the reaction prevented the production of a labeled band migrating near 198 bases on the denaturing gel (Fig. 12, *lanes 18* and *19*, respectively). Breakage, as described above, occurs when short heterology is present at the 5′ end of an otherwise homologous duplex DNA but not to a significant extent when the heterology is on the 3′ end, suggesting that homologous sequences positioned 3′ to the heterology may be important.

The effect of recA protein-mediated ATP hydrolysis on strand breakage was examined with the experiment in Fig. 13. The standard reaction that produces the (+) strand break is shown in *lanes 2* and *3*. The substitution of ATPγS for ATP prevented strand breakage (Fig. 13, *lanes 4* and *5*). Additionally, strand breakage did not occur under the reaction conditions used by Menetski *et al.* (1990) to obtain extensive heteroduplex formation with ATPγS (including 4 mM magnesium acetate) (Fig. 13, *lanes 6* and *7*). We confirmed independently the results of Menetski *et al.* (1990) with our DNAs and recA preparation (data not shown), and the results in *lanes 6* and *7* were obtained under conditions in which substantial strand exchange occurs when the two DNA substrates were completely homologous. The strand breaks clearly require ATP hydrolysis. It is unclear whether ATP hydrolysis is used directly for breakage or whether ATP hydrolysis plays an indirect role such as facilitating the formation of a DNA structure that is more susceptible to breakage. In general, the requirements for breakage outlined in Figs. 12 and 13 are very similar to those for recA protein-promoted strand exchange, suggesting that the two reactions are closely related.

Further denaturing gel assays were used to determine

whether the position at which strand breakage occurs reflects the heterology/homology junction or some other DNA structural feature. In Fig. 14, strand breakage was examined for substrates with different lengths of 5′ heterology and different heterology/homology junction sequences. As described above for the case of 198 by of 5′ heterology, breakage occurs between 201 and 217 bases from the 5′ end of the (+) of the duplex (Fig. 10, *lanes 2–6*; Fig. 11, *lanes 2–4*; Fig. 12, *lanes 5–7, 11–13*; Fig. 13, *lanes 2* and *3*; Fig. 14, *lanes 2* and *3*). When the heterology at the 5′ end of the dsDNA was 121 bp long instead of 198 bp, strand exchange resulted in the production of fragment bands migrating at 110–123 bases on the denaturing gel (Fig. 14, *lanes 4* and *5*). When this same 121 bp of heterology was located near but not at the 5′ end of the duplex substrate so that 317 bp of homology were present on the 5′ side of the heterology, a band migrating at >404 bases was observed. In this last substrate, the 3′ end of the heterology was 121 + 317 = 438 bp from the 5′ end of the molecule. Breakage therefore occurs on the 3′ side of the heterology near the heterology/homology junction in all of these cases. These results also argue against the possibility that the fragment bands seen in Figs. 10–14 were produced by breakage of the labeled (−) strand (at the opposite end of the molecule) rather than breakage of the (+) strand.

