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# Characterization of Strand Exchange Activity of Yeast Rad51 Protein

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**The *Saccharomyces cerevisiae RAD51* gene product takes part in genetic recombination and repair of DNA double strand breaks. Rad51, like *Escherichia coli* RecA, catalyzes strand exchange between homologous circular single-stranded DNA (ssDNA) and linear double-stranded DNA (dsDNA) in the presence of ATP and ssDNA-binding protein. The formation of joint molecules between circular ssDNA and linear dsDNA is initiated at either the 5′ or the 3′ overhanging end of the complementary strand; joint molecules are formed only if the length of the overhanging end is more than 1 nucleotide. Linear dsDNAs with recessed complementary or blunt ends are not utilized. The polarity of strand exchange depends upon which end is used to initiate the formation of joint molecules. Joint molecules formed via the 5′ end are processed by branch migration in the 3′-to-5′ direction with respect to ssDNA, and joint molecules formed with a 3′ end are processed in the opposite direction.**

The *Saccharomyces cerevisiae RAD51* gene is a member of the so-called *RAD52* epistasis group, which has been implicated in various recombination and repair processes. Both genetic and biochemical evidence suggests the existence of molecular interactions among several of the proteins comprising this group: Rad51 and Rad52 (9, 26, 34), Rad51 and Rad55 (9, 15), Rad51 and Rad54 (12), Rad52 and Rfa1 (or RPA, single-stranded DNA [ssDNA] binding protein) (9a), and Rad55 and Rad57 (9, 15). However, the surface has barely been scratched concerning the specific biochemical activities of individual members of the group, and still less is known about their molecular complexes.

The earliest insight into the possible biological function of the Rad52 group proteins came from the striking sequence similarity between the critical bacterial recombination protein RecA and Rad51 (1, 3, 34). Furthermore, the two proteins were shown to form structurally similar nucleoprotein filaments with ssDNA and double-stranded DNA (dsDNA) (28, 39). Soon thereafter, Sung (38) demonstrated that purified yeast Rad51 could emulate RecA's ability to promote homologous pairing and strand exchange between a single-stranded circular DNA and its double-stranded linear DNA homolog. His laboratory has characterized this reaction in detail, pointing out similarities and differences between Rad51- and RecA-mediated reactions. One striking conclusion was that Rad51 initiates joint molecule formation exclusively from the 5′ end of the linear dsDNA's complementary strand and that the ensuing strand exchange proceeds in the direction opposite to RecA (39, 40).

We have investigated the strand exchange reaction with Rad51 purified from insect cells infected with recombinant baculovirus that expresses the *RAD51* gene. Our results demonstrate that Rad51 initiates joint molecule formation from either end of the linear DNA's complementary strand, provided it exists as an overhanging single strand. 3′ overhanging ends are more efficient than a 5′ overhang, while blunt or recessed ends are not used. Consequently, strand exchange proceeds in either the 5′-to-3′ or 3′-to-5′ direction, depending upon whether the 3′ or the 5′ end of the complementary strand initiates the exchange.

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## MATERIALS AND METHODS

**Cell culture and media.** *Spodoptera frugiperda* (Sf9 and Sf21) cells were maintained and propagated in suspension in SF900 II SFM medium (GIBCO/BRL) at 27°C with constant shaking at 150 rpm.

**Construction of recombinant baculoviruses.** Plasmid pRad51, which contains the yeast *Rad51* gene cloned into the *Bam*HI-*Pst*I sites of YCplac33, was digested with *Stu*I and *Dra*I endonucleases, and the resulting 1.4-kb fragment was isolated and inserted into the *Sma*I site of a pSLH148 plasmid. PCR was used to fuse the 3′ terminus of the *Rad51* gene to a sequence encoding the $His_6$ tag to produce pSLH154. Expression of this modified Rad51 protein containing the C-terminal $His_6$ tag complemented the X-ray sensitivity of a yeast strain with the *rad51* deletion. The transfer vector pVRad51His was constructed by inserting a *Kpn*I/*Xba*I fragment of Rad51 $His_6$ from pSLH154 into the *Bam*HI/*Xba*I sites of pVL1393 (PharMingen). Recombinant baculovirus was generated by cotransfection of the pVRad51His plasmid with linear baculovirus DNA into Sf9 cells. The recombinant virus expressed the Rad51 protein under the control of the polyhedrin promoter. (pRad51, pSLH148, and pSLH154 plasmids were constructed by Sharon Hays, and they and their structures can be obtained on request.)

**Baculovirus infection, extract preparation, and purification of Rad51 from infected cells.** Sf21 cells growing in SF900 II SFM medium (3 liters) containing 5% heat-inactivated fetal bovine serum (HyClone), yeastolate, and lactalbumin hydrolysate (GIBCO/BRL) were infected with recombinant baculovirus at a multiplicity of infection of 2 to 5. Cells were grown at 27°C with constant rotation at 150 rpm for 60 h. The cells were harvested, fast-frozen, and stored at −70°C until required. The following procedures were carried out at 4°C.

Frozen cells were thawed at 4°C and then homogenized 20 times with a B-type pestle in lysis buffer (20 mM $KH_2PO_4$ [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 5-μg/ml leupeptin, 2-μg/ml pepstatin A, 2-μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride), after which NaCl was added to a final concentration of 0.15 M. After 30 min of incubation, the homogenate was centrifuged at 100,000 × *g* for 45 min, and ammonium sulfate (0.242 g/ml) was added to the supernatant. The resulting precipitate was collected by centrifugation, resuspended in buffer A (20 mM $KH_2PO_4$ [pH 7.5], 2 mM EDTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol) plus 100 mM NaCl, and dialyzed against the same buffer (fraction I; ~350 mg). Fraction I was applied to a 50-ml phosphocellulose column equilibrated with buffer A plus 100 mM NaCl. The flowthrough proteins were diluted with buffer A to 50 mM NaCl (fraction II; ~150 mg) and loaded onto two Econo-Pac Q cartridges (each 5 ml; Bio-Rad Laboratories) equilibrated with the same buffer. The proteins were eluted with a 100-ml linear gradient of 0.05 to 0.8 M NaCl in buffer A. Fractions containing Rad51, which eluted at approximately 0.15 M NaCl, were pooled and dialyzed against buffer B (10 mM $KH_2PO_4$ [pH 7.2], 40 mM KCl, 1 mM DTT, 10% glycerol) (fraction III; ~60 mg). This fraction was then loaded onto two hydroxyapatite Econo-Pac CH-II cartridges (each 5 ml; Bio-Rad Laboratories)

equilibrated with buffer B, and the protein was then eluted with a 75-ml linear gradient of 0.01 to 0.5 M potassium phosphate (pH 7.2) in 40 mM KCl–0.5 mM DTT–10% glycerol. The Rad51 protein (fraction IV; ~25 mg) which had been eluted at approximately 0.1 M phosphate was dialyzed against buffer A plus 50 mM NaCl and then loaded onto a Mono Q 5/5 column (Pharmacia). Proteins were eluted with a 25-ml linear gradient of 0.05 to 0.8 M NaCl in buffer A. Rad51 eluted from the Mono Q column at 350 mM NaCl and was stored at −70°C in this buffer at a concentration of 4.8 mg/ml (fraction V; 14.4 mg).

The detection and quantitation of Rad51 protein relied on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis with antibody raised against Rad51 protein (21, 27).

