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A Reverse DNA Strand Exchange Mediated by recA Protein and Exonuclease I

THE GENERATION OF APPARENT DNA STRAND BREAKS BY recA PROTEIN IS EXPLAINED*

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The combined action of exonuclease I and recA protein leads to a kind of reverse DNA strand exchange in which joint molecules formed on the "wrong" or distal end of a linear duplex in the presence of ATP are stabilized by exonuclease I degradation of the displaced (+) strand. Continued pairing and degradation of the displaced strand leads to strand exchange that appears to progress with a polarity opposite that of the normal recA protein promoted reaction (*i.e.* 3′-5′ with respect to the (+) strand). However, in contrast to the normal 5′-3′ strand exchange, the displaced strand is completely degraded in the process. When the linear duplex DNA substrate has a heterologous region at the 5′ (proximal) end, the major product (described in a previous study (Bedale, W. A., Inman, R. B., and Cox, M. M. (1991) *J. Biol. Chem.* 266, 6499–6510)) is a circular duplex DNA molecule with a double-stranded tail whose length corresponds closely to the heterologous segment of the substrate. The origin of this product is here shown to be the result of the exonuclease activity of exonuclease I (either added exogenously or present as a trace contaminant of recA protein or SSB protein preparations), as opposed to endonucleolytic or mechanical breakage. The levels of exonuclease I required to generate these products are sufficiently low that they are undetected by assays for exonuclease contamination in recA protein preparations. These results demonstrate that the interplay of recA protein with other enzymes can have a profound effect on both the mechanism and outcome of recA protein-promoted DNA strand exchange. They also demonstrate that the (+) strand of the duplex DNA substrate is at least transiently displaced in recA protein-mediated pairing even when joint molecules are limited to the distal end.

The recA protein of *Escherichia coli* is required for homologous recombination *in vivo* and is also able to promote DNA strand exchange reactions *in vitro* (Cox *et al.*, 1987; Radding, 1989; Roca and Cox, 1990). Probably the most common *in vitro* assay used to study recA protein-promoted DNA strand exchange is the three-strand exchange reaction depicted in Fig. 1*A*. RecA protein forms a filament on the circular single-stranded DNA substrate in the first step of this reaction. SSB protein of *E. coli* is also included in the *in vitro* reactions and probably facilitates formation of a uniform and stable filament of recA protein on the ssDNA[1] (Cox *et al.*, 1987; Kowalczykowski, 1991; Radding, 1989). Homologous linear duplex DNA can then pair with the nucleoprotein filament. These pairing intermediates may involve regions of triple-helical DNA (Hsieh *et al.*, 1990; Jain *et al.*, 1992; Rao *et al.* 1993). In reactions between completely homologous DNA substrates, strand exchange begins at the 5′ (proximal) end of the (+) strand of the duplex and branch migration continues in the 5′-3′ direction (Cox and Lehman, 1981a; Kahn *et al.*, 1981; West *et al.*, 1981).

Joint molecules are also formed efficiently on the opposite (distal) end of the linear duplex but are generally not converted to strand exchange products because of the polarity of the branch migration phase of the reaction (Cox and Lehman, 1981a). Upon removal of recA protein, the joint molecules formed at the distal end of the duplex in the presence of ATP yield triplex DNA structures which are resistant to nuclease degradation and thermal denaturation (Hsieh *et al.*, 1990; Rao *et al.*, 1991a, 1991b). It has been postulated (Hsieh *et al.*, 1990; Rao *et al.*, 1991a; Stasiak, 1992) that the stability of these triplex joints reflects an inability to displace the (+) strand from the joint at the 3′ end.

RecA protein is a DNA-dependent ATPase, and ATP hydrolysis is required during the branch migration phase of the reaction for efficient DNA strand exchange (Cox and Lehman, 1981b). While the molecular role of this ATP hydrolysis is not entirely clear, it has recently been demonstrated that ATP hydrolysis is required to drive strand exchange through short heterologous insertions (Kim *et al.*, 1992b; Rosselli and Stasiak, 1991), to render the reaction unidirectional[2] and to carry out strand exchange reactions involving four DNA strands (Kim *et al.*, 1992a).

The ability of recA protein-promoted DNA branch migration to overcome structural barriers in the DNA is critical to the cell for processes such as recombinational DNA repair. The mechanism by which one type of barrier to recombination (short heterologous insertions located near the center of an otherwise homologous duplex) is overcome has been addressed in a number of studies (Bianchi and Radding, 1983; Jwang and Radding, 1992; Kim *et al.*, 1992b; Rosselli and Stasiak, 1991). In a previous paper (Bedale *et al.*, 1991), we investigated

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[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); PAGE, polyacrylamide gel electrophoresis; bp, base pairs.

[2] S. Jain, C. Cowan, and J. Kim, unpublished results.

the effect of heterologous sequences located at the ends of linear duplex DNA on recA protein-promoted DNA strand exchange. We found in the case of 3′ (distal) heterology (with respect to the (+) strand) that strand exchange resulted in branched products (Fig. 1*B*), indicating that normal strand exchange was halted by the heterologous regions. In the case of 5′ (proximal) heterology, we predicted strand exchange would be blocked. Instead, these reactions were surprisingly efficient in the formation of joint molecules, and many of the products had undergone complete strand exchange. Interestingly, these products had double-stranded tails that corresponded to the length of heterology present in the original duplex DNA (Fig. 1*C*). Since recA-protein-promoted branch migration proceeds 5′-3′ with respect to the (+) strand in completely homologous strand exchange reactions, we proposed that these unusual σ-like products resulted from a break or nick in the (+) strand of the duplex at the junction of the heterology and the homology. We have used the term "breakage" to refer to the production of these circular duplex molecules with short dsDNA tails (or σ-like molecules) since their formation requires at least one scission in the (+) strand of the duplex DNA. However, the exact process by which these products were generated was unknown (Bedale *et al.*, 1991). In this paper we present results that indicate that the term breakage is a misnomer. The σ-like products are formed as a result of the presence of exonuclease I acting processively from the 3′ end of the (+) strand of the duplex. Furthermore, the combination of exonuclease I (which is a trace contaminant of some recA and SSB protein preparations generated by a variety of published procedures) and recA protein leads to a kind of reverse strand exchange reaction which mimics the action of some eukaryotic strand exchange proteins and may have significance *in vivo*. This result also provides evidence that the (+) strand of the duplex DNA substrate is at least transiently displaced when pairing occurs at the distal end of the duplex and is accessible to exonuclease I.

While this paper was in preparation, Konforti and Davis (1992) reported that the addition of exonuclease I to recA protein-mediated DNA strand exchange reactions facilitates the formation of joint molecules containing short regions of homology at the 3′ end of the (+) strand and presented evidence suggesting that the 3′ end of the (+) strand of the duplex had been displaced to some degree. The results presented here confirm and greatly expand on these observations.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—E. coli recA protein was purified as described (Cox *et al.*, 1981). Other preparations of recA protein were generously provided by Dr. Stephen Brenner (E. I. du Pont de Nemours & Co, Wilmington, DE), Dr. Stephen Kowalczykowski (University of California, Davis, CA), and Dr. Charles Radding (Yale University, New Haven, CT). Additional recA protein was overproduced and purified using plasmid pGE226 in *E. coli* strain SK4642, which was the generous gift of Dr. Jack Griffith (University of North Carolina, Chapel Hill, NC). This host strain is a bacteriophage P2 eductant in which the entire *sbcB* gene region has been deleted (Phillips *et al.*, 1988). RecA protein that was purified from a strain lacking exonuclease I was also purchased from United States Biochemical Corp. RecA protein concentrations were determined using an extinction coefficient of $\epsilon_{280} = 0.58\ A_{280}\ mg^{-1}ml$ (Craig and Roberts, 1981). All recA protein preparations were at least 95% pure as judged by SDS-PAGE. *E. coli* SSB protein was purified as described (Lohman *et al.*, 1986). The SSB protein concentration was determined by measuring the absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}\ mg^{-1}ml$ (Lohman and Overman, 1985). T4 DNA ligase was purified as described (Davis *et al.*, 1980). Restriction nucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Exonuclease I (1 mg/ml and 131 units/μg; 1 unit releases 10 mononucleotides in 30 min at 37 °C using boiled [^{3}H]T7 DNA as a substrate (Lehman and Nussbaum, 1964)) was the generous gift of Dr. Sidney Kushner (University of Georgia, Athens, GA). Additional exonuclease I and *Micrococcus luteus* ATP-dependent DNase were purchased from United States Biochemical Corp. Exonuclease VII (0.225 mg/ml and 6.3 units/μg; 1 unit releases 1 nmol of ssDNA to a trichloroacetic acid-soluble form in 30 min at 37 °C (Vales *et al.*, 1982)) was the generous gift of Dr. John Chase (U. S. Biochemical Corporation). Additional exonuclease VII was purchased from Bethesda Research Laboratories. Calf intestinal alkaline phosphatase and Tris buffer were purchased from Boehringer Mannheim. T7 endonuclease I was purchased from Pharmacia LKB Biotechnology Inc. *E. coli* RecBCD (46 μg/ml and 2.4×10^6 units/mg; 1 unit releases 1 nmol of nucleotides from dsDNA in 20 min at 37 °C (Eichler and Lehman, 1977)) was the generous gift of Dr. Gerald Smith (Fred Hutchinson Cancer Center, Seattle, WA). Rabbit anti-exonuclease VII antiserum was the generous gift of Dr. Paul Modrich (Duke University, Durham, NC). Rabbit anti-exonuclease I antiserum was obtained by Hazleton Research Products (Denver, PA) using conventional procedures. Preimmune serum and anti-exonuclease I antiserum were purified by protein A column chromatography (Pierce Scientific). This procedure eliminated all detectable contaminating nuclease activities (data not shown). Nitrocellulose (0.1-μm pore size) was from Schleicher and Schuell. Proteinase K, creatine kinase, phosphocreatine, ATP, goat anti-rabbit IgG (coupled to alkaline phosphatase), Ponceau S, and 5-bromo-4-chloro-3-indolyl phosphate were purchased from Sigma. Protein molecular weight markers were purchased from Bio-Rad. Radionucleotides were purchased from Amersham Corp.