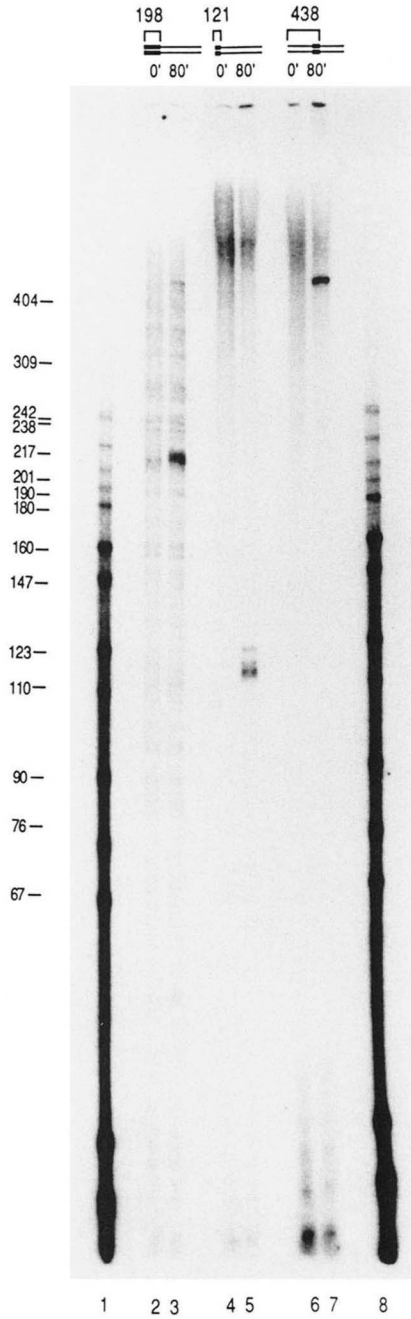


FIG. 14. **Strand breakage with other lengths and positions of heterology in the duplex DNA.** Strand exchange reactions were performed with 5′ end-labeled DNA as described under "Experimental Procedures" and the legend to Fig. 3. The markers (*lanes 1* and *8*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. The time points for each reaction are 0 and 80 min. The single-stranded DNA in each reaction is single-stranded M13mp8 (+). *Lanes 2* and *3* correspond to the reaction with M13mp8.198 (*Eco*RI) (198 bp of heterologous DNA at the 5′ end). *Lanes 4* and *5* correspond to the reaction with M13mp8.121 (*Eco*RI) (121 bp of heterologous DNA at the 5′ end). *Lanes 6* and *7* correspond to the reaction with M13mp8.121 (*Ava*II) (121 bp of heterologous DNA beginning 317 bp from the 5′ end).

Breakage does not occur at a single site but at several sites with various efficiencies within a small (approximately 15-base) region near the heterology/homology junction (Fig. 15). Breakage also does not always occur at the same sequence. The 198- and 121-bp insertions in bacteriophages M13mp8.198 and M13mp8.121 were cloned into different sites of M13mp8 so the sequences at the heterology/homology junctions are substantially different (Fig. 15). Breakage is therefore not the result of a sequence-specific nuclease.

Tests for Nuclease Activity in Protein Preparations—Because cleavage of the DNA by a contaminating nuclease represents one possible molecular mechanism for the strand breaks, all reagents used in this study were analyzed extensively for the presence of nuclease activities. Each of the proteins used (recA protein, SSB protein, pyruvate kinase, creatine kinase, and proteinase K) was incubated separately for 3 h with FI, FIII, or single-stranded DNA (all at 20 μM) at concentrations equivalent to twice those used in these experiments. No significant degradation of the DNA was observed; although a small amount of FI DNA was nicked after 3 h of incubation with each of these proteins, no nicking was observed after 90 min under normal reaction conditions (data not shown). In addition, the recA protein-mediated strand breakage described here was observed with two recA protein preparations purified by different procedures (Fig. 12, *lanes 5–7* and *11–13*), three SSB protein preparations purified in two different laboratories, and two different ATP-regenerating systems (as well as without any regenerating system) (data not shown).

ATP Levels Do Not Affect DNA Strand Breakage—Concern arose in the course of these investigations regarding the level

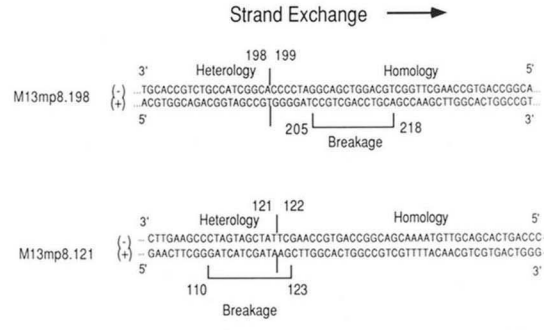


FIG. 15. **Approximate sequence positions of DNA strand breakage.** The regions at which DNA strand breakage is occurring (as determined by denaturing polyacrylamide gels) for M13mp8.198 (*Eco*RI) and M13mp8.121 (*Eco*RI) in reactions with M13mp8 (+) are indicated. The exact nucleotide positions at which breakage occurs have not yet been determined.