**Preparation of DNA substrates for joint molecule formation and strand exchange reactions.** The concentrations of the DNA substrates are expressed as nucleotide equivalents. Plasmid, viral, and supercoiled M13 dsDNAs were prepared as described previously (31). M13T597 was constructed by ligation of a *Bam*HI-*Nru*I 597-bp fragment from pBR322 with *Bam*HI- and *Hin*cII-digested M13mp18 dsDNA, and M13T653 was made by ligation of an *Eco*RI-*Sal*I 653-bp fragment from pBR322 with *Eco*RI- and *Sal*I-digested M13 dsDNA. The ϕX174 dsDNA and viral ssDNA were purchased from GIBCO/BRL. Unless otherwise noted, linear dsDNA was obtained by digestion of supercoiled DNA with *Pst*I endonuclease, followed by extraction with phenol-chloroform, ethanol precipitation, and resuspension in Tris-EDTA buffer. Linear ϕX174 dsDNAs with different types of termini were prepared by single cleavage of ϕX174 dsDNA with a particular restriction endonuclease; linear dsDNAs with 3′ overhanging termini were made with *Pst*I endonuclease, linear dsDNAs with 5′ overhangs were produced with *Xho*I or *Bss*HII endonucleases; and *Stu*I and *Ssp*I endonucleases were used to create linear dsDNA with blunt ends. Linear M13mp18 dsDNAs with 3′, 5′, and blunt termini were prepared with *Pst*I, *Bam*HI, and *Hin*cII endonucleases, respectively. 3′ or 5′ overhanging termini were converted to blunt ends by incubation of the linear duplex DNA with T4 DNA polymerase and nucleoside triphosphates. Linear dsDNAs with single-strand 3′ overhangs of 4, 3, 2, 1, and 0 nucleotides were prepared by cleavage of ϕX174 dsDNA with *Pst*I, *Dra*III, *Ksp*I, *Ahd*I, and *Fsp*I endonucleases, respectively. $^{32}$P-5′-labeled linear duplex DNA was obtained following dephosphorylation by incubation with polynucleotide kinase and [γ-$^{32}$P]ATP. $^{32}$P-3′-labeled linear duplex DNA was obtained by incubation with Klenow fragment DNA polymerase and [α-$^{32}$P] dCTP.

**Standard joint molecule formation and strand exchange assays.** The DNA substrates, joint molecules, and complete strand exchange products are shown in Fig. 2A. Unless otherwise noted, the standard reaction buffer used in all experiments contained 40 mM K-MES (morpholineethanesulfonic acid) (pH 6.5), 12 mM $MgCl_2$, 1 mM DTT, 0.5 mM ATP, and 5% glycerol, and the incubation temperature was 37°C. For the study of joint molecule formation, the DNA substrates were obtained from M13mp18 phage, while the studies of complete strand exchange were done with ϕX174 DNA.

In the standard reaction, 20 μM ssDNA substrate (viral positive-strand ϕX174 or M13mp18) was preincubated with 5.6 μM Rad51 in the presence of 2 mM $MgCl_2$ and 0.5 mM ATP for 10 min. Then yeast RPA (a gift from S. C. Kowalczykowski) or *Escherichia coli* ssDNA-binding protein (SSB; Pharmacia) was added to concentrations of 0.7 and 2 μM, respectively. After 10 min, the reaction was started by the addition of linear ϕX174 or M13 dsDNA to 20 μM and of $MgCl_2$ to 12 mM. The reaction was terminated at the indicated times by the addition of SDS and proteinase K to final concentrations of 0.5% and 0.5 mg/ml, respectively, and deproteinized by incubation at 37°C for 1 h. Samples (10 μl) were loaded on a 0.9% agarose gel in TAE buffer (40 mM Tris acetate [pH 7.5], 0.5 mM EDTA), and electrophoresis was performed at 1.2 V/cm for 15 h. DNA was visualized by ethidium bromide staining.

**Determination of what kinds of ends of the linear dsDNA's complementary strand are required for joint molecule formation (see Fig. 5A).** Different linear dsDNAs with homology at one end of the negative strand and heterology at the other as well as those with different types of termini were prepared from M13 dsDNA which contained insertions of a segment of pBR322 DNA. M13T597 dsDNA was cleaved at either the *Pst*I or the *Kpn*I site so that the negative strand of the linear dsDNA contained 3′ overhanging or 5′ recessed homologous ends. M13T653 dsDNA was cut at either the *Sal*I or the *Eco*RI site to create 3′ recessed or 5′ overhanging homologous ends in the linear dsDNA negative strand. Linear dsDNA with heterology at both ends was prepared from M13T653 dsDNA by digestion with *Bam*HI endonuclease.

To examine which end of the linear dsDNA's negative strand is utilized in the formation of joint molecules, 5.6 μM Rad51 was preincubated with 20 μM M13 viral positive ssDNA and 0.7 μM RPA protein. The reaction was started by the addition of 20 μM various linear dsDNAs. After 2 h of incubation at 37°C, the reaction was stopped by the addition of SDS and proteinase K, and the products of the reaction were separated by agarose gel electrophoresis as described above.

**Determination of the polarity of strand exchange (see Fig. 6A).** Different linear dsDNAs with a blunt terminus at one end and an overhang at the other were prepared from ϕX174 dsDNA. The linear dsDNAs with blunt ends at different sites were generated by digestion of ϕX174 dsDNA with *Bss*HII (followed by incubation with T4 DNA polymerase), *Fsp*I, or *Bsr*BI endonuclease. Then, the linear dsDNAs with *Bss*HII- or *Fsp*I-generated blunt ends were cleaved with *Pst*I endonuclease; this sequence of digestions generated linear dsDNAs with a blunt terminus at one end and a 3′ overhanging end at the other.

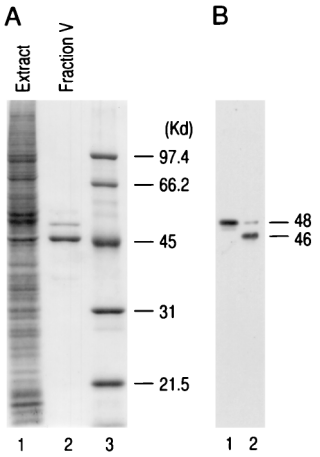


FIG. 1. Purification of Rad51 protein. (A) Rad51 was analyzed by SDS-PAGE followed by Coomassie blue staining. Lane 1, total cell lysate of Sf21 cells expressing Rad51; lane 2, fraction V showing two polypeptides of 46 and 48 kDa; lane 3, molecular mass markers. (B) Immunoblot probed with Rad51-specific antibodies. Lane 1, Rad51 purified from yeast; lane 2, Rad51, fraction V.

A second pair of the linear dsDNAs with blunt ends at the *Fsp*I or *Bsr*BI site were digested by *Xho*I endonuclease to produce linear dsDNAs with one blunt end and a 5′ overhang at the other end. Linear ϕX174 dsDNAs, both ends of whose negative strand are overhanging or recessed, were produced by double digestions at the *Bss*HII and *Pst*I sites or at the *Pst*I and *Xho*I sites, respectively.

In the reaction testing the polarity of the strand exchange reaction, 5.6 μM Rad51 was preincubated with 20 μM ϕX174 positive-strand ssDNA and 0.7 μM RPA. The reaction was started by the addition of 20 μM various linear ϕX174 dsDNAs. After 2 h at 37°C, the reaction was stopped and analyzed as described before.

**dsDNA exonuclease assay.** 5′-to-3′ and 3′-to-5′ exonuclease activities were monitored by the loss of $^{32}$P from $^{32}$P-end-labeled M13 linear dsDNA. The reaction mixture was electrophoresed in a 10% polyacrylamide gel, and the gel was dried on DEAE paper and autoradiographed at −70°C with X-ray film. There was less than 0.1% $^{32}$P released after 2 h of incubation of 20 μM 5′- or 3′-$^{32}$P-labeled linear DNA with 5.6 μM Rad51 under the conditions for strand exchange reaction.

**ssDNA exonuclease assay.** Exonuclease activity was monitored by the loss of $^{32}$P from $^{32}$P-end-labeled poly(dT) (Pharmacia) as measured by acid precipitation. 5′-$^{32}$P-labeled poly(dT) was prepared with polynucleotide kinase and [γ-$^{32}$P]ATP. The reactions were performed as described above. Less than 1% of $^{32}$P was released after 2 h of incubation of 20 μM 5′-$^{32}$P-labeled poly(dT) with 5.6 μM Rad51.