DNA—Duplex and ssDNA were derived from bacteriophage M13mp8 (Messing and Vieira, 1982). The bacteriophage M13mp8.198 (7427 bp total length) contains a 198-bp heterologous insertion at the *Sma*I restriction site in the polylinker of M13mp8 (Bedale *et al.*, 1991). Duplex and ssDNA were prepared using previously described methods (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). Oligonucleotides were synthesized at the University of Wisconsin-Madison Biotechnology Center. Purification of oligonucleotides, restriction digestion of supercoiled DNA, and phosphatasing and radiolabeling of DNA with T4 polynucleotide kinase were performed as described (Sambrook *et al.*, 1989).

The concentrations of ssDNA and dsDNA were determined by measuring the absorbance at 260 nm, using 36 and 50 $\mu g\ ml^{-1}\ A_{260}^{-1}$, respectively, as conversion factors. All DNA concentrations are expressed in moles of nucleotides.

Linearization of ssDNA—Linear M13mp8 ssDNA was obtained by restriction digestion of circular ssDNA to which a complementary oligonucleotide had been annealed. An 18-nucleotide oligomer complementary to the (+) strand of M13mp8 at the polylinker region was synthesized and gel-purified. Circular M13mp8 ssDNA (present at 500 μM) was added to the oligonucleotide (present at 50 μM) in standard restriction enzyme buffer. The mixture was incubated at 70 °C for 15 min and was allowed to cool very slowly to room temperature. *Eco*RI restriction enzyme was added (2 units/μg of DNA) and the reaction was incubated at 37 °C. The extent of digestion was monitored by electrophoresis on 1.4% agarose minigels. After digestion was completed, residual protein was removed by 1:1 extraction of the reaction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), followed by ethanol precipitation. The resuspended DNA was incubated at 65 °C for 10 min to restore normal DNA secondary structure and stored on ice.

Preparation of "Endless" DNA—DNA with hairpin oligonucleotides annealed at the ends was prepared by a procedure provided by Dr. Paul Modrich and Dr. Deani Cooper (Duke University, Durham, NC). A 24-nucleotide oligomer that could form an internally base-paired hairpin and that would form a single *Eco*RI half-site when self-annealed was synthesized, gel purified, and kinased. The oligonucleotide was then ligated to M13mp8.198 DNA that had been linearized with *Eco*RI. Ligation was monitored by resistance to digestion with *E. coli* recBCD enzyme or *M. luteus* ATP-dependent DNase. The final ligation mixture was then treated with recBCD enzyme or ATP-dependent DNase to remove any DNA molecules that did not have hairpins at both ends. The hairpin DNA was then extracted 1:1 with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The reaction volume was reduced by repeated extractions with *n*-butanol and then dialyzed extensively against TE (25 mM Tris·HCl, pH 7.5, 1 mM EDTA).

Single-stranded Exonuclease Assay—Unlabeled linear M13mp8 ssDNA was diluted to 30 μM in the same reaction buffer used for strand exchanges (25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol). In some reactions, SSB protein (3 μM) was also present. RecA protein or purified exonuclease was added, and the mixture was

incubated at 37 °C for 90 min. An aliquot of each reaction (10 μl) was mixed with 2.5 μl each of gel loading buffer (Type II, (Sambrook *et al.*, 1989)) and 10% SDS and subjected to agarose gel electrophoresis as described (Bedale *et al.*, 1991).

Strand Exchange Reaction Conditions—Reactions contained 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 (12 mM phosphocreatine, and 10 units/ml creatine kinase). 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 reaction.

Immunoblots—Proteins were separated by SDS-PAGE (9% acrylamide). Immunoblots were performed by the procedure of Walczak *et al.* (1993).

Denaturing Gel Assays—Strand exchange reactions were performed with 5′ end-labeled dsDNA that contained 198 bp of 5′ heterology. To determine whether breakage of the (+) strand of the duplex occurs during strand exchange, the labeled products were denatured and electrophoresed on denaturing 6% acrylamide gels (Bedale *et al.*, 1991). Breakage at the heterology/homology junction results in the production of discrete bands at about 200 bases on such gels (Bedale *et al.*, 1991).

Other Methods—Agarose gel electrophoresis and electron microscopy were performed as described previously (Bedale *et al.*, 1991).

RESULTS

Experimental Strategy—This project was an outgrowth of our attempts to determine the origin of the σ-like products we had observed previously in reactions where the dsDNA substrate contained 5′ (proximal) heterology (Fig. 1C) (Bedale *et al.*, 1991). This σ-like product could only be generated by cleavage or breakage of the (+) strand of the duplex DNA substrate near the heterology/homology junction. There were two major possibilities; the activity responsible for the production of the σ-like molecules was either intrinsic to recA protein itself or involved a contaminating nuclease. Since the recA protein preparations that we used lacked detectable nuclease activity against both linear and circular ss or dsDNA, one possibility was a nuclease that was specific for intermediates in recA protein-mediated recombination. In subsequent experiments, a recA protein preparation was identified that lacked the ability to generate σ-like molecules in reactions where the duplex DNA substrate had 5′ heterologous sequences, but was still fully active in promoting strand exchange and joint molecule formation between completely homologous DNA substrates. This supported the idea that the ability to generate σ-like products was the result of a contaminating cryptic nuclease found in some (but not all) recA protein preparations. We then screened many additional recA protein preparations purified by a variety of procedures for the ability to generate σ-like molecules. We attempted to restore this activity to preparations that did not normally promote σ-like product formation by the addition of purified nucleases. To determine whether those enzymes which could restore this activity were actually contaminants of those recA or SSB protein preparations that promoted the generation of σ-like molecules, biochemical and immunological tests were employed. This analysis ultimately identified exonuclease I as the responsible contaminating activity. Finally, the mechanism by which the contaminating nuclease facilitates formation of σ-like molecules was addressed by electron microscopy of reaction intermediates.

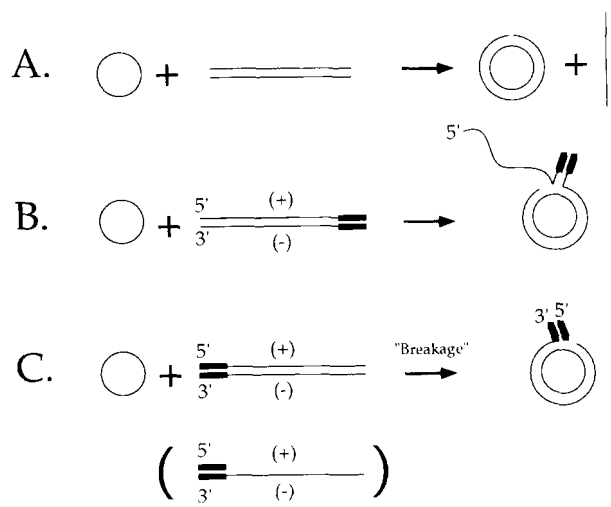


FIG. 1. **RecA protein-promoted three strand exchange reactions.** *A*, a three strand exchange reaction between completely homologous circular ssDNA and linear duplex DNA, leading to the production of a nicked circular duplex and the displaced linear (+) strand. *B*, a short heterology (200 bp) at the 3′ end of the (+) strand of the duplex (distal heterology) results in the formation of a circular duplex molecule with a short double-stranded tail and a long single-stranded tail (Bedale *et al.*, 1991). *C*, a short heterology (200 bp) at the 5′ end of the (+) strand of the duplex (proximal heterology) results in the production of a circular duplex with a short double-stranded tail corresponding to the length of heterology with certain recA protein preparation. The homologous part of the (+) strand of the duplex is absent in this product molecule, as though it were derived from the molecule shown in *parenthesis*.

When σ-like molecules are generated from 5′end-labeled duplex DNA and subsequently denatured and electrophoresed on a denaturing polyacrylamide gel, a labeled band corresponding to the remaining 5′ heterologous portion of the (+) strand is observed (Bedale *et al.*, 1991). In some of the experiments presented in this paper, the appearance of this labeled band will be used as evidence that σ-like products were formed, since electron microscopic confirmation of the formation of such products in every experiment would be very labor intensive.

Not All RecA Protein Preparations Promote Breakage—We reported previously that the apparent breakage was observed in reactions containing any of three different recA protein preparations and with two different SSB protein preparations (Bedale *et al.*, 1991). As indicated in the Introduction, the phenomenon to which the term breakage has been applied is the generation of the σ-like products shown in Fig. 1*C*; the term breakage itself is a misnomer as will be shown below. We extended our screen of recA protein preparations to include a total of 10 different recA protein preparations (Fig. 2, *lanes 2–11*). The recA protein was purified in five different laboratories and by several different procedures (Table I). We found that not every recA protein preparation resulted in the generation of the σ-like products. Since each of the recA protein preparations appeared otherwise indistinguishable (as judged by SDS-PAGE and the ability to promote strand exchange between completely homologous DNA substrates, data not shown), this strongly suggested that a contaminating nuclease was responsible for the apparent breakage.

Interestingly, we noticed that one of the recA protein preparations that we had previously reported as promoting the generation of σ-like products (Bedale *et al.*, 1991) did not exhibit this activity in this experiment (Table I, recA protein preparation 9; Fig. 2, *lane 10*). The original result reported (Bedale *et al.*, 1991) was traced to an exonuclease I contaminant in an SSB preparation generated by others in this laboratory which had not been tested for single-stranded exonuclease activity.[3] When we used this recA protein prep-

[3] This was an unintended oversight by another researcher in this laboratory. It was not picked up by MMC, with whom the responsibility lies. We do not know what error occurred in the generation of the preparation that resulted in the observed contamination. It is *not* due to the procedure used (that of Lohman, *et al.*, 1986). A total of four other SSB protein preparations generated in this laboratory by this same procedure before and since the preparation in question have exhibited no exonuclease I contamination.