of ATP present at the time of DNA strand breakage. The high recA protein and ssDNA concentrations used in these experiments lead to rapid depletion of the ATP supply, even when a phosphoenolpyruvate/pyruvate kinase ATP regeneration system is provided. ATPase assays were performed showing that under the conditions used here the standard ATP regeneration system (with 2.3 mM phosphoenolpyruvate) is exhausted after 11 min of reaction. This is consistent with known rates of ATP hydrolysis by recA protein under the conditions used. Because DNA strand breakage was not observed until about 20 min of reaction (Fig. 10, *lane 4*) it was possible that breakage only occurred when ADP levels were rising. As ADP/ATP ratios increase, recA protein/DNA complexes gradually dissociate (Cox *et al.*, 1983; Lee and Cox, 1990), leaving the status of the recA protein filament in these reactions questionable. It was therefore important to learn whether breakage could occur under conditions in which ATP hydrolysis is still occurring, which should correspond to conditions in which the recA protein filament is intact. We therefore carried out these reactions under two sets of conditions in which ATP is regenerated for longer periods of time. These experiments were done with the duplex substrates containing a 121-bp 5′ heterologous sequence. First, we have performed reactions with 12 mM phosphocreatine and creatine kinase. Calculations and ATPase assays demonstrated that ATP was regenerated (ADP is minimal) for 60 min under these conditions.[2] Second, we performed reactions in which we decreased the recA protein, SSB protein, ssDNA, and the dsDNA concentrations by a factor of 3.33. In this case, ATP was regenerated for 37 min. In both of these sets of reactions, DNA breakage was observed prior to 30 min (Fig. 16, and data not shown), indicating that breakage does not depend upon net dissociation of recA protein such as that observed when ADP levels are allowed to rise (Lee and Cox, 1990).

Note the presence of a second band in the experiments in Fig. 16, reflecting a second break location in the (+) strand about 200 bp in the 3′ direction from the one described above. This band can also be seen in other experiments and is generally found 200 bp from the heterology/heterology junction. For example, when the duplex substrate with a 198-bp 5′ heterology is used, a minor break point is sometimes observed in the (+) strand about 400 nucleotides from the 5′ end (see Fig. 9*B* and Fig. 10, *lane 6*). The amount of this product varies greatly in different experiments. The band is especially prominent in the experiment of Fig. 16. Efficient breakage at the heterology/homology junction, of course, would obscure detection of breaks at this second site (it is seen to diminish as the junction break increases in Fig. 16, *lanes 2–5*). We do not know the significance of this secondary break site except that it indicates that breaks are not restricted entirely to the heterology/homology junction.