**Helicase assay.** The 20-mer universal sequencing primer was hybridized to M13mp18 ssDNA, and the primer was elongated with T4 DNA polymerase by using dGTP and [α-$^{32}$P]dCTP. The assay for DNA helicase activity was performed under the same conditions as strand exchange. After incubation of 5.6 μM Rad51 with 20 μM $^{32}$P-labeled DNA substrate for 2 h, the reaction was stopped by addition of SDS, EDTA, and proteinase K to final concentrations of 0.5%, 20 mM, and 0.5 mg/ml, respectively. After 30 min of incubation at 37°C, the reaction products were separated by electrophoresis on a 10% native polyacrylamide gel in Tris-borate-EDTA buffer. The gel was dried onto DEAE paper and autoradiographed at −70°C with X-ray film.

**Other methods.** Protein concentrations were determined by using the bicinchoninic acid protein assay kit (Pierce) with bovine serum albumin as a standard. Photographic images were scanned directly from autoradiograms or from photographs by using Adobe Photoshop.

## RESULTS

**Overexpression and purification of yeast Rad51 protein from insect cells.** The baculovirus expression system was used for the production of large quantities of biologically active Rad51. Rad51 protein was expressed to levels of 20 mg/liter in Sf21 insect cells infected with the recombinant baculovirus. Fraction V contained two polypeptides of 46 and 48 kDa (Fig. 1A) that interacted with antibody against Rad51 (Fig. 1B). Amino-terminal sequencing of the 46-kDa polypeptide, which constitutes ~80% of the total protein, matches the sequence of Rad51 starting from the second ATG located 22 amino acids

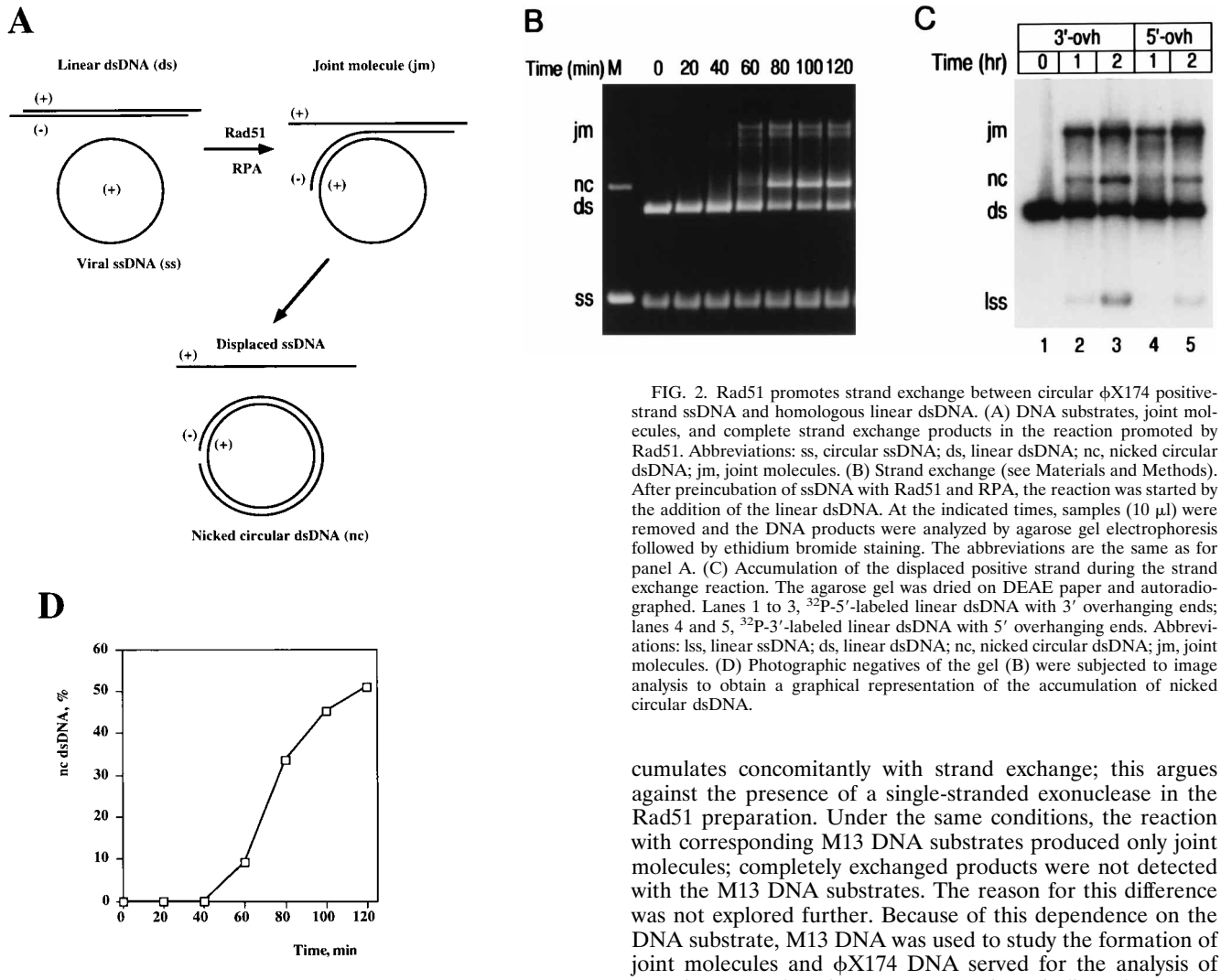


FIG. 2. Rad51 promotes strand exchange between circular ϕX174 positive-strand ssDNA and homologous linear dsDNA. (A) DNA substrates, joint molecules, and complete strand exchange products in the reaction promoted by Rad51. Abbreviations: ss, circular ssDNA; ds, linear dsDNA; nc, nicked circular dsDNA; jm, joint molecules. (B) Strand exchange (see Materials and Methods). After preincubation of ssDNA with Rad51 and RPA, the reaction was started by the addition of the linear dsDNA. At the indicated times, samples (10 μl) were removed and the DNA products were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. The abbreviations are the same as for panel A. (C) Accumulation of the displaced positive strand during the strand exchange reaction. The agarose gel was dried on DEAE paper and autoradiographed. Lanes 1 to 3, $^{32}$P-5′-labeled linear dsDNA with 3′ overhanging ends; lanes 4 and 5, $^{32}$P-3′-labeled linear dsDNA with 5′ overhanging ends. Abbreviations: lss, linear ssDNA; ds, linear dsDNA; nc, nicked circular dsDNA; jm, joint molecules. (D) Photographic negatives of the gel (B) were subjected to image analysis to obtain a graphical representation of the accumulation of nicked circular dsDNA.

downstream from the first ATG. No sequence information could be obtained for the 48-kDa protein (~15% of the total) due to a blocked amino terminus. The mobility of the 48-kDa protein on SDS-PAGE was similar to the mobility of yeast Rad51 expressed in yeast cells (Fig. 1B). We presume that the 48-kDa polypeptide is translated from the first AUG and is the same as the wild-type Rad51. Presumably, translation from the second AUG is preferred in baculovirus translation, and there is no information available as to the preferred context for translation initiation at AUGs in this organism. However, sequence analysis of Rad51 proteins from different organisms indicates considerable differences in the N-terminal part of the proteins. We surmise, and our data indicate (see later), that removal of 22 amino acids from the N terminus of Rad51 does not affect its functional activity. Fraction V did not contain any detectable helicase, ligase, endo-, and exonuclease activities.

Sung and others have shown (38–40) that yeast Rad51 catalyzes a yeast RPA-dependent homologous pairing and strand exchange reaction similar to that catalyzed by *E. coli* RecA. Our preparation of Rad51 also promotes both joint molecule formation and strand exchange between circular ssDNA and linear duplex DNA (Fig. 2A). Rad51 converted about 50% of linear ϕX174 dsDNA to nicked circular dsDNA in 2 h (Fig. 2B and D). Figure 2C shows that the displaced single strand accumulates concomitantly with strand exchange; this argues against the presence of a single-stranded exonuclease in the Rad51 preparation. Under the same conditions, the reaction with corresponding M13 DNA substrates produced only joint molecules; completely exchanged products were not detected with the M13 DNA substrates. The reason for this difference was not explored further. Because of this dependence on the DNA substrate, M13 DNA was used to study the formation of joint molecules and ϕX174 DNA served for the analysis of complete strand exchange. Except where indicated, M13 or ϕX174 linear duplex DNAs were prepared by cleavage of the circular dsDNA with *Pst*I endonuclease.