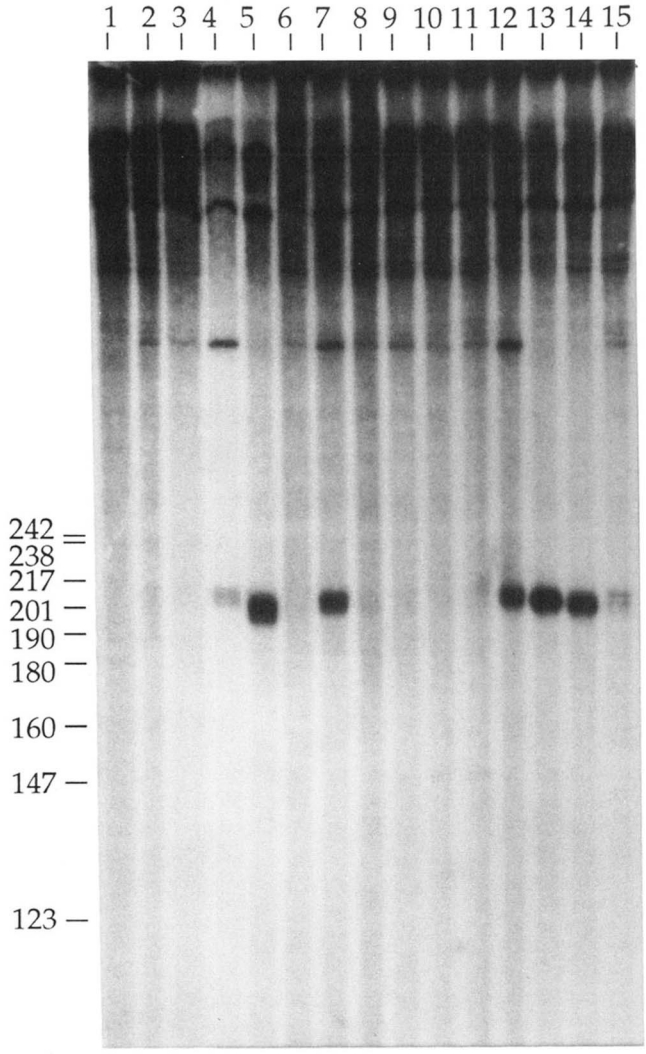


FIG. 2. **Denaturing gel assay to screen different recA protein preparations.** Strand exchange reactions and electrophoresis were performed as described (Bedale *et al.*, 1991) with 5′ end-labeled DNA containing 198 bp of 5′ heterology. Denaturation of the σ-like products shown in Fig. 1*C* results in the production of a labeled DNA fragment ~200 bases long, corresponding to the 5′ end of the (+) strand of the original dsDNA. The DNA size markers (lane not shown) were derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. *Lane 1* is a 0 min time point. All other lanes are 90 min time points corresponding to different reactions. The reactions shown in *lanes 2–11* were performed with recA protein preparations 1–10, respectively, described in Table I. The reactions in *lanes 12*, *13*, and *15* were all performed with recA protein preparation 1, which was purified from an exonuclease I-free strain of *E. coli*. *Lane 12* corresponds to a reaction that was performed with an SSB protein preparation known to be contaminated with a low level of exonuclease I. *Lane 13* corresponds to a 20-μl reaction to which 0.13 units of exonuclease I were added. *Lane 14* corresponds to a reaction in which partially purified recA protein (Fraction III) was substituted for purified recA protein (Cox *et al.*, 1981). *Lane 15* corresponds to a 20-μl reaction to which 0.14 units of exonuclease VII were added.

aration with the original SSB protein preparation, generation of the σ-like products was observed (data not shown). In addition, the original SSB protein was able to restore generation of the σ-like products to other recA protein preparations that lacked this activity (Fig. 2, *lane 12*). We previously had examined several different recA protein preparations and several SSB preparations, but we did not try every combination of recA protein preparation with SSB protein preparation. It appears, therefore that the generation of the σ-like products was due to a contaminating enzymatic activity that can be present in some recA protein preparations and was present in at least one SSB preparation.

RecA Protein Preparations That Promote the Generation of σ-like Products Possess Single-stranded Exonuclease Activity—A clue to determining how the generation of σ-like products occurs came from completely homologous strand exchange reactions where 5′ end-labeled linear duplex DNA was used. Two labeled products are usually generated in this reaction: a nicked circular duplex and the displaced linear ssDNA. However, in reactions involving recA protein preparations that promoted the formation of σ-like molecules, we found that the labeled linear ssDNA product was not observed (data not shown). Circular ssDNA in these reactions was not degraded (data not shown). This observation suggested that a contaminating single-stranded exonuclease may either remove the 5′ label or completely degrade the displaced ssDNA.

This exonuclease contaminant is not readily detected in the nuclease screens routinely performed on all of our recA protein preparations. When the recA preparations that promote the generation of σ-like molecules were screened for single-stranded exonuclease activity under conditions similar to those used in DNA strand exchange, no degradation of unlabeled linear ssDNA was observed (data not shown). The SSB protein present in normal strand exchange experiments is not normally added to these nuclease assays, however. When the assay for single-stranded exonuclease activity was repeated in the presence of SSB protein, single-stranded exonuclease activity was observed for each of the recA protein preparations that promoted the generation of σ-like products (Fig. 3, *lanes 5*, *6*, and *8*). RecA protein preparations that did not promote the generation of σ-like products did not possess single-stranded exonuclease activity even in the presence of SSB (Fig. 3, *lanes 3–4*, *7*, and *9–12*). In addition, the SSB protein preparation that promoted the generation of σ-like products possessed single-stranded exonuclease activity (in the absence of recA protein) while other SSB preparations did not (Fig. 3. *lanes 2* and *13*). The very strong correlation between single-stranded exonuclease activity and the ability to generate σ-like molecules suggested that the two activities might be related. However, it was not obvious how a single-stranded exonuclease might be be responsible for what appeared to be endonucleolytic cleavage of double-stranded DNA.

Addition of Exonuclease I or Exonuclease VII Can Restore Formation of σ-like Molecules to RecA Protein Preparations That Lack This Activity—The inability of certain recA protein preparations to promote the formation of σ-like molecules provided a way to identify the contaminating activity responsible for this activity. We screened a number of purified DNA recombination and repair enzymes for the ability to generate σ-like products. Of all the purified enzymes tested, only exonuclease I and VII were capable of restoring this activity to recA protein preparations that lacked this activity. Fig. 4, *lane 6*, demonstrates that 0.1 units of exonuclease VII in a 20-μl reaction is sufficient to restore the ability to generate σ-like products to the levels seen with the recA protein preparations that had this activity. Maximum product formation (conversion of about 15% of substrate dsDNA into σ-like products in this experiment) is observed at about 1 unit of exonuclease VII/20-μl reaction. Additional exonuclease VII does not increase the amount of σ-like products observed (data not shown). Comparatively less exonuclease I is needed to restore the formation of σ-like products. Generation of σ-like products occurs with the addition of 0.01 units of exonuclease I in a 20-μl reaction (Fig. 5, *lane 6*). This amount of exonuclease I is equivalent to approximately 1 molecule of exonuclease I/20,000 recA protein molecules. We note that this level of exonuclease I contamination in recA protein preparations is undetectable unless SSB is added to standard single-stranded exonuclease assays. In contrast, the amount

TABLE I

RecA protein preparations used in this study

Ten different recA protein preparations were screened for the ability to generate σ-like products as discussed in the Introduction (see also Fig. 1*C*). These preparations were also screened for single-stranded exonuclease activity and the presence of exonuclease I by immunoblotting. In addition, those recA protein preparations that exhibited single-stranded exonuclease activity were incubated with anti-exonuclease I antibodies to determine whether all of the single-stranded exonuclease activity present could be attributed to exonuclease I (see "Experimental Procedures"). Purification protocols used to obtain the recA preparations were: A, Griffith and Shores (1985); B, Shibata *et al.* (1981); and C, Cox *et al.* (1981). The results described here should *not* be taken to indicate the presence of exonuclease I in the recA preparations used to generate published data from any laboratory. Note that in many cases the recA protein preparations were not obtained from the laboratory whose purification procedure is cited. The preparation used in line 6 was a preparation known to contain exonuclease I and provided by C. Radding at our request.

RecA protein preparation	Breakage	ss exonuclease activity	ss exo inhibited by anti-*Exo*I	*Exo*I immunoblot	Method of purification
1	−	−	NA[a]	−	Cox *et al.*, 1981
2	−	−	NA	−	Purchased from USB
3	+	+	+	+	A
4	+	+	−	+	A
5	−	−	NA	−	A
6	+	+	+	+	Unpublished procedure
7	−	−	NA	−	B
8	−	−	NA	−	C
9	−	−	NA	−	C
10	−	−	NA	−	Kowalczykowski[6]

[a] NA, not applicable.

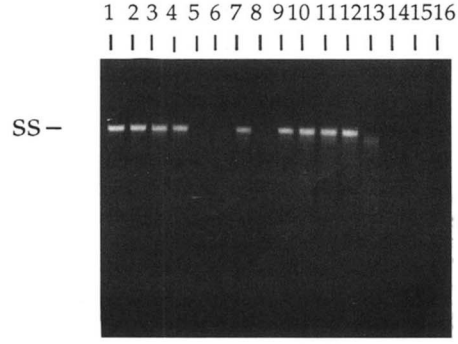


FIG. 3. **Agarose gel assay of single-stranded exonuclease activity found in different recA protein preparations.** Single-stranded DNA was incubated with recA protein and other proteins as described under "Experimental Procedures." All reactions contain SSB protein at 3 μM. *Lane 1* is a 0 min time point. All other lanes are 90 min time points. No additional proteins were added to the reaction shown in *lane 2*. The conditions and protein preparations for each reaction shown in *lanes 3–12* were identical to those shown in *lanes 2–11*, respectively, in Fig. 2 (preparations 1–10, respectively, in Table I). *Lane 13* corresponds to a reaction that was performed with an SSB protein preparation known to be contaminated with a low level of exonuclease I. *Lane 14* corresponds to a 10-μl reaction to which 0.13 units of exonuclease I were added. *Lane 15* corresponds to a reaction in which partially purified recA protein (Fraction III) was substituted for purified recA protein (Fraction IV) (Cox *et al.*, 1981). *Lane 16* corresponds to a 10-μl reaction to which 0.14 units of exonuclease VII were added.

of exonuclease VII needed to restore formation of σ-like products is much higher, at about 1 molecule of exonuclease VII/200 recA protein molecules. Other preparations of both exonuclease I and exonuclease VII exhibited similar capabilities to generate σ-like products (data not shown). It is unlikely that either of these exonucleases is contaminated with the other. Exonuclease VII is purified from a strain that has the *sbcB* (encoding exonuclease I) gene deleted (Chase and Richardson, 1974, Chase and Vales, 1981), and Western analysis of purified exonuclease I with anti-exonuclease VII antibodies did not detect contamination with exonuclease VII (data not shown).