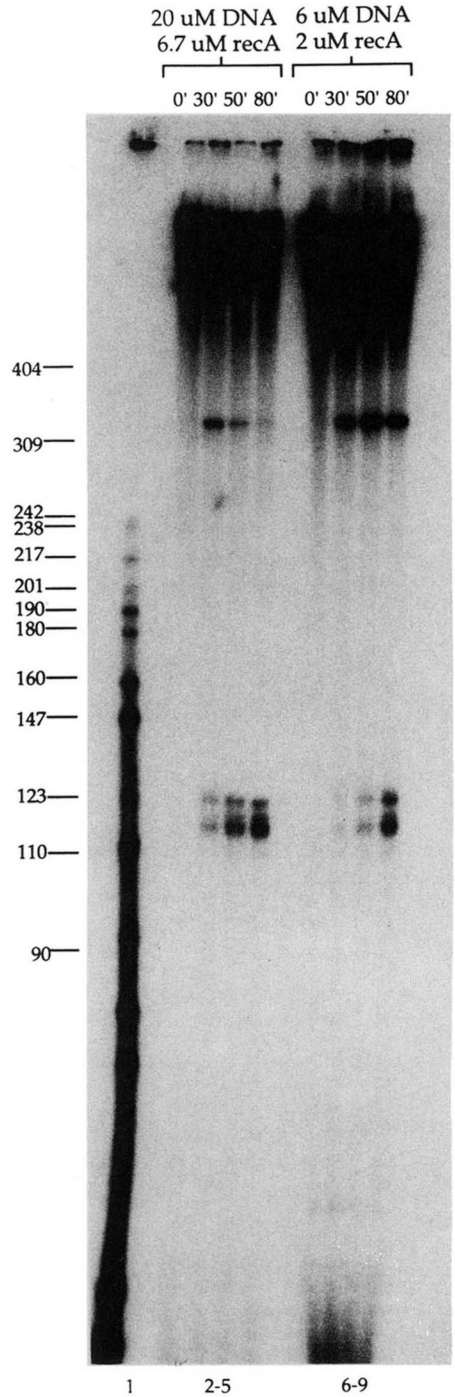


FIG. 16. **Strand breakage with longer lasting ATP regeneration.** Strand exchange reactions were performed with 5′ end-labeled M13mp8.121 (*Eco*RI) and single-stranded M13mp8 (+) as described under "Experimental Procedures" except the protein and DNA concentrations were varied as indicated. The time points for each reaction are 0, 30, 50, and 80 min. The markers (*lane 1*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. *Lanes 2–5* correspond to 6.7 μM recA protein, 2 μM SSB protein, 20 μM ssDNA and dsDNA. *Lanes 6–9* correspond to 2 μM recA protein, 0.6 μM SSB protein, 20 μM ssDNA and dsDNA.

DISCUSSION

Our primary conclusion is that DNA strand breakage represents a major mechanism by which recA protein promotes DNA strand exchange past heterologous insertions in one of the DNA substrates. The breaks occur exclusively in the displaced (+) strand of the duplex, and most are found near the heterology/homology junction. Breakage requires homologous sequences on the 3′ side of the heterology and also requires ATP hydrolysis, at least indirectly. The location of the break permits a complete strand exchange reaction to ensue. This phenomenon is illustrated in Fig. 17, with the mechanism of breakage unspecified. Bianchi and Radding (1983) originally observed and characterized recA protein-mediated strand exchange through heterologous insertions in the duplex DNA. In general, the insertions analyzed by these workers were located in the center of the duplex, and they suggested that the bypass involved unwinding of the heterologous DNA. In the present experiments, DNA unwinding clearly represents no more than a minor mechanism for

[2] W. A. Bedale and M. M. Cox, unpublished data.