**Requirements for Rad51-promoted formation of joint molecules and complete strand exchange.** Under our conditions, both joint molecule formation and strand exchange require a stoichiometric amount of Rad51; the optimal ratio is one monomer of Rad51 per 3.6 nucleotides of ssDNA (Fig. 3A). Neither joint molecules nor nicked circular dsDNA is formed until the amount of Rad51 reaches a minimal concentration, following which the yield of joint molecules and nicked circular dsDNA rises sharply to a maximum value. Quite strikingly, halving or doubling this ratio virtually eliminates the reactions.

The reactions promoted by Rad51 are strongly stimulated by the addition of yeast ssDNA-binding protein, RPA (Fig. 3B). Omission of RPA reduced the amount of joint molecule formed from 40 to about 5%; however, no nicked circular dsDNA was detected without RPA protein (data not shown). *E. coli* ssDNA-binding protein (SSB) can substitute for RPA but is about half as effective. The optimal molar ratio of RPA to ssDNA (expressed as nucleotides) is about 30 and for SSB is about 10 (Fig. 3B).

The reactions promoted by Rad51 require $Mg^{2+}$. The reactions were most efficient when the Rad51-ssDNA mixture was preincubated with the single-stranded binding protein at 2 to 4 mM $Mg^{2+}$ and then the concentration of $Mg^{2+}$ was increased

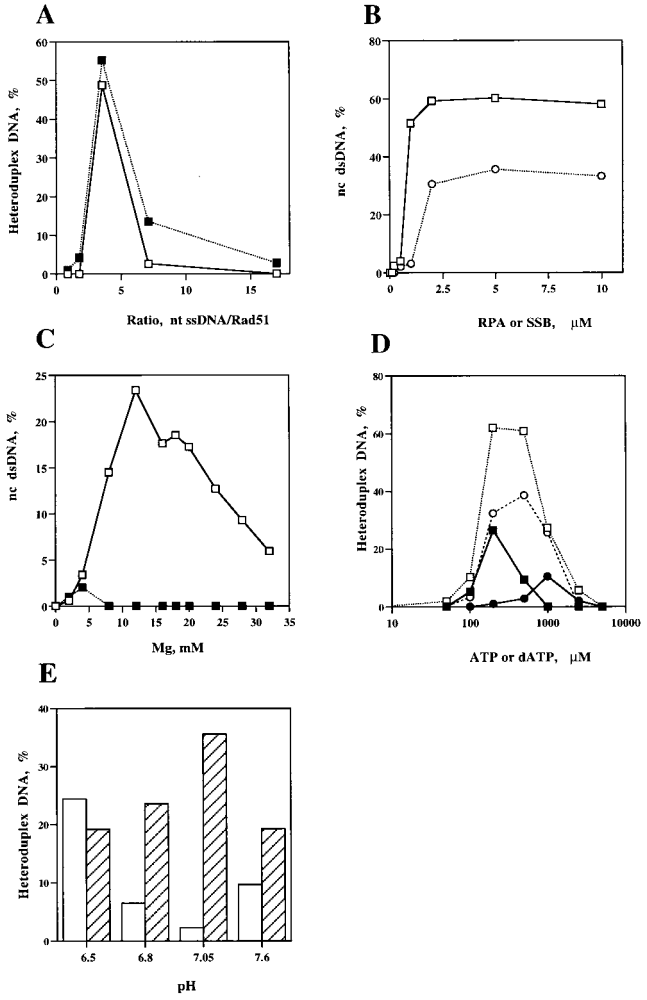


FIG. 3. Requirements for joint molecule formation and strand exchange by Rad51. The reactions were performed as described in Materials and Methods. The products of the reaction were analyzed by agarose gel electrophoresis followed by image analysis. (A) Requirement for stoichiometric amounts of Rad51. A total of 20 μM ϕX174 positive-strand ssDNA was preincubated with various amounts of Rad51. After the addition of 0.7 μM RPA, the reactions were started by the addition of linear dsDNA and incubated for 2 h at 37°C. Measurements of strand exchange (□), in which nicked circular dsDNA is the principal product, were done with ϕX174 DNA substrates, while M13mp18 DNA substrates were used to measure the formation of joint molecules (■). (B) Requirement for ssDNA-binding protein for strand exchange. A total of 20 μM ϕX174 $^{32}$P-5′-labeled linear dsDNA with 3′ overhanging ends was incubated with 5.6 μM Rad51 and different amounts of yeast RPA (□) or *E. coli* SSB (○). The reactions were initiated by the addition of ϕX174 linear dsDNA and incubated for 2 h at 37°C. (C) Dependence on $Mg^{2+}$ concentration. The effect of $Mg^{2+}$ concentration on strand exchange was determined by two different protocols. In the first, ϕX174 positive-strand ssDNA was preincubated with Rad51 and SSB with different concentrations of $MgCl_2$, and the reaction was started by the addition of linear dsDNA (■). In the second, ϕX174 positive-strand ssDNA, Rad51, and SSB were preincubated with 2 mM $MgCl_2$, and the reaction was started by the simultaneous addition of ϕX174 linear dsDNA and different amounts of $MgCl_2$ (□). After 2 h of incubation, the amount of nicked circular dsDNA was determined. (D) Dependence on the concentration of ATP or dATP. Strand exchange (■ and ●) and joint molecule formation (□ and ○) were carried out in the presence of SSB and different amounts of ATP (■ and □) or dATP (● and ○) for 2 h. (E) Dependence on pH. Measurement of strand exchange (□) and joint molecule formation (▨) in the presence of SSB was performed for 2 h in four different buffer systems: 40 mM MES-KOH (pH 6.5), 40 mM MOPS (morpholinepropanesulfonic acid)-KOH (pH 6.8), 20 mM HEPES-KOH (pH 7.05), and 20 mM Tris-HCl (pH 7.6).

following the addition of dsDNA: the optimum concentration of $Mg^{2+}$ in the final reaction mixture is 10 to 20 mM (Fig. 3C). Preincubation of the ssDNA and Rad51 with SSB at $Mg^{2+}$ concentrations higher than 4 mM is inhibitory.

*E. coli* RecA-dependent homologous pairing requires the presence of nucleotide cofactors. Homologous pairing and strand exchange mediated by Rad51 also require ATP; dATP also serves, albeit not as effectively (Fig. 3D). The formation of joint molecules and nicked circular dsDNA was maximal at concentrations of 0.2 to 0.5 mM ATP. The optimum with dATP was somewhat higher (0.5 to 1 mM). Increasing concentrations of the nucleotides up to 5 mM decreased the yield of joint molecules and nicked circular dsDNA to 1 to 2%. Whereas *E. coli* RecA actively hydrolyzes ATP during the strand exchange and requires the use of an ATP-regenerating system to prevent inhibition by ADP, Rad51 promotes strand exchange at a relatively low concentration of ATP, and an ATP-regenerating system is unnecessary even after 2 h of incubation.

Rad51 promotes strand exchange most effectively at pH 6.5; at pHs 6.8, 7.05, and 7.6, the efficiency of complete strand exchange is reduced to about 20, 10, and 30%, respectively (Fig. 3E). However, the yield of joint molecules is relatively unchanged over the same pH range. Irrespective of the pH, the requirement for preincubation with 2 mM $Mg^{2+}$ and the need for higher levels afterward remain.

**Strand exchange depends on the type of terminus of linear duplex DNA.** RecA can utilize linear duplex DNA with any type of terminus (3′ or 5′ overhanging or blunt), although there is some preference for duplex DNA with 3′ overhanging ends (35, 36). In Sung's studies of Rad51-mediated strand exchange (38–40), the linear dsDNA had 3′ overhanging termini. To determine if that was an exclusive requirement, we measured joint molecule formation and strand exchange with linear duplex DNAs having different kinds of termini. Linear dsDNAs with different termini were generated by cleavage of viral RFI dsDNAs with different restriction endonucleases. *Pst*I endonuclease was used to generate 3′ overhangs, *Bam*HI and *Xho*I endonucleases created 5′ overhangs, and *Hin*cII, *Stu*I, and *Ssp*I endonucleases produced blunt termini.