Exonuclease I (Not Exonuclease VII) Is Responsible for the Formation of σ-like Molecules Observed with Certain RecA Protein Preparations—We next wanted to learn whether exonuclease I or VII contaminates those recA protein preparations that promote formation of σ-like products and if either of these enzymes is in some way responsible for this activity. Several experimental results provided evidence that exonuclease VII does not contaminate any of these recA protein preparations. First, exonuclease VII possesses a 5′-3′ as well as a 3′-5′ single-stranded exonuclease activity. In recA protein-promoted DNA strand exchange reactions where the duplex DNA has 3′ heterology with respect to the (+) strand, the major product has a long single-stranded tail with a 5′ end (Bedale *et al.*, 1991), (Figs. 1*B* and Fig. 6, *reaction A*). The addition of purified exonuclease I, a 3′-5′ single-stranded exonuclease, has no effect on the mobility of this product band (Fig. 6, *reaction C*). The addition of purified exonuclease VII (at the minimal level required to restore formation of σ-like molecules) results in an increase in mobility of this product band (Fig. 6, *reaction D*). This change in mobility is consistent with degradation of the long single-stranded tail by 5′-3′ exonuclease activity. Since no such mobility change is noticed in those reactions with recA protein preparations that can promote formation of σ-like products (Fig. 6, *reaction B*), this result suggests exonuclease VII does not contaminate those recA protein preparations that have this activity.

In addition to this biochemical evidence, immunological evidence also argues against exonuclease VII being the enzyme responsible for the production of σ-like molecules. First, Western analysis with anti-exonuclease VII antibodies did not detect exonuclease VII in any of the recA protein preparations tested (data not shown). Second, anti-exonuclease VII antibodies (at concentrations which can inhibit exonuclease VII activity, data not shown) do not block the generation of σ-like products that is observed with those recA preparations that have this activity intrinsically (Fig. 7, *lanes 3–5*). These antibodies are, however, capable of blocking the generation of σ-like molecules that result from the addition of purified exonuclease VII (Fig. 7, *lanes 10–12*).

Exonuclease I, in contrast to exonuclease VII, is present in those recA protein preparations that promote the formation of σ-like products. RecA protein purified from an *E. coli* host strain in which the chromosomal region surrounding the *sbcB* gene had been deleted does not promote the formation of σ-like products (Fig. 2, *lanes 2* and *3*), yet the addition of purified exonuclease I is sufficient to restore this activity to these recA protein preparations (Fig. 2, *lane 13*). Western analysis using

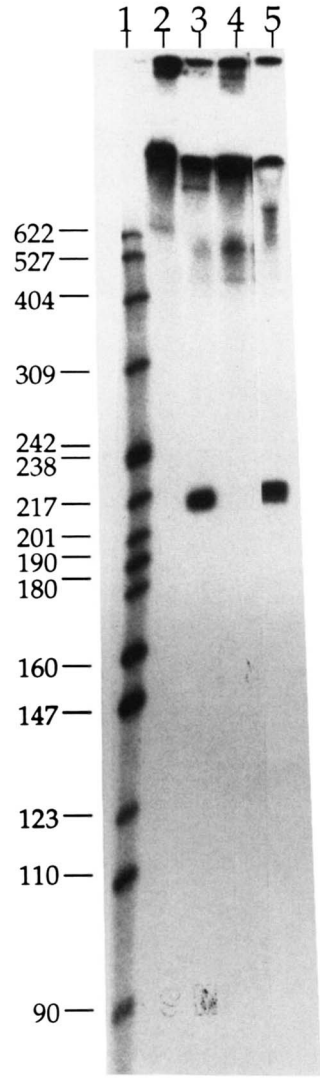


FIG. 4. **Exonuclease VII can restore the ability to generate σ-like molecules to recA protein preparations that lack this activity.** Strand exchange reactions and electrophoresis were carried out with 5′ end-labeled DNA containing 198 bp of 5′ heterology as described under "Experimental Procedures." The markers (*lane 1*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. *Lane 2* is a 0 min time point. All other lanes are 90 min time points. *Lane 3* corresponds to a reaction that was carried out with recA protein preparation 4 (Table I). *Lane 4* corresponds to a reaction that was performed with recA protein preparation 8 (Table I). *Lane 5* corresponds to a 20-μl reaction containing recA protein preparation 8 (Table I) to which 1.2 units of exonuclease VII were added.

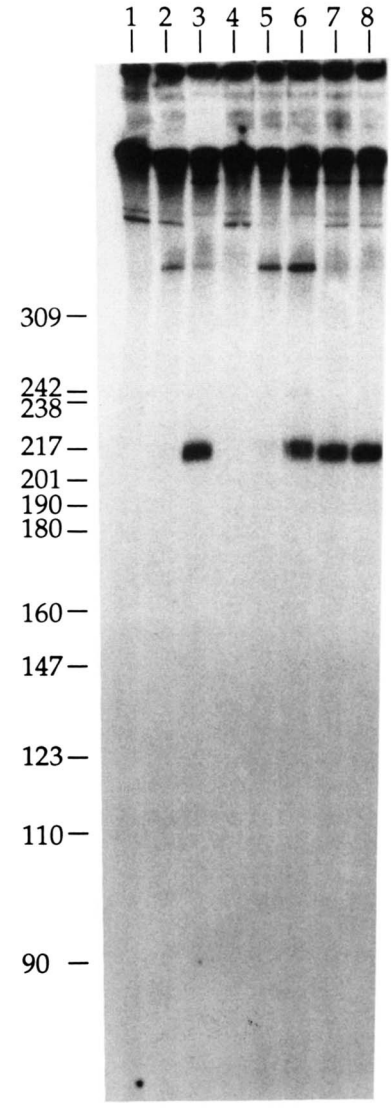


FIG. 5. **Exonuclease I can restore the ability to generate σ-like molecules to recA protein preparations that this activity.** Strand exchange reactions and electrophoresis were carried out with 5′ end-labeled DNA containing 198 bp of 5′ heterology as described under "Experimental Procedures." The DNA size markers (lane not shown) were derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. *Lane 1* is a 0 min time point. All other lanes correspond to 90 min time points. All reaction volumes were 20 μl. *Lane 2* corresponds to a reaction that was performed with recA protein preparation 8. *Lane 3* corresponds to a reaction that was performed with recA protein preparation 4. *Lane 4* corresponds to a reaction from which recA protein was omitted to which 1 unit of exonuclease I was added. *Lanes 5–8* correspond to reactions containing recA protein preparation 8 to which 0.001, 0.01, 0.1, and 1 units of exonuclease I were added, respectively.

anti-exonuclease I antibodies demonstrated that those recA protein preparations that promote the generation of σ-like products contain contaminating exonuclease I (Fig. 8, *lanes 3, 4,* and *6*). The amounts of contaminating exonuclease I are very low, on the order of one molecule of exonuclease I for every 7000–30,000 recA protein molecules. This amount of contaminating enzyme is similar to the amount of pure exonuclease I that is needed to restore the production of σ-like molecules to those recA protein preparations that lack this activity. In addition, the amounts of contaminating exonuclease I present in recA protein preparations correlates very well with the level of σ-like products observed with each protein preparation (compare Fig. 8 with Fig. 2) and with the presence of single-stranded exonuclease activity (Fig. 3). Purified anti-exonuclease I antibodies can inhibit purified exonuclease I, although relatively large amounts of the antibodies are required (data not shown). Preimmune serum does not have any effect on the single-stranded exonuclease activity (data not shown). The antibodies that inhibit purified exonuclease I can also inhibit the single-stranded exonuclease activity found in all but one of the recA protein preparations that promote the formation of σ-like molecules, but have no effect on the single-stranded exonuclease activity of exonuclease VII (data not shown). Exonuclease I also appears to be a contaminant of the original SSB protein preparation that had the ability to generate σ-like molecules (Fig. 8, *lane 11*), and the single-stranded exonuclease activity in the SSB protein preparation can also be inhibited by the anti-exonuclease I antibodies (data not shown).

Exonuclease I Allows RecA Protein-promoted DNA Strand Exchange to Proceed with a Reverse Polarity—How could a

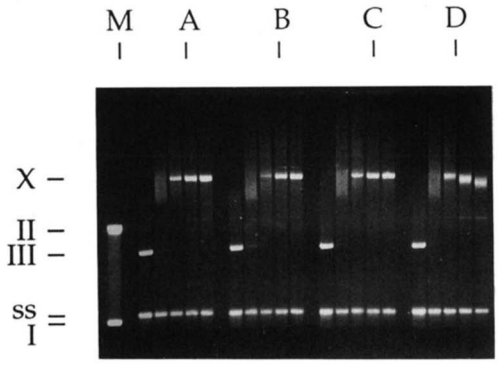


FIG. 6. **Effects of the addition of exonucleases on the mobility of branched products from reactions with 3′ (distal) heterology.** Strand exchange reactions were carried out with single-stranded M13mp8 and linear dsDNA containing 198 bp of 3′ heterology as described under "Experimental Procedures." Such reactions result in the efficient formation of the product shown in Fig. 1*B*, which migrates on agarose gels at the position labeled *X*. For each *reaction A-D*, the time points shown correspond to 0, 10, 20, 40, and 60 min of reaction. The marker (*lane M*) is M13mp8 (*FI* and *FII*) DNA. *Reactions A*, *C*, and *D* were performed using recA protein preparation 8 (Table I). *Reaction B* was performed using recA protein preparation 4. Exonuclease I (1 unit in an 80-μl reaction) was added to *reaction C* at the same time as the recA protein. Exonuclease VII (0.1 unit in an 80-μl reaction) was added to *reaction D* at the same time as the recA protein.