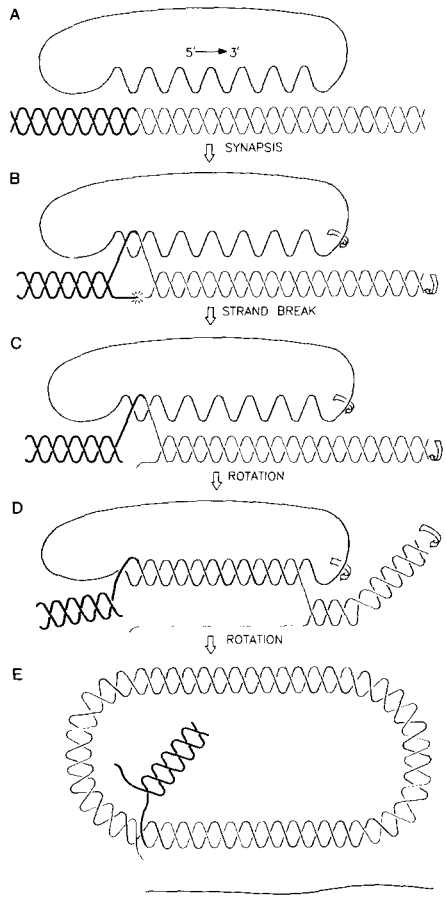


FIG. 17. **Model for DNA strand exchange after strand breakage when 5′ heterology is present in the duplex DNA.** *Heavy lines* correspond to heterology. *A*, original substrates (circular ssDNA, linear duplex DNA with 5′ heterology). *B*, pairing of homologous sequences. *C*, strand breakage at the heterology/homology junction in the (+) strand of the duplex DNA permits strand exchange to occur. *D*, DNA strand rotation allows continued DNA strand exchange. *E*, final products are a double-stranded circular molecule with a double-stranded tail (corresponding to the heterology in the original duplex substrate) and the displaced linear ssDNA.

heterology bypass. At present, the strand breakage result applies only to linear duplex DNA substrates with a short heterologous insertion at or near the 5′ end (relative to the (+) strand). Little, if any, breakage involving recA protein is seen when the heterology is at the 3′ end. We have not examined the mechanism by which heterologies located near the center of the duplex are bypassed and we do not know whether these events involve unwinding of the duplex DNA or strand breakage. We note, however, that the experimental procedures used by Bianchi and Radding would not have detected strand breaks and that their data can be explained alternatively by a strand breakage mechanism.

Our second conclusion is that strand exchange produces the expected product when the heterology is at the 3′ end of the duplex but that strand exchange appears to halt about 500 base pairs in front of the homology/heterology junction (Fig. 7). This halt in exchange short of the heterologous barrier reflects the extent to which heteroduplex DNA has been produced which can be cross-linked by the psoralen derivative AMT. Cross-linking was used in this study to prevent spontaneous branch migration during the spreading process for electron microscopy. Some random breakage of the displaced (+) strand was seen in the electron microscopy experiments involving duplex substrates with 3′ heterology, but this was associated with sample preparation procedures rather than any reaction related to strand exchange. Noncross-linked products were qualitatively similar to cross-linked products but did exhibit a wider range of heteroduplex lengths. We have recently used AMT cross-linking to trap DNA structures formed within the first pairing intermediate (paranemic joint) formed by recA protein during DNA strand exchange (Umlauf *et al.*, 1990). This work reinforced the idea that pairing involves the formation of a triple helical DNA structure, as also seen in several other electron microscopy studies (Register *et al.*, 1987; Stasiak *et al.*, 1984). The complex structures trapped with AMT when strand exchange is topologically blocked (Umlauf *et al.*, 1990) are not observed during normal strand exchange[3] (this work) although a short region with some form of triple helix might be expected to occur at the branch point during strand exchange. The absence of heteroduplex DNA that can be cross-linked near the heterologous barrier may reflect the presence of some form of a triple helix or other DNA structure which is present ahead of the migrating branch during strand exchange. This structure could be related to the structure that apparently lingers and slows the separation of products after strand exchange is complete (Rao *et al.*, 1990). This result with 3′ heterology is quite distinct from the strand break phenomenon observed with 5′ heterology, but it is included here because we feel the side-by-side comparison of strand exchange with 3′ or 5′ heterology in the duplex DNA is useful. In particular, the absence of specific strand breaks with 3′ heterology indicates that the reactions do not contain a contaminating nuclease that cleaves branched DNA structures or any structure that is created in the course of this particular strand exchange reaction.

Since we cannot yet specify the actual mechanism by which the strand breaks occur, this report is admittedly phenomenological. The phenomenon itself, however, suggests mechanisms by which some recombinational repair events might proceed *in vivo* and provides a useful clue about the structure of early pairing intermediates in DNA strand exchange. At a minimum, the strand breaks indicate that the (+) strand of the incoming duplex is being strained or exposed in a specific region near the heterology/homology junction. There is no sequence specificity to these events, and the requirements for breakage indicate clearly that the location of the breaks reflects some structural or energetic aspect of the DNA pairing activity. Since the (−) strand of the duplex is not broken, this may reflect a significant degree of strand separation or at least an asymmetric placement of the two strands of the duplex substrate in the paired intermediate (paranemic joint) that precedes strand exchange itself.