Rad51-promoted joint molecule formation and strand exchange were assayed with dsDNA with either 3′ or 5′ overhangs (4 nucleotides) or blunt termini (Fig. 4A and B). The results show that Rad51 promotes joint molecule formation and strand exchange with dsDNA with 3′ overhanging termini about three to four times more efficiently than with dsDNA with 5′ overhangs (Fig. 4A and B; compare lanes 2 and 3 and lanes 7 and 8). No joint molecules or strand exchange was observed when the dsDNA had blunt ends (Fig. 4A, lanes 5, 6, 10, and 11, and B, lanes 6 and 11). The trace of product in the reaction with linear dsDNA with blunt termini (Fig. 4A, lanes 5 and 10) can be explained by a small amount of contaminating exonuclease in the commercial *Stu*I endonuclease preparation. To determine if this result reflects the type of terminus and not its sequence, linear dsDNAs with blunt ends were made by using T4 DNA polymerase to fill in a 5′ overhang or to remove ssDNA from a 3′ overhang. Filling in a 5′ overhang or removing ssDNA from a 3′ overhang eliminated joint molecule formation and strand exchange by Rad51 (Fig. 4A, compare lanes 2 and 4; B, compare lanes 2 and 4 and lanes 3 and 5). The same dependence on the type of terminus was found with either RPA or SSB. These data indicate that the reactions promoted by Rad51 require that the linear dsDNA must have single-strand ends and that the formation of joint molecules and strand exchange are more efficient with linear dsDNA having a 3′ overhang than substrates with a 5′ overhang.

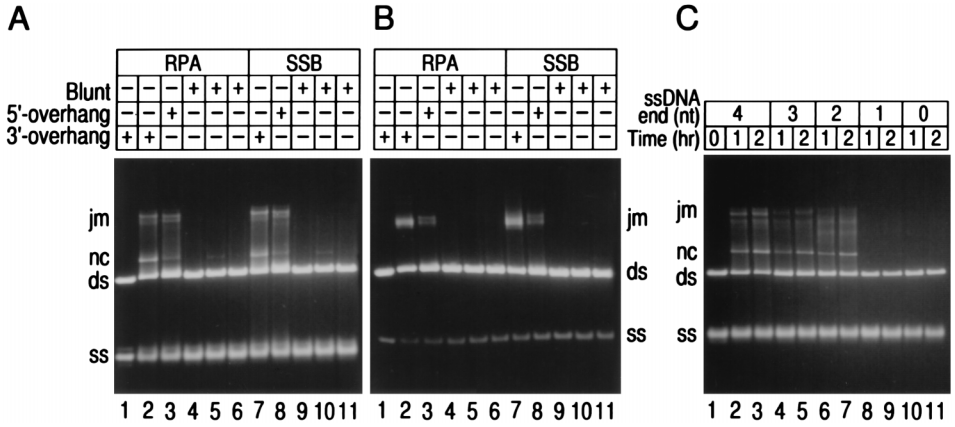


FIG. 4. Rad51 promotes strand exchange and joint molecule formation only with linear dsDNA having 5′ or 3′ overhanging termini. ϕX174 positive-strand ssDNA or M13mp18 ssDNA was preincubated with Rad51 and RPA, and the reaction was initiated by the addition of the linear dsDNAs with different termini. After 2 h, the products of the reaction were analyzed by agarose gel electrophoresis followed by ethidium bromide staining. (A) Strand exchange with ϕX174 linear dsDNAs with different types of termini. Lane 1, complete reaction mixture without Rad51; lanes 2 and 7, linear ϕX174 dsDNA with 3′ overhanging termini; lanes 3 and 8, linear dsDNA with 5′ overhanging termini; lanes 4 and 9, dsDNA containing blunt ends generated by removing the 3′ overhanging termini with T4 DNA polymerase; lanes 5 and 10, linear dsDNA with blunt ends generated by digestion with *Stu*I endonuclease; lanes 6 and 11, linear dsDNA with blunt ends generated by *Ssp*I endonuclease. (B) Joint molecule formation with linear M13mp18 dsDNAs having different types of termini. Lane 1, complete reaction mixture without Rad51; lanes 2 and 7, linear dsDNA with 3′ overhanging termini; lanes 3 and 8, linear dsDNA with 5′ overhanging termini; lanes 4 and 9, linear dsDNA with blunt ends generated by removing 3′ overhanging termini with T4 DNA polymerase; lanes 5 and 10, linear dsDNA with blunt ends generated by filling in 5′ overhanging termini with T4 DNA polymerase; lanes 6 and 11, linear dsDNA with blunt ends generated by *Hin*cII endonuclease. (C) Strand exchange with 3′ termini of different lengths of single-stranded overhangs. Lanes 1 to 3, linear ϕX174 dsDNA with 4-nucleotide ssDNA ends; lanes 4 and 5, with 3 nucleotides; lanes 6 and 7, with 2 nucleotides; lanes 8 and 9, with 1 nucleotide; lanes 10 and 11, with blunt ends. Abbreviations: nt, nucleotide; jm, joint molecules; nc, nicked circular dsDNA; ds, linear dsDNA; ss, circular ssDNA.

To determine the minimum number of nucleotides in the single-strand overhang that is needed for the reactions, we generated linear dsDNAs with 4-, 3-, 2-, 1-, and 0-nucleotide 3′ overhanging termini. For that purpose, ϕX174 dsDNA was cleaved with *Pst*I, *Dra*III, *Ksp*I, *Ahd*I, and *Fsp*I endonucleases, respectively. The results in Fig. 4C show that Rad51 promotes strand exchange about equally efficiently with termini of 2 to 4 nucleotides, but there is no reaction with a single-nucleotide overhang or a blunt end.

**Rad51 forms joint molecules only with linear dsDNA whose single-strand overhang is complementary to the circular ssDNA.** The results cited above indicate that joint molecule formation and strand exchange mediated by Rad51 require a single-strand overhang at either the 3′ or the 5′ end of linear dsDNA, but with a preference for the 3′ overhang. Accordingly, we sought to determine if joint molecule formation is initiated from the 3′ or the 5′ end of the linear dsDNA. To do this, linear duplex DNAs with different types of termini were prepared as well as ones with homology at only one end of the negative strand (see Materials and Methods) (Fig. 5A).

Figure 5B summarizes the extent of joint molecule formation with linear dsDNAs having the kinds of termini shown in Fig. 5A. First, note that no joint molecules were formed when both ends of the linear dsDNA were heterologous with respect to the circular ssDNA (Fig. 5B, lane 16). Furthermore, the results show clearly that Rad51 formed joint molecules only with linear dsDNA whose 3′ overhanging terminus was complementary to the single-strand circle (Fig. 5B, lane 2). No joint molecules were detected with linear dsDNA whose complementary strand's 3′ terminus was recessed (Fig. 5B, lane 10). Similarly, when the homology was at the 5′ end of the negative strand, joint molecules were formed only when there were 5′ overhanging ends (Fig. 5B, lane 12). Furthermore, no joint molecules were detected if the linear dsDNA had complementary but 5′ recessed termini (Fig. 5B, lane 4). These data indicate that Rad51 can form joint molecules from either the 3′ or the 5′ termini of the linear duplex DNA but only if the overhanging ends are complementary to the circular ssDNA.