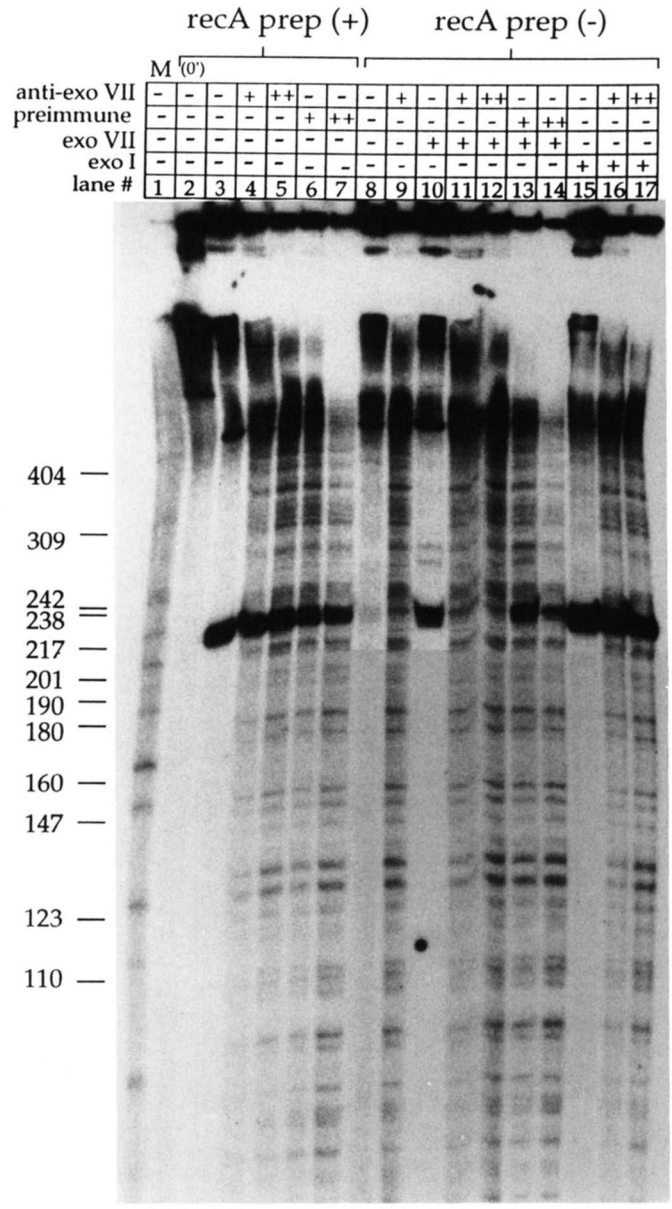


FIG. 7. **Anti-exonuclease VII antibodies inhibit exonuclease VII, but do not inhibit the activity responsible for the production of σ-like products present in recA protein preparations.** Strand exchange reactions and electrophoresis were performed with 5′ end-labeled DNA containing 198 bp of 5′ heterology as described under "Experimental Procedures." The markers (*lane 1*) are derived from a ^{32}P end-labeled *Msp*I digest of pBR322 DNA. All reaction volumes were 20 μl. *Lane 2* is a 0 min time point. All other lanes are 90 min time points. Reactions were performed with recA protein preparation 4 (*lanes 2–7*) or with recA protein preparation 8 (*lanes 8–17*) (Table I). The designations "recA protein (+)" and "recA protein (−)" in the figure refer to the presence or absence of contaminating exonuclease I in these preparations based on immunoblot analysis. All additions of exonuclease VII, exonuclease I, anti-exonuclease VII antibodies, and preimmune serum were done immediately prior to the initiation of strand exchange by the addition of ATP and SSB protein. Exonuclease VII (0.14 units) was added to the reactions shown in *lanes 10–14*. Exonuclease I (0.3 units) was added to the reactions shown in *lanes 15–17*. Anti-exonuclease VII antibodies were added to *lanes 4*, *9*, *11*, and *16* (0.5 μg) and to *lanes 5*, *12*, and *17* (2 μg). Preimmune serum was added to *lanes 6* and *13* (0.5 μg) and to *lanes 7* and *14* (2 μg).

single-stranded exonuclease generate an apparently internal scission in duplex DNA? One possibility is that the single-stranded exonuclease has a latent endonuclease activity (perhaps specific for recombination intermediates) in addition to its well characterized single-stranded exonuclease activity. Another possibility is shown in Fig. 9*B*. Homologous sequences at the 3′ (distal) end of the duplex DNA with respect to the (+) strand first could pair with the recA protein-coated ssDNA (Fig. 9*B*, *i*). If the 3′ end of the (+) strand of the duplex is displaced, exonuclease I could catalyze the degradation of this strand and stabilize the nascent joint (Fig. 9*B*, *ii*). SSB protein may be present on this displaced single strand, which would stimulate the action of exonuclease I (Molineux and Gefter, 1975). Pairing adjacent to the stabilized joint would displace more of the (+) strand of the original duplex, making it progressively accessible to exonuclease I (Fig. 9*B*, *iii*). Pairing and strand displacement must precede exonuclease I action; the (-) strand is already annealed when exonuclease I acts. Exonucleolytic degradation of the (+) strand of the original duplex would continue (Fig. 9*B*, *iv*) until the paired region reached the heterology/homology junction and no further displacement of the (+) strand could occur (Fig. 9*B*, *v*).

To distinguish between these two possible mechanisms for exonuclease I action, we first decided to examine early reaction intermediates by electron microscopy. We reasoned that if the endonuclease model was correct, products with two double-stranded tails should be present at early time points. One of the tails would be short and would correspond to the length of heterology, while the other tail should begin long and would shorten with time (Fig. 9*A*). In this endonuclease model, the displaced ssDNA should still be present at early time points. Its 3′ end is still base paired to the (-) strand and should therefore remain resistant to exonuclease I until complete strand displacement has occurred. In the case of the exonuclease model, only one double-stranded tail should be present at any time point, and its length should be long at early time points but very short (corresponding to the length of the heterology) at later time points (Fig. 9*B*). According to this model, the displaced ssDNA should be immediately degraded by exonuclease I and therefore should not be observed. Strand exchange reactions were performed with duplex DNA that had 198 bp of 5′ heterology with respect to the (+) strand. Aliquots were withdrawn at early time points of re-

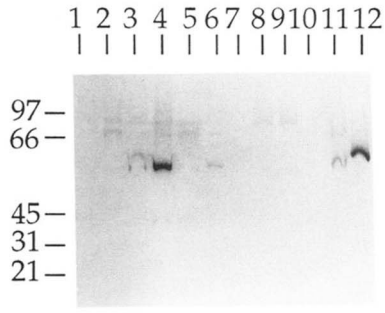


FIG. 8. **Exonuclease I is present in recA protein preparations that can generate σ-like molecules.** Proteins were separated by SDS-PAGE and transferred to nitrocellulose paper as described (Walczak *et al.*, 1993). To remove antibodies from the anti-exonuclease I antiserum that react with bacterial proteins other than exonuclease I that are present in recA protein preparations, the antiserum was preabsorbed to nitrocellulose paper that had been prebound with 300 μg of denatured recA protein purified from an exonuclease I-free strain of *E. coli*. Immunoblotting was performed as described (Walczak *et al.*, 1993). Protein molecular weight markers were electrophoresed and transferred at the same time, and their positions on the gel as determined by Ponceau S staining are marked. *Lanes 1–10* each contain 10 μg of recA protein from 10 different recA protein preparations in the order described in Table I. *Lane 11* contains 10 μg of the SSB protein preparation that was used for the reactions in an earlier paper (Bedale *et al.*, 1991). *Lane 12* contains 2 ng of purified exonuclease I.

action and prepared for electron microscopy. The results of these experiments are summarized in Table II. Very few intermediates had two double-stranded tails at any time point (Table II, DNA species 7), while many molecules had a single, long, double-stranded tail at early time points (Table II, DNA species 3, 5, and 6; *left panel* of Fig. 11). The lengths of these double-stranded tails at early time points were quite random (Fig. 10*A* and *left panels* of Fig. 11), but at later time points became very short and uniform, reaching a limit at the length of the heterologous region (Fig. 10*B* and *right panels* of Fig. 11). The numbers of molecules with long double-stranded tails compared with the numbers of those with short double-stranded tails at various time points (Fig. 11) suggest that an intermediate/product relationship exists between these two classes of molecules. In addition, very few molecules that had long ssDNA tails were observed at any time point. These electron microscopy results, therefore, support the exonuclease model (Fig. 9*B*) which shows how exonuclease I can facilitate recA protein-mediated strand exchange with substrates that have a blocked 5′ (proximal) end.

In all of the electron microscopy experiments, molecules were not scored as "σ-like products" unless they had an appropriately sized duplex tail and the circular portion of the product had no visible single-stranded gaps (except for short, <200 base, single-stranded regions occasionally seen near the junction of the tail with the circle). Note that some molecules (a fraction varying greatly from one experiment to the next) resulted in the production of gapped circles, some of which had short double-stranded tails. Although only 14% of the duplex DNA was converted into the product σ-like molecules in the experiment summarized in Table II and Fig. 12, many of these gapped circular products (50% of the total at 50 min) are very similar to these products. A short single-stranded gap or a missing short duplex tail relegates a molecule to this category. These products presumably arose from nicked double-stranded substrate DNA. The double-stranded substrate DNA in this experiment was later shown to contain more nicks than usual (data not shown). In the experiment described in Table III (which was performed with different double-stranded substrate DNA), nearly 60% of the DNA was converted to scorable σ-like molecules.

Another set of experiments also supports the exonuclease model as opposed to the endonuclease model. In these experiments, a short oligonucleotide hairpin was ligated to the ends of the duplex DNA, generating in effect an "endless" DNA molecule (Fig. 13*A*). If an endonucleolytic nick in the duplex DNA was responsible for the initiation of strand exchange, then the absence of a free end on the duplex DNA would not be expected to affect the reaction. If, however, the end of the DNA is important, then the presence of the hairpin would be expected to block the reaction. Strand exchange reactions with duplex DNA (with or without hairpins) were performed using recA protein (preparation 4 in Table I), which promoted the generation of σ-like molecules and was found by immunoblots to be contaminated with exonuclease I. The products of the reaction were analyzed by agarose gel electrophoresis and by electron microscopy. The presence of hairpins greatly decreased the amount of duplex DNA that formed joint molecules in the presence of exonuclease I (Fig. 13*B*, Table III). The very small percentage of duplex DNA with hairpins that did form joint molecules in the presence of exonuclease I (Table III, "blocked types") could have arisen from strand exchange initiated at nicks located at random sites in the duplex DNA.