There is clearly a potential for DNA strand breaks inherent to most, if not all, recA protein preparations. This must be kept in mind when designing and interpreting recA protein experiments that involve substrates with heterologous inserts. Breaks were not observed in duplex DNA with heterologous ends in the experiments of Umlauf *et al.* (1990), but the reactions in that study were generally run for only a few minutes.

There are at least three possibilities for the actual mechanism by which the breaks occur: 1) cleavage by a contaminating nonspecific nuclease; 2) cleavage by a recombination-specific nuclease that interacts with recA protein and has some specificity for DNA structures produced as a result of recA protein action; or 3) direct mechanical shearing by the recA protein nucleoprotein filament itself, in a reaction distinct from nuclease activity. Given the extensive study of the recA protein, we do not consider the possibility that recA

[3] S. W. Umlauf and M. M. Cox, unpublished data.

protein possesses an intrinsic and heretofore unobserved nuclease activity to be plausible.

As described under "Results," we have not been able to detect in our reagents any nonspecific nuclease that exhibits significant activity on any DNA substrate within the time scale and conditions of these experiments. The specific breakage also occurs when we use recA protein and SSB protein preparations obtained from other laboratories, and within the limits of these measurements, the amount of breakage does not vary significantly from one protein preparation to the next. Some nonspecific nuclease activity can be found in almost any protein or DNA preparation with sufficiently long incubations and high reagent concentrations, however, and we cannot entirely eliminate this possibility for the origin of the breaks.

The idea that an associated and recombination-specific nuclease may be responsible is more interesting. Attempts to separate the strand exchange and breakage activities have not been successful to date although much more work is required. At present, we do not believe this phenomenon is related to the recA protein-associated resolvase identified by West and coworkers (Connolly and West, 1990), again because we do not observe specific cleavage of the branched products formed when the heterology is on the 3′ end of the duplex.

The third possibility, that the breaks are caused by a recA protein-mediated shearing process, is intriguing even though it is unprecedented. It could occur if the (−) strand of the duplex is fixed in some manner by the pairing process and strain applied to the (+) strand. The strain could occur if the DNA rotation indicated in Fig. 17 is promoted directly by recA protein and coupled to ATP hydrolysis. In this case the torsional stress applied to the (+) strand would be generated effectively by ATP hydrolysis throughout the nucleoprotein filament. Models for how recA protein might actively facilitate DNA rotation have been reviewed recently (Roca and Cox, in press). The observed requirement for ATP hydrolysis in this phenomenon is significant. Whatever the mechanism of strand breakage, chemical energy is being used either to induce the formation of a DNA structure susceptible to cleavage by a nuclease or to create sufficient torsional stress to produce a mechanical break.

We currently have no complete explanation for several experimental observations related to the strand breaks. For example, reactions between circular single-stranded DNA and covalently closed duplex DNA do not result in strand breakage or strand exchange[2] (Shibata *et al.*, 1979). We note that the pairing between two circular substrates is not nearly as extensive as is the case with the substrates used in this study (Umlauf *et al.*, 1990), and the lack of breaks may reflect a barrier to a required buildup of some form of torsional stress. It is also not clear why the location of the breakage is as specific as it is. If pairing can occur anywhere in the homologous region, breakage leading to strand exchange might be expected to occur at a much wider distribution of sites. The specific strand and position at which breakage predominantly occurs suggest that a force is applied directly at this site. It is possible that some of the minor products we observe reflect breakage at other sites, and we noted at the end of "Results" the presence of at least one additional break site.