To examine this point further, we cut the linear dsDNA again so that the ends complementary to the circular ssDNA were changed from recessed to overhanging and from overhanging to recessed. Thus, linear dsDNAs whose complementary but recessed 3′ ends failed to react (Fig. 5B, lane 10) became effective in promoting joint molecule formation (Fig. 5B, lane 14), when the linear dsDNA was cut by *Pst*I so as to generate an overhanging 3′ complementary end. The same type of activation of previously inactive linear dsDNA whose homologous negative-strand 5′ end was recessed (Fig. 5B, lane 4) occurred after a second cut by *Eco*RI created an overhanging 5′ complementary end (Fig. 5B, lane 8). There was also a significant decrease in the yield of joint molecules when the negative strand's homologous 3′ overhanging end was changed to a recessed terminus by *Hin*dIII cleavage (compare Fig. 5B, lanes 2 and 6); the low level of the joint molecule formation remaining is the result of the incomplete removal of the 3′ overhanging ends by the second cleavage.

**The polarity of Rad51-promoted strand exchange depends on the kinds of ends on the complementary strand of the linear dsDNA.** Our findings indicate that the formation of joint molecules between a circular ssDNA and a linear dsDNA occurs via the complementary overhanging 3′ or 5′ end. Once initiated by pairing of the complementary strands, complete strand exchange is presumed to occur by branch migration. Therefore, the polarity of the strand exchange will depend on which end of the complementary strand is overhanging (Fig. 6A). If the linear dsDNA's complementary strand has a 5′ overhang, the polarity of strand exchange will be in the 3′-to-5′ direction with respect to the ssDNA, and if the linear dsDNA's complementary strand has a 3′ overhang, branch migration will be in the opposite direction. To examine these predictions, we studied the complete strand exchange reaction with linear ϕX174 dsDNA with either a 5′ (cut with *Xho*I) or a 3′ (cut with *Pst*I)

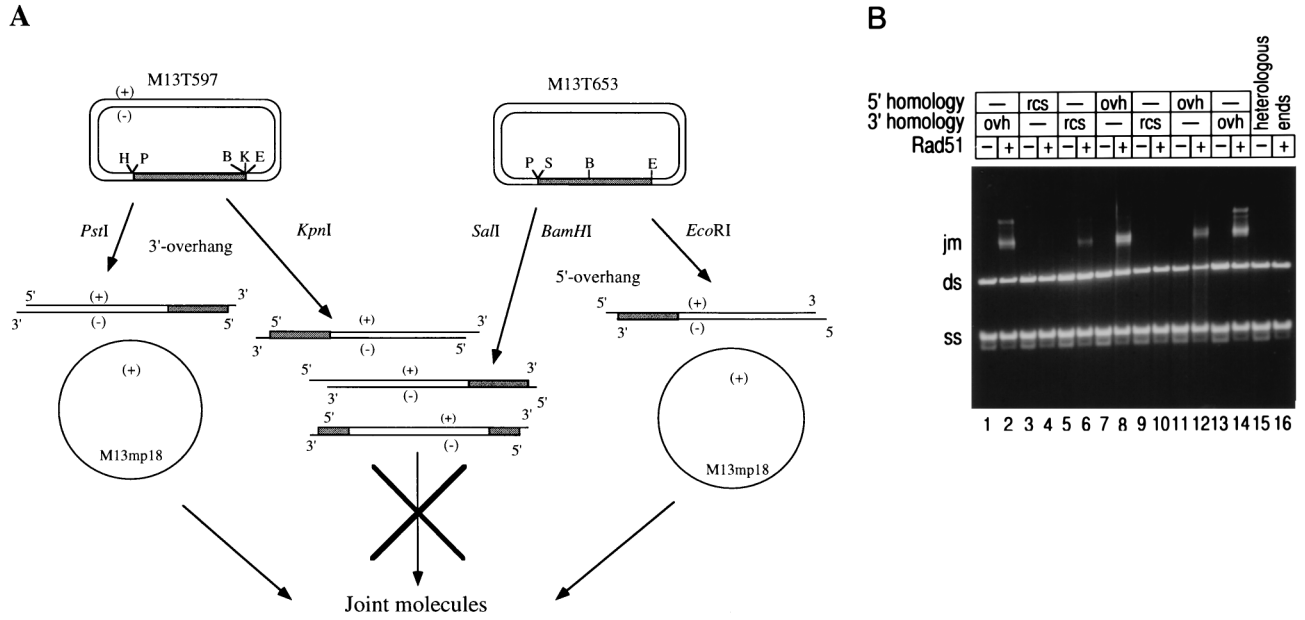


FIG. 5. Rad51 initiates formation of joint molecules only at the overhanging ends of the linear dsDNA's complementary negative strand. (A) The different linear dsDNAs with the negative strand having homology at one end and heterology at the other and different types of termini were prepared from M13 dsDNA having an insertion of pBR322 DNA (filled-in segment). Abbreviations: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I, S, *Sal*I. (B) The viral M13mp18 positive-strand ssDNA was incubated with Rad51 and RPA. After addition of the linear dsDNA, the incubation was continued for 2 h. The formation of joint molecules was detected as described in Materials and Methods. Lanes 1 and 2, linear dsDNA whose negative strand has a homologous 3′ overhanging end, with and without Rad51; lanes 3 and 4, the linear dsDNA's negative strand having a homologous 5′ recessed end, with and without Rad51; lanes 5 and 6, the homologous 3′ overhanging end of the linear dsDNA's negative strand (lane 2) changed to a 3′ recessed terminus by an additional cleavage; lanes 7 and 8, the homologous 5′ recessed end of the linear dsDNA's negative strand (lane 4) changed to a 5′ overhanging terminus by an additional cleavage; lanes 9 and 10, the linear dsDNA's negative strand with homology at the 3′ recessed end; lanes 11 and 12, the linear dsDNA's negative strand with homology at the 5′ overhanging end; lanes 13 and 14, the homologous 3′ recessed end of the linear dsDNA's negative strand (lane 10) changed to a 3′ overhanging end by the additional cleavage; lanes 15 and 16, linear dsDNA with the negative strand having both heterologous 5′ overhanging and 3′ recessed ends. Abbreviations: ovh, complementary overhanging end; rcs, complementary recessed end; ss, circular ssDNA; ds, linear dsDNA; jm, joint molecules.

overhanging end and a blunt end at the other (see Materials and Methods and Fig. 6A). If the joint molecule is initiated at either of the complementary overhanging ends (blunt ends are inactive) and branch migration can proceed in either direction, the final product in each case will be a circular duplex DNA with a gap.

Figure 6B, lane 3, shows that Rad51 promotes complete strand exchange with linear ϕX174 dsDNA containing 5′ overhanging ends. If the 5′ complementary overhanging end is removed by cleavage with *Bss*HII (Fig. 6A), the strand exchange is prevented (Fig. 6B; compare lanes 3 and 5). By contrast, if the same *Xho*I-cut linear dsDNA is then cut at the *Bsr*BI site, there is no effect on the formation of circular dsDNA (Fig. 6B, lane 7). These data show that, when strand exchange is initiated at the 5′ overhanging end, branch migration must proceed in the 3′-to-5′ direction with respect to the ssDNA. Similarly, if the linear ϕX174 dsDNA with a 3′ complementary overhanging end is cut at the *Bss*HII site, the complementary 3′ overhanging end remains and strand exchange is unaffected (Fig. 6C; compare lanes 3 and 5). In this case, strand exchange was initiated at the 3′ end and proceeded 5′ to 3′ on the ssDNA. However, removal of the 3′ complementary overhanging end by cleavage with *Fsp*I endonuclease prevents strand exchange from occurring (Fig. 6C; compare lanes 3 and 7).

To verify that Rad51 initiates strand exchange only from the complementary overhanging end of the linear dsDNA, we prepared linear ϕX174 dsDNAs with both 3′ and 5′ recessed complementary ends by successive cleavages with *Pst*I and *Xho*I endonucleases as well as linear dsDNAs with both 3′ and 5′ overhanging complementary ends by using cleavages with *Pst*I and *Bss*HII endonucleases. Rad51 promoted efficient strand exchange reaction with linear dsDNA having complementary overhangs at both ends (Fig. 6D, lanes 2 and 3), but no circular dsDNA was detected when both of the complementary ends were recessed (Fig. 6D, lanes 5 and 6).