The Presence of Exonuclease I Increases the Efficiency of Joint Molecule Formation by RecA Protein—The results presented here and in a previous paper (Bedale *et al.*, 1991) show that the presence of very small amounts of single-stranded exonuclease allows recA protein to promote strand exchange with DNA substrates that are normally poor substrates for *in vitro* recombination. This idea is substantiated in Table III and in Fig. 13*B*. In reactions with linear duplex DNA that contains 198 bp of 5′ heterology (no hairpins), the presence of exonuclease I allows essentially all of the linear duplex DNA substrate to be incorporated into joint molecules at 90 min. The limited strand exchange in the absence of exonuclease I that occurs with these substrates appears to be largely limited to substrate molecules containing nicks.

DISCUSSION

Our major conclusion is that the combination of a 3′-5′ single-stranded exonuclease and recA protein leads to a kind of reverse DNA strand exchange that allows recombination to occur much more efficiently in reactions where the 5′ (proximal) end of the DNA is blocked from initiating strand exchange by the presence of a heterologous sequence. Previous work from this laboratory (Bedale *et al.*, 1991), which demonstrated that strand exchange between circular single-stranded DNA and dsDNA containing short heterologous sequences at the 5′ (initiating) end were surprisingly efficient, can be explained by this exonuclease-assisted strand transfer. A model illustrating how an exonuclease could facilitate this reaction is shown in Fig. 9*B*. The exonuclease-enhanced reaction begins at the 3′ end of the (+) strand of the incoming duplex, which is the opposite end of the duplex DNA from which strand exchange between completely homologous substrates normally proceeds (Cox and Lehman, 1981a; Kahn *et al.*, 1981; West *et al.*, 1981). The strand transfer in the presence of single-stranded exonuclease may occur in this "backwards" direction (3′-5′ with respect to the (+) strand) by the combined (and possibly synergistic) actions of recA protein and exonuclease. RecA protein would pair the homologous regions of the dsDNA to the ssDNA. If some displacement of the 3′ end of the dsDNA occurs at this time, a 3′-5′ exonuclease could attack this displaced end. Pairing must be a prerequisite for exonuclease I action, since no exonucleolytic degradation occurs with completely heterologous duplex DNA (Bedale *et al.*, 1991, data not shown). Additional cycles of

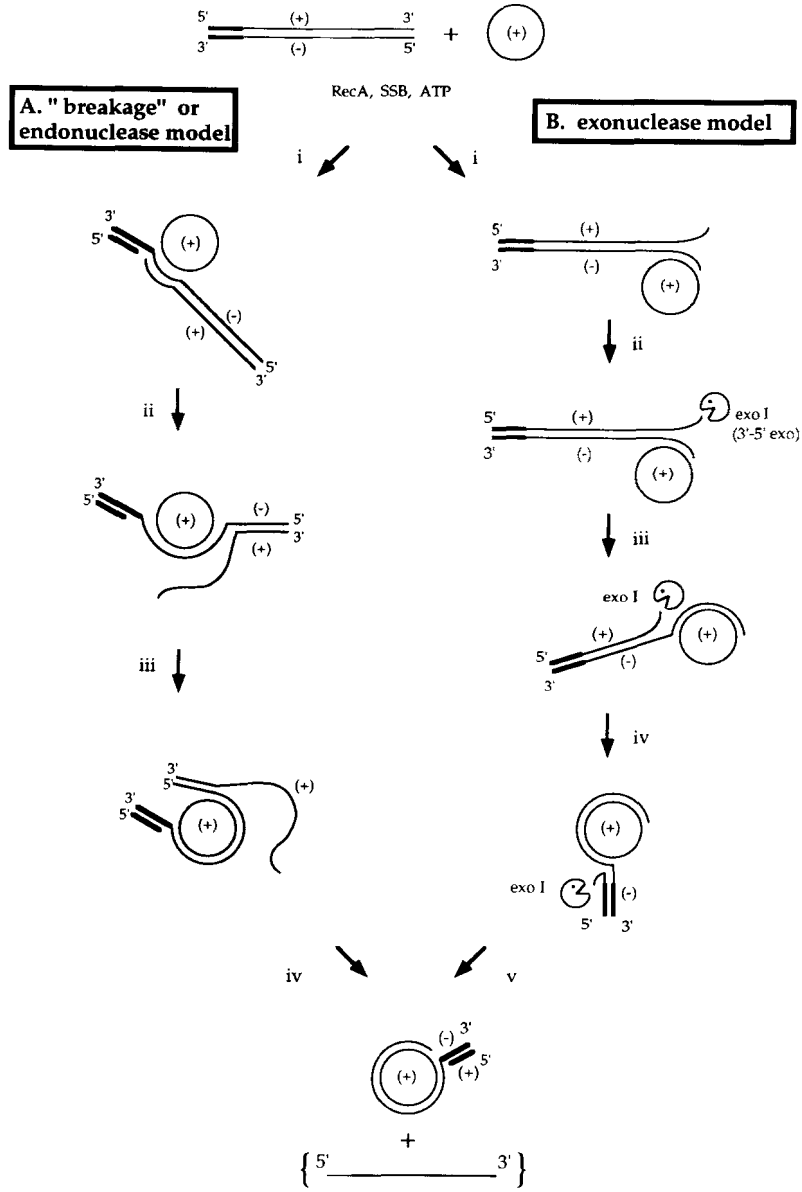


FIG. 9. **Models for the role of exonuclease I in facilitating recA protein-promoted DNA strand exchange in reactions involving 5′ (proximal) heterology.** *A*, original breakage or endonucleolytic model. *i*, pairing of homologous sequences and strand breakage at the heterology/homology junction in the (+) strand of the duplex DNA permits strand exchange to occur. *ii* and *iii*, DNA strand exchange continues 5′-3′ with respect to the (+) strand. *iv*, 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. *B*, alternative exonucleolytic model. *i*, pairing of homologous sequences results in the displacement of the 3′ end of the (+) strand of the dsDNA. Exonuclease I can then attack this strand (*ii*). *iii* and *iv*, continued transfer of the (−) strand to the circular ssDNA accompanied by exonucleolytic digestion of the displaced (+) strand of the original duplex. *v*, when transfer of the homologous region of (−) strand is complete, no more displacement of the (+) strand of the original duplex can occur and exonuclease I digestion halts. The final product is a double-stranded circular molecule with a double-stranded tail (corresponding to the heterology in the original duplex substrate).

pairing, displacement, and exonucleolytic degradation could occur and would effectively transfer all of the homologous portion of the (−) strand of the dsDNA to the ssDNAs. The final product of this reaction is a double-stranded circle with a short double-stranded tail corresponding to the length of heterology present at the 5′ end of the original dsDNA substrate. With our best DNA preparations, 60% or more of the input duplex DNA can be converted to scorable σ-like molecules by the combined action of recA protein and exonuclease I. These experiments confirm and extend the observations of Konforti and Davis (1992) that exonuclease I can facilitate formation of joint molecules on the 3′ end of the (+) strand of the duplex. When much longer homologous regions are provided in the duplex DNA and the reactions then analyzed by electron microscopy, a dynamic interplay of recA protein and exonuclease I is revealed that leads to the formation of discrete and predictable products.

In an earlier paper (Bedale *et al.*, 1991), the production of this unusual DNA species was attributed to either an endonucleolytic or mechanical break in the dsDNA at the heterology/homology junction that would allow strand exchange to initiate and proceed in the normal 5′-3′ direction from this break (Fig. 9*A*). Biochemical evidence presented here, however, indicates that the presence of a single-stranded exonuclease is required for the production of this species in a recA protein-promoted reaction. In addition, the electron microscopy and agarose gel results described here support the idea that strand exchange begins at an end of the original linear DNA substrate rather than at an internal nick or break generated specifically at the heterology/homology junction.

The apparent ability of exonuclease I to act on joint molecules formed when homology is limited to the distal end of the DNA is relevant to the current discussion of the proposed three-stranded structure of such distal joint molecules (Hsieh *et al.*, 1990; Rao *et al.*, 1991a; Stasiak, 1992). Whereas joint molecules formed at the proximal end of the duplex DNA participate in strand exchange with displacement of the (+) strand of the original dsDNA, distal joints can be isolated (after recA removal) in the form of stable DNA triplexes as determined by their thermal stability and sensitivity to strand-specific nucleases (Hsieh *et al.*, 1990; Rao *et al.*, 1991a).

A major rationale for the formation of stable triplexes exclusively at the distal end is that the (+) strand of the duplex, lacking a free 5′ end here, is not readily displaced (Hsieh *et al.*, 1990; Rao *et al.*, 1991a; Stasiak, 1992). It would seem unlikely that such distal joint molecules would be sus-

TABLE II

Electron microscopy of reactions with 5′ (proximal heterology) as a function of time

A strand exchange reaction was performed with dsDNA containing 198 bp of 5′ heterology as described (Bedale *et al.*, 1991). Aliquots were withdrawn at 0, 3, 8, 15, 24, and 50 min of reaction, and prepared for microscopy as described (Bedale *et al.*, 1991). Complex species (those involving >2 substrate DNA molecules) were not included in the counts.

DNA species[a]	Min						Total no. counted
	0	3	8	15	24	50	
	%						
1. ds linear (substrate)	99	83	65	21	16	20	447
2. ds circle with short ds tail (product)[b]	0	0	0	2	14	14	52
3. Gapped circle with long ds tail[c] (presumed intermediate)	0	10	27	18	14	7	89
4. Gapped circle with and without short ds tail[d]	0	1	2	50	43	50	239
5. Gapped circle with long ds tail[c] and long ss tail[e]	0	1	0	2	4	1	13
6. Gapped circle with long ds tail[c] and short ss tail[f]	0	1	2	3	4	2	16
7. Gapped circle with two ds tails[g]	1	3	0	0	0	1	9
8. Miscellaneous[h]	0	1	4	4	5	5	27
Total molecules counted	121	239	49	126	56	301	892

[a] Complex species (containing >2 substrate molecules), broken intermediates or products, and ss circles were not counted; ss circles were in excess and represented >50% of all molecules.