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REFERENCES

Bianchi, M., Das Gupta, C., and Radding, C. M. (1983) *Cell* **34,** 931–939
Bianchi, M., Riboli, B., and Magni, G. (1985) *EMBO J.* **4,** 3025–30
Bianchi, M. E., and Radding, C. M. (1983) *Cell* **35,** 511–520
Connolly, B., and West, S. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 8476–8480
Cox, M. M., and Lehman, I. R. (1981a) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6018–6022
Cox, M. M., and Lehman, I. R. (1981b) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3433–3437
Cox, M. M., and Lehman, I. R. (1987) *Annu. Rev. Biochem.* **56,** 229–262
Cox, M. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256,** 4676–4678
Cox, M. M., Soltis, D. A., Lehman, I. R., DeBrosse, C., and Benkovic, S. J. (1983) *J. Biol. Chem.* **258,** 2586–2592
Craig, N. L., and Roberts, J. W. (1981) *J. Biol. Chem.* **256,** 8039–8044
DasGupta, C., and Radding, C. M. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 762–766
Davis, R. W., Botstein, D., and Roth, J. R. (1980) in *Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Griffith, J., and Shores, C. G. (1985) *Biochemistry* **24,** 158–162
Hahn, T.-R., West, S., and Howard-Flanders, P. (1988) *J. Biol. Chem.* **263,** 7431–7436
Hsieh, P., Meyn, M. S., and Camerini-Otero, R. D. (1986) *Cell* **44,** 885–894
Inman, R. B., and Schnos, M. (1970) *J. Mol. Biol.* **49,** 93–98
Kahn, R., Cunningham, R. P., Das Gupta, C., and Radding, C. M. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 4786–4790
Kolodner, R., Evans, D. H., and Morrison, P. T. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 5560–5564
Lee, J., and Cox, M. M. (1990) *Biochemistry* **29,** 7666–7676
Lichten, M., and Fox, M. S. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 7180–7184
Lindsley, J. E., and Cox, M. M. (1990) *J. Biol. Chem.* **265,** 10164–10172
Littlewood, R. K., and Inman, R. B. (1982) *Nucleic Acids Res* **10,** 1691–1706
Livneh, Z., and Lehman, I. R. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 3171–3175
Lohman, T. M., and Overman, L. B. (1985) *J. Biol. Chem.* **260,** 3594–3603
Lohman, T. M., Green, J. M., and Beyer, R. S. (1986) *Biochemistry* **25,** 21–25
Menetski, J. P., Bear, D. G., and Kowalczykowski, S. C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 21–25
Messing, J. (1983) *Methods Enzymol.* **101,** 20–78
Messing, J., and Vieira, J. (1982) *Gene (Amst.)* **19,** 269–276
Neuendorf, S. K., and Cox, M. M. (1986) *J. Biol. Chem.* **261,** 8276–8282
Radding, C. M. (1989) *Biochim. Biophys. Acta.* **1008,** 131–145
Rao, B. J., Jwang, B., and Radding, C. M. (1990) *Molecular Mechanisms in DNA Replication and Recombination*, pp. 387–398, Alan R. Liss, Inc., New York
Register, J. C., III, Christiansen, G., and Griffith, J. (1987) *J. Biol. Chem.* **262,** 12812–12820
Roca, A. I., and Cox, M. M. (1990) *CRC Crit. Rev. Biochem.* **25,** 415–456
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, rev. ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Senecoff, J. F., Bruckner, R. C., and Cox, M. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7270–7274
Shibata, T., Das Gupta, C., Cunningham, R. P., and Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 1638–1642
Stasiak, A., Stasiak, A. Z., and Koller, T. (1984) *Cold Spring Harbor Symp. Quant. Biol.* **49,** 561–570
Umlauf, S. W., Cox, M. M., and Inman, R. B. (1990) *J. Biol. Chem.* **265,** 16898–16912
West, S. C., Cassuto, E., and Howard-Flanders, P. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 6149–6153