The preferred polarity of RecA-promoted strand exchange is presumed to be driven by the polymerization-driven binding of RecA protein in the 5′-to-3′ direction on the displaced single strand; support for this view stems from the observation that degradation of the displaced strand by a single-strand specific exonuclease eliminated the preferred polarity (18). Because Rad51 promoted strand exchange in either direction, we sought to determine if this effect was due to the presence of single-strand exonuclease in our Rad51 preparation. As already mentioned, the Rad51-promoted strand exchange involving a circular ssDNA and a linear dsDNA with 3′ or 5′ overhanging ends is accompanied by the accumulation of the displaced single strand (Fig. 2C). Thus, the potential bidirectional nature of the Rad51-promoted strand exchange is not due to exonuclease degradation of the displaced strand.

Taken together, these results indicate that joint molecules are initiated only if the complementary strand of the linear dsDNA is single and that such joint molecules are then resolved to fully strand-exchanged product by branch migration in either direction. Therefore, the polarity of strand exchange promoted by Rad51 depends on which end of the complementary strand is used to initiate the formation of joint molecules.

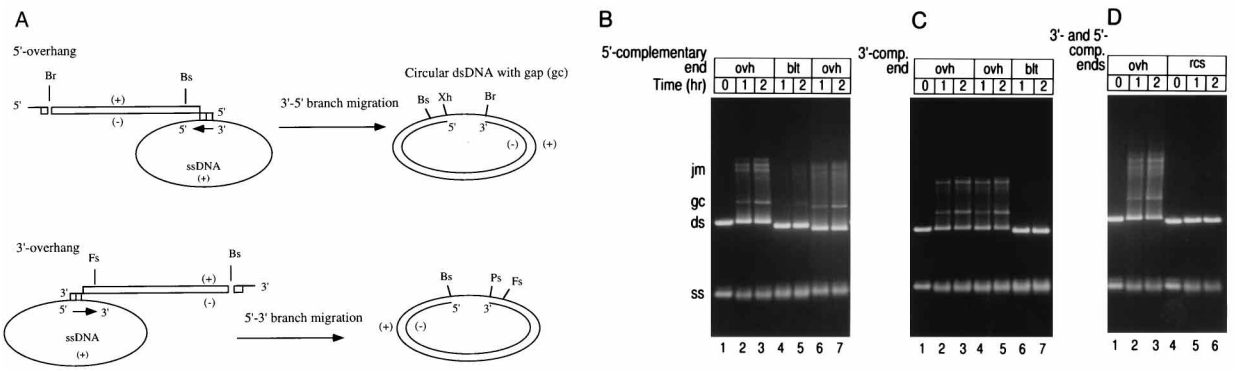


FIG. 6. The direction of branch migration promoted by Rad51 depends on which end of the linear dsDNA's complementary strand is used to initiate joint molecule formation. (A) The different linear dsDNAs with an overhang at one end and blunt at the other were prepared from ϕX174 dsDNA as described in Materials and Methods. Joint molecules between the ϕX174 positive ssDNA and the linear dsDNA's negative strand's overhanging ends are then processed by unidirectional branch migration to produce the gapped circular dsDNA. Branch migration proceeds in the 3′-to-5′ direction with respect to the ssDNA if the linear dsDNA's negative strand has a 5′ overhanging end and in the opposite direction with linear dsDNAs having a negative strand with a 3′ overhanging end. The reactions were performed as described in Materials and Methods. Abbreviations: Br, *Bsr*BI; Bs, *Bss*HII; Fs, *Fsp*I; Xh, *Xho*I; Ps, *Pst*I. (B) Strand exchange reaction in the 3′-to-5′ direction with respect to the ssDNA. Lanes 1 to 3, linear dsDNA's negative strand with 5′ overhanging and 3′ recessed ends; lanes 4 and 5, linear dsDNA whose negative strand has 5′ blunt (*Bss*HII) and 3′ recessed (*Xho*I) ends; lanes 6 and 7, linear dsDNA whose negative strand contains 5′ overhanging (*Xho*I) and 3′ blunt (*Bsr*BI) ends. (C) Strand exchange in the 5′-to-3′ direction with respect to the ssDNA. Lanes 1 to 3, linear dsDNA whose negative strand has 5′ recessed and 3′ overhanging ends prepared by *Pst*I endonuclease cleavage; lanes 4 and 5, linear dsDNA's negative strand with 5′ blunt (*Bss*HII) and 3′ overhanging (*Pst*I) ends; lanes 6 and 7, linear dsDNA whose negative strand has 5′ recessed (*Pst*I) and 3′ blunt (*Fsp*I) ends. (D) Failure to use linear dsDNA whose negative strand ends are both recessed. Lanes 1 to 3, linear dsDNA's negative strand with both overhanging ends; lanes 4 to 6, linear dsDNA's negative strand with both recessed ends. Abbreviations: ovh, overhanging negative end; rcs, recessed negative end; ss, circular ssDNA; ds, linear dsDNA; gc, circular dsDNA with gap; jm, joint molecules, blt, blunt.

## DISCUSSION

Apropos of Rad51's implied role in recombinational repair of double-strand breaks in DNA, we examined its activity in promoting the strand exchange reaction first described by Sung (38). Rad51 was expressed in insect cells infected with baculovirus and purified extensively through several steps from extracts of the infected cells (Fig. 1). Rad51 promoted limited strand exchange with M13 circular ssDNA and M13 linear dsDNA, the products being joint molecules and little or none of the completely exchanged product, nicked double-stranded circles. With ϕX174 circular single-strand and linear duplex DNA substrates, the reaction yielded fully exchanged product. The reason for this difference is unknown and was not investigated further.

Our studies show that the formation of joint molecules can be initiated only if the linear dsDNA contains an overhanging single strand complementary to the circular ssDNA. Indeed, Rad51 can initiate joint molecule formation most efficiently with a complementary 3′ overhanging end, less efficiently with a complementary 5′ overhanging end, and not at all if the dsDNA ends are blunt or if the complementary 3′ or 5′ ends are recessed. Noncomplementary overhanging 3′ or 5′ ends fail to initiate joint molecule formation. The minimal length of a complementary overhanging end for initiating joint molecule formation is 2 nucleotides, indicating that that length of heteroduplex formation is sufficient to permit the ensuing branch migration and complete strand exchange.

Given that Rad51 forms joint molecules initiating at either a complementary 3′ or 5′ overhanging single strand, the formation of nicked double-stranded circular DNA as the product implies that branch migration can proceed in either direction. Thus, when initiation occurs with a 3′ end of the dsDNA, branch migration proceeds in the 5′-to-3′ direction with respect to the ssDNA's polarity, and if joint molecule formation is initiated by the 5′ end of the dsDNA, branch migration is in the opposite direction. Sung et al. have reported previously (39, 40) that Rad51 promotes strand exchange in a direction opposite to RecA. Their work suggested that strand exchange is initiated exclusively by the 5′ terminus of the linear dsDNA's complementary strand and, therefore, proceeds in only the 3′-to-5′ direction relative to the ssDNA. In those experiments, the polarity of strand exchange was studied exclusively with dsDNA having complementary 5′ overhangs (created by *Bss*HII endonuclease cleavage). This experimental design obscured the fact that joint molecules can be initiated at either a 5′ or a 3′ end of the complementary strand and that branch migration can proceed in either direction.

In commenting on our results, it is useful to compare features of the Rad51-promoted strand exchange reaction with the same overall reaction carried out by the better-known bacterial protein, RecA, with which Rad51 has extensive sequence homology.

**(i) Mg and ATP requirements.** $Mg^{2+}$ and ATP are necessary components of strand exchange by both RecA (8) and Rad51. However, the optimal amounts of these for the Rad51-mediated reaction differed somewhat from those with RecA. The optimal $Mg^{2+}$ concentration for strand exchange by RecA is about 6 to 12 mM. At low $Mg^{2+}$ (less than 2 mM), RecA binds weakly to ssDNA and cannot displace SSB from ssDNA to form the active filament (19, 20). In the Rad51-mediated strand exchange reaction, we found two different optima for $Mg^{2+}$ (Fig. 3C); Rad51 seems to form an active filament with ssDNA in the presence of SSB only at low $Mg^{2+}$ (2 to 4 mM), perhaps because Rad51's higher affinity for ssDNA at low $Mg^{2+}$ allows it to displace SSB from the ssDNA. However, the actual formation of the joint molecule and subsequent branch migration require a higher concentration of $Mg^{2+}$ (12 to 20 mM). Different optima for the two stages of strand exchange have also been noted with a mutant, RecA K72R (32).