[b] ds tail corresponds roughly to the length of heterology, about 200 bp.

[c] ds tail is significantly longer than 200 bp; tail lengths are not uniform.

[d] These products presumably arose as a result of nicks in the duplex substrate.

[e] ss tail is approximately the length of the region that has undergone strand exchange.

[f] ss tail is significantly shorter than the length of the region that has undergone strand exchange; tail lengths are not uniform.

[g] Tail lengths are not uniform.

[h] None of the miscellaneous species had 2 ds tails; none were observed more than 5 times for all the time points combined.

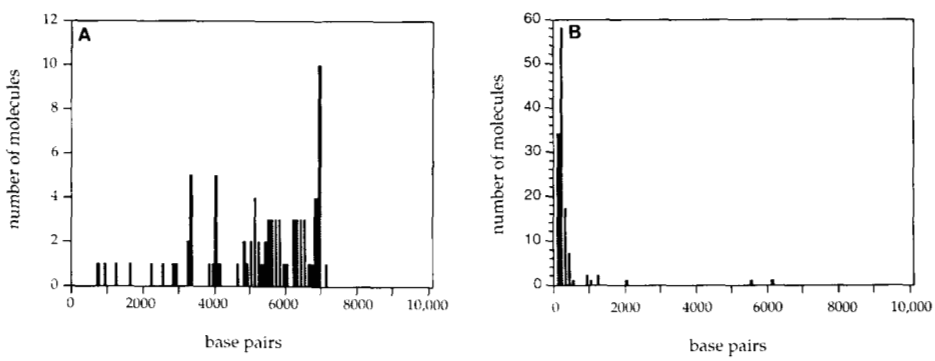


FIG. 10. **Distribution of double-stranded tail lengths after 15 and 50 min of reaction.** Strand exchange reactions between single-stranded M13 mp8 (+) and M13mp8. 198 (*Eco*RI) were performed as described under "Experimental Procedures." The reaction was stopped after 15 min (*A*) and 50 min (*B*) and prepared for electron microscopy as described (Bedale *et al.*, 1991). Double-stranded tail lengths for 82 molecules (*A*) and 125 molecules (*B*) were measured. The data were normalized on the assumption that the dsDNA substrate was 7427 bp in length.

ceptible to attack by exonuclease I, since this enzyme is extraordinarily specific for ssDNA (Lehman and Nussbaum, 1964; Prasher *et al.*, 1983). All three strands of the joints isolated by Rao *et al.* (1991) were demonstrably intact, indicating that the stable joints did not arise as an artifact associated with nuclease contamination. The results presented here, however, demonstrate that the 3′ end of the (+) strand of the duplex in distal joints is accessible to degradation by added exonuclease I when recA is present. A limited degradation of this strand by added exonuclease I has recently been reported by Rao *et al.* (1993) in isolated triplex joints (recA removed). This could indicate an inherent sensitivity of this strand to exoI degradation in recA triplexes and suggest that the (+) strand need not be displaced to produce the σ-like molecules we observe. However, the degradation reported by Rao *et al.* (1993) is much less than is observed for single-stranded DNA and substantially less than we observe in the present studies carried out in the presence of recA protein. In Rao *et al.* (1993), 25 units of exonuclease I removed the end label from about 0.05 pmol (10% of the total) of isolated triplexes in 2 min. In our experiments, 0.1 units of exonuclease I removes 7000 bases of the (+) strand from a similar number of molecules in 50–90 min. The extensive degradation of this (+) strand when recA remains bound is likely to reflect displacement of the strand.

If this interpretation is correct, the rationale that the stability of triplexes at the distal end reflects an inability to displace the (+) strand needs to be reexamined. Other work has also hinted that the 3′ end of the (+) strand in distal joints is displaced at least some of the time (Adzuma, 1992; Stasiak, 1992).[4] ATP hydrolysis appears to be required for the sensitivity of distal joint molecules to exonuclease I; σ-like products are not generated in the presence of ATPγS (Bedale *et al.*, 1991). The (+) strand of the original duplex substrate in these experiments is sensitive to exonuclease I in the presence of ATP but not in the presence of ATPγS, providing new evidence that the displacement of this strand is one function of ATP hydrolysis.[5] Clearly, the structure and characteristics of distal joints need to be elucidated before a resolution to these issues can be obtained.

The evident sensitivity of the duplex (+) strand to exonuclease I does not preclude the current interpretation of several studies (Hsieh *et al.*, 1990; Rao *et al.*, 1991a) that provide evidence for the formation of stable triplex structures in distal joints. For example, there could be a dynamic equilibrium between a triplex intermediate and products (with the (+)

[4] S. Jain and M. Cox, unpublished results.

[5] W. Bedale and M. Cox, unpublished results.

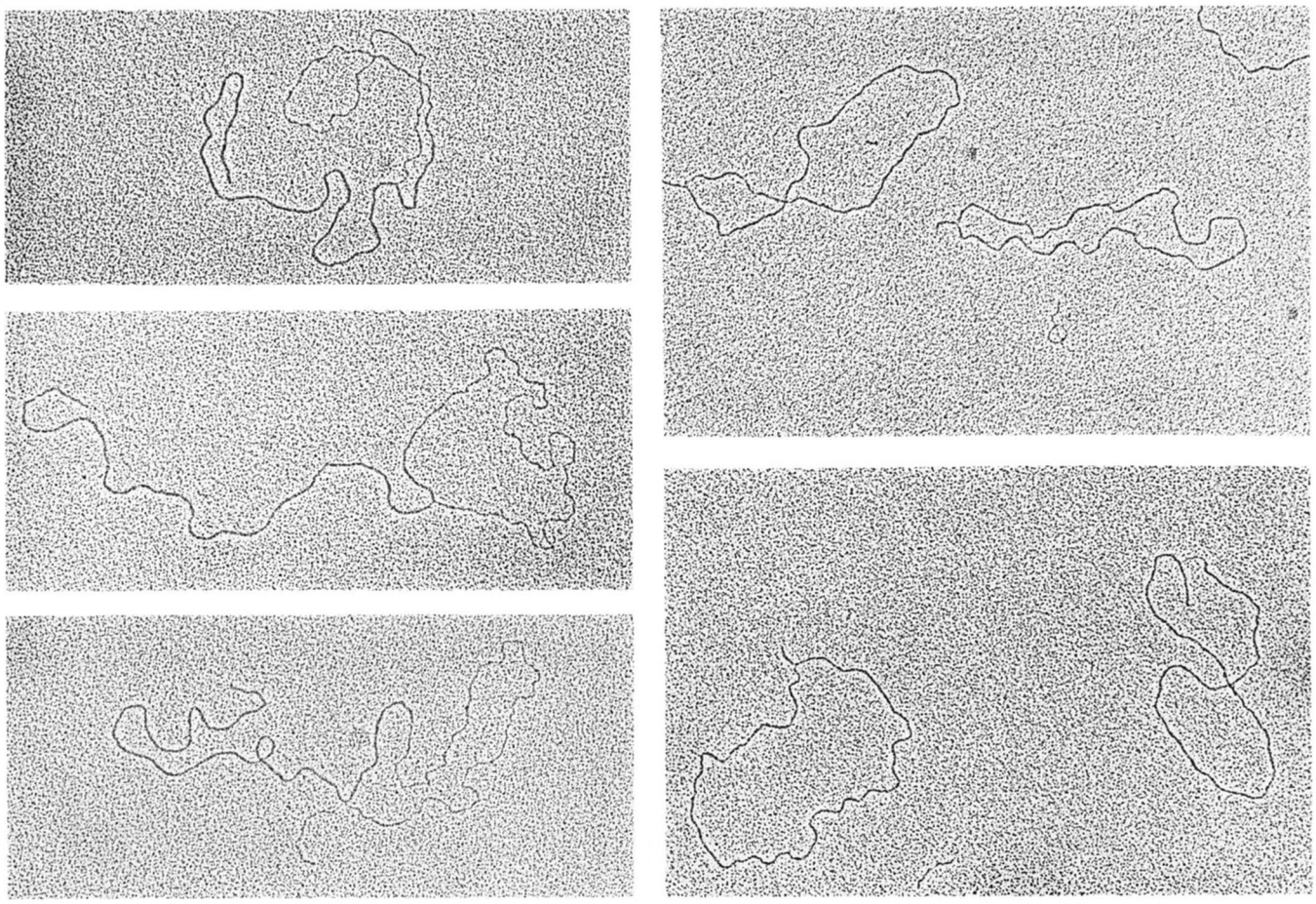


FIG. 11. **Electron microscopy of double-stranded tailed species at 15 and 50 min of reaction.** Strand exchange reactions between single-stranded M13 mp8 (+) and M13mp8.198 (*Eco*RI) were performed as described under "Experimental Procedures." The reaction was stopped at various times and prepared for electron microscopy as described (Bedale *et al.*, 1991). Electron micrographs of representative molecules after 15 min (*left panels*) and 50 min (*right panels*) are shown.

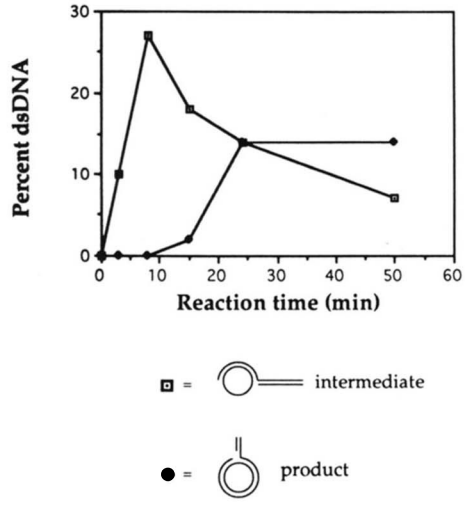


FIG. 12. **Time course of formation of double-stranded tailed products and intermediates.** Strand exchange reactions between single-stranded M13 mp8 (+) and M13mp8. 198 (*Eco*RI) were performed as described under "Experimental Procedures" and prepared for electron microscopy as previously described (Bedale *et al.*, 1991). Total numbers of molecules counted are shown in Table II.

strand displaced) in these joints that permits the isolation of triplexes when recA protein is removed. The apparent displacement of the (+) strand in joints formed at both ends of the duplex, however, makes it much harder to explain why joint molecules (and triplexes) appear to be more stable when formed at the distal end.

An additional conclusion of our work is that recA protein preparations can often be contaminated with exonuclease I, and that trace, almost undetectable amounts of exonuclease I present in recA protein preparations can have dramatic effects on certain recA protein-promoted reactions. Three of 10 recA protein preparations examined by immunoblots contained exonuclease I (Fig. 8). The contaminating exonuclease I was present at very low levels, on the order of one molecule exonuclease I for every 20,000 recA protein molecules. When these recA protein preparations were screened for single-stranded exonuclease activity, none was detected unless SSB protein was also present. SSB protein has been shown to interact physically with exonuclease I and to stimulate its activity (Molineux and Gefter, 1975). The amount of single-stranded exonuclease activity present in recA protein preparations (as judged by the amount of purified exonuclease I that is needed to restore production of σ-like molecules to those preparations that lack this activity, Fig. 5) is comparable to the amount of exonuclease I present in those preparations (as judged by immunoblots, Fig. 8), suggesting that exonuclease I is the enzyme actually responsible for the formation of σ-like molecules.

Exonuclease I was not present in any recA protein preparations tested that were purified by the method of Cox *et al.* (1981) (Table I). This purification procedure consists of polymin P precipitation, phosphocellulose chromatography, and ssDNA cellulose chromatography. The ssDNA cellulose col-

TABLE III

Electron microscopy of strand exchange reactions in the presence of exonuclease I with endless DNA

Strand exchange reactions were performed with DNA containing 198 bp of 5′ heterology or with endless DNA as described under "Experimental Procedures." Reactions were performed using recA protein preparations that contained exonuclease I or that were free of exonuclease I. After 90 min of reaction, products were prepared for electron microscopy as described (Bedale *et al.*, 1991). Single-stranded circles and complex species (those involving >2 substrate DNA molecules) were not included in the counts.

DNA species[a]	Closed ends, + exo I	Closed ends, − exo I	Open (normal) ends, + exo I	Open (normal) ends, − exo I
			%	
ds linear (substrate)	68	73	<1	50
ds circle with short ds tail (product)[b]	4	<1	59	<1
Gapped circle	6	9	25	20
Gapped circle with short ds tail[b]	4	<1	7	5
ds circles with short ss tail	0	<1	1	4
ds circle	3	6	7	18
Gapped circle with long ss tail	0	4	0	0
Blocked type	10	0	0	0
Miscellaneous	5	6	1	2
Total molecules counted	273	135	246	144

[a] Reactions were spread for microscopy after 90 min of reaction, complex species (containing >2 substrate molecules) and single-stranded circles were not counted.

[b] ds tail corresponds roughly to the length of heterology, about 200 bp.

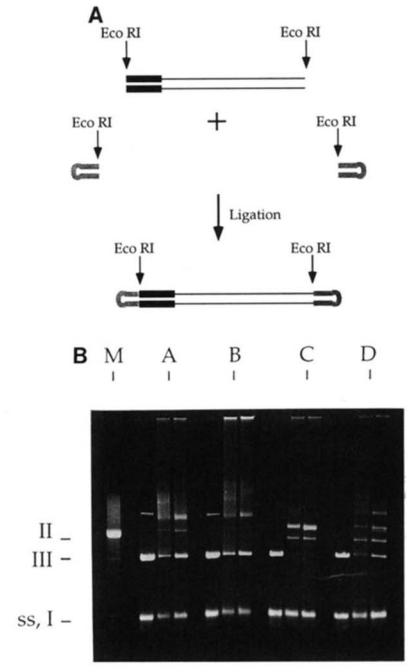


FIG. 13. **Strand exchange reactions in the presence of exonuclease I with endless DNA.** *A*, scheme for making endless DNA. Hairpin oligos were ligated to M13mp8.198 (*Eco*RI) DNA as described under "Experimental Procedures." *B*, strand exchange reactions and agarose gel electrophoresis were performed as described under "Experimental Procedures." The marker (*lane M*) is M13mp8 DNA (FI–III). For each *reaction A-D*, the time points shown correspond to 0, 45, and 90 min of reaction. *Reactions A* and *B* contain the endless DNA shown in *A* and produced as described under "Experimental Procedures." *Reactions C* and *D* contain ssM13mp8 and M13mp8.198 (*Eco*RI); the latter has 198 bp of 5′ (proximal) heterology. *Reactions A* and *C* were performed with recA protein preparation 4, which is contaminated with exonuclease I. *Reactions B* and *C* were performed with recA protein preparation 1, which was purified from an *E. coli* strain that is exonuclease I-free.

umn (with ATP elution) seems to effectively remove exonuclease I, as exonuclease I is present in the fraction just prior to this column (data not shown). The purification method of Griffith and Shores (1985) is similar to that of Cox *et al.* (1981) except that spermidine acetate precipitation is used instead of ssDNA cellulose chromatography. This procedure has many advantages in terms of speed and simplicity, and it generates very pure recA protein. It does not appear to be as efficient as other protocols in removing exonuclease I, however. One of three recA protein preparations tested that were purified by this procedure was free of single-strand exonuclease. The other two contained trace amounts of exonuclease I (Table I). We also tested one recA protein preparation purified by the method of Shibata *et al.* (1981) and found it to be free of exonuclease I. This procedure includes polymin P precipitation, hydroxyapatite, Sephacryl S200, and DEAE-cellulose chromatography. RecA protein purified by another procedure[6] was free of contaminating exonuclease I (Table I). We note that this protocol also uses a spermidine acetate precipitation step, but exonuclease I is removed by additional procedures. If spermidine acetate precipitation is to be used, we recommend this protocol.[6] A straightforward ramification of these results is that every recA protein preparation should be screened for single-stranded exonuclease activity *in the presence* of SSB protein. The presence of exonuclease I in one SSB protein preparation was apparently an anomaly that has not recurred. The method of Lohman *et al.* (1986) for preparing SSB protein routinely provides material free of exonuclease I.[3]

The observation that exonuclease I can increase the efficiency of recA protein-mediated DNA stand exchange when a heterologous sequence is present at the 5′ end of the duplex DNA *in vitro* suggests a possible role for exonuclease I in *in vivo* recombination. Exonuclease I is the primary single-stranded exonuclease in *E. coli* (Chase and Richardson, 1974) and is specific for ssDNA, which it degrades processively in the 3′-5′ direction (Lehman and Nussbaum, 1964; Weiss, 1981). The enzyme will degrade single-stranded tails on dsDNA molecules to within six to eight nucleotides of the base-paired region (Brutlag and Kornberg, 1972). Exonuclease I is the product of the *sbcB* gene, and *sbcB* mutations suppress the recombination deficiency and UV light and mitomycin C sensitivity of *recB* and *recC* mutations (Kushner *et al.*, 1971), suggesting a possible role for exonuclease I in DNA recombination or repair (Kushner *et al.*, 1971). Mutants which lack exonuclease I show no detectable defects in DNA metabolism, however, so its precise role remains unclear (Weiss, 1981). Interestingly, properties of another class of mutations in the gene for exonuclease I suggest that the enzyme may have an additional activity. This class of mutations, *xonA*, suppresses the DNA repair but not the recombinational deficiencies in *recBC* mutants. Phillips *et al.* (1988) suggested that the *xonA* gene product might block the ability of enzymes from an alternative recombination pathway to recognize the DNA substrate. This might occur if exonuclease I interacted with other ssDNA binding proteins, such as SSB protein and recA protein (Phillips *et al.*, 1988). An interaction of exonuclease I and recA may be especially important in the process of recombinational repair. Interestingly, it was our attempts to understand how recA protein facilitated bypass of structural barriers in the DNA during strand exchange (an activity important for repair rather than recombination) that initially led to these studies.

[6] S. Kowalczykowski, personal communication.

The proposed action of an exonuclease in homologous recombination has precedent in eukaryotic DNA strand transferases. Sep1 protein is a strand transferase from yeast which possesses an intrinsic 5′-3′ exonuclease activity (Johnson and Kolodner, 1991). The action of this nuclease is necessary for the initiation of strand exchange. The exonuclease degrades the ends of the dsDNA substrate so that 3′ single-stranded tails are generated. These single-stranded tails can pair with complementary ssDNA; branch migration can then occur. Under conditions that prevent the nuclease activity, strand exchange can occur if the ends of the DNA are resected by an exogenous exonuclease (Johnson and Kolodner, 1991). The polarity of Sep1 protein promoted DNA strand exchange is a consequence of polarity of the nuclease activity itself and not of branch migration. If the exogenous exonuclease leaves 3′ single-stranded tails, strand exchange proceeds 5′-3′(with respect to the (+) strand), while if it leaves 5′ single-stranded tails, strand exchange proceeds 3′-5′ (Johnson and Kolodner, 1991).

The ability of recA protein, SSB protein, and exonuclease I to function together *in vitro* during homologous recombination makes it tempting to speculate that they may cooperate similarly *in vivo*. Exonuclease I can greatly improve the efficiency of *in vitro* DNA strand exchange reactions in situations where the 5′ (proximal) end is blocked by heterology. SSB protein stimulates recA protein-promoted strand exchange dramatically (Cox and Lehman, 1981b, 1982; Cox *et al.*, 1983) and probably enhances the activity of exonuclease I by binding to the strand that exonuclease I degrades. Although exonuclease I was not observed to bind directly to a recA protein affinity column (Freitag and McEntee, 1988), the observation that exonuclease I and recA protein copurify through many purification steps suggests that the two proteins may interact physically. Exonuclease I and SSB are also known to interact and copurify under certain conditions (Molineux *et al.*, 1974). Many important questions still remain, such as whether or not exonuclease I plays a similar role with recA protein and SSB protein *in vivo* as it appears to play *in vitro*, and if it is the only single-stranded exonuclease that affects recA protein-promoted strand exchange. The ability of at least one other single-stranded exonuclease, exonuclease VII, to promote this reaction makes the assigning of specific roles difficult. It is at least possible that one of several functions of exonuclease I *in vivo* is to facilitate homologous recombination in situations where recA protein does not work efficiently, and that other 3′-5′ single-stranded exonucleases can promote similar reactions.

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