The role of ATP in RecA-promoted strand exchange reaction is the subject of considerable discussion in the literature (11, 17, 24, 30). Compared with RecA, Rad51 has a very low ATPase activity (38). Rad51 (40), like RecA (24), can promote strand exchange in the presence of the nonhydrolyzable analog ATPγS. In our assays, Rad51 promotes strand exchange at a lower concentration of ATP than does RecA (Fig. 3D). The

optimum concentration of ATP for Rad51-promoted strand exchange is 200 to 500 μM; dATP can substitute for ATP but is only half as effective. We have obtained preliminary data indicating that ATP promotes the formation of an active Rad51 conformation which is essential for binding with ssDNA.

**(ii) Stoichiometry with respect to ssDNA and SSBs.** Sung and Robberson (39) demonstrated that preincubation of stoichiometric amounts of Rad51 and ssDNA in the presence of yeast RPA leads to efficient strand exchange with dsDNA. We estimate that the initiation of joint molecules as well as the complete strand exchange reaction is optimal with a ratio of one Rad51 monomer per 3.6 nucleotides of ssDNA and that exceeding that ratio even slightly inhibits joint molecule formation. We suspect that, when the amount of Rad51 exceeds the amount required for saturating ssDNA, free Rad51 binds to the linear dsDNA and prevents the formation of joint molecules. Perhaps, as is the case with RecA (10, 22, 42), Rad51 has at least two DNA binding sites: one for binding to ssDNA to form the ssDNA-protein filament and a second site for binding to dsDNA. That property could facilitate the homology search which enables the two DNAs to initiate joint molecule formation.

Rad51-promoted joint molecule formation and complete strand exchange are stimulated almost equally well by yeast RPA and by *E. coli* SSB. This suggests that specific interaction between Rad51 and RPA is not an essential feature of the strand exchange reaction. The optimal ratio for SSB in the strand exchange reaction (one SSB per 10 nucleotides) is about the same as the size of the SSB's binding site on ssDNA (29). Previous studies reported that the size of the RPA binding site is 90 to 100 nucleotides of ssDNA (2), but our data based on the optimum ratio of RPA to ssDNA suggest a binding site size of 30 nucleotides, similar to that found for human RPA (6, 16). The same optimal ratio for SSB and RPA in the strand exchange reaction promoted by Rad51 purified from yeast has been recently reported (37). The fact that Rad51's ssDNA-dependent ATPase activity is stimulated by RPA but is independent of RPA if the ssDNA is devoid of secondary structure (37) suggests that the principal contribution of RPA and SSB to promoting strand exchange is to eliminate secondary structures in the ssDNA (19, 20, 37).

**(iii) Requirement of Rad51 for complementary overhanging ends and lack of activity with blunt ends.** RecA has the unique ability to unwind duplex DNA and to promote heteroduplex formation with complementary ssDNA. This property of RecA is reflected in its ability to promote joint molecule formation between circular ssDNA and linear dsDNA having any type of terminus. Rad51, like RecA, appears to be able to form joint molecules and effect complete strand exchange if the linear dsDNA has overhanging ends, with a preference for 3′ overhanging termini (35, 36). However, unlike RecA, Rad51 cannot initiate joint molecules with dsDNA having blunt ends (Fig. 4A and B).

Rad51 initiates joint molecules from either 3′ or 5′ ends of linear dsDNA's complementary strand, but only if the ends of the complementary strand are overhanging (Fig. 5). However, dsDNA whose complementary strand's termini are recessed cannot be used by Rad51. It is quite striking that an overhanging end with as few as 2 nucleotides is sufficient to promote joint molecule formation and strand exchange (Fig. 4C).

Thus, our data show that Rad51 alone cannot melt a blunt or complementary strand's recessed terminus to initiate joint molecule formation. Rather, Rad51 must first form an initial heteroduplex between a complementary single-strand end and the ssDNA which can then be extended by branch migration.

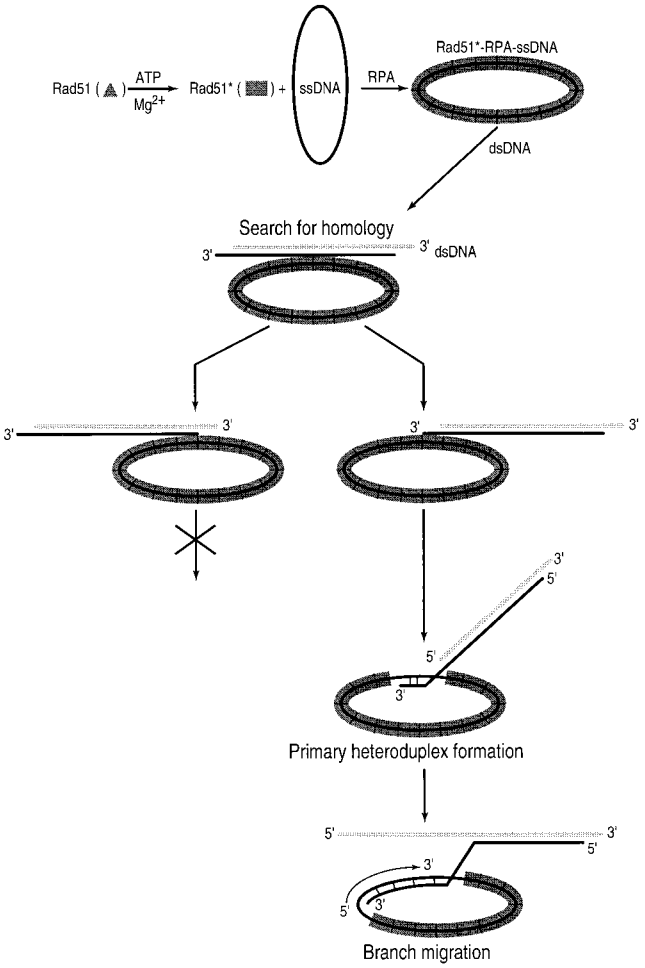


FIG. 7. Model for the mechanism of strand exchange promoted by Rad51. See text for details.

Studies with SepI (13, 14) indicate that 2 nucleotides are too small for a simple noncatalytic annealing-extension mechanism; indeed, strand exchange promoted by SepI proceeds only when ssDNA tails are on the order of 20 nucleotides long.

**(iv) The direction of branch migration in Rad51-mediated strand exchange depends only on whether joint molecule formation is initiated via the 3′ or the 5′ end of the complementary strand.** RecA-mediated DNA strand exchange in the presence of ATP is unidirectional and slow, proceeding in the 5′-to-3′ direction with respect to the ssDNA (4, 7). However, in the presence of ATPγS, RecA resembles the activity of mutant RecA K72R in the presence of dATP; the reactions proceed bidirectionally and is incapable of bypassing heterologous structural barriers in the duplex DNA (11, 32). Sung et al. (39, 40) have recently reported that the Rad51-promoted strand exchange proceeds only in the 3′-to-5′ direction along the ssDNA. Our data establish, however, that Rad51 can initiate joint molecule formation from either a complementary 3′ or a 5′ overhanging end, that such joint molecules proceed to complete strand exchange, and that branch migration can proceed in either direction. Thus, the polarity of strand exchange promoted by Rad51 depends upon which end, 3′ or 5′, is used to initiate joint molecule formation (Fig. 6B and C). Understanding the nature of the lack of specific polarity requires more detailed study.

**Mechanism of Rad51-promoted strand exchange in vitro.** Our working model to explain how Rad51 promotes strand exchange between circular ssDNA and linear dsDNA in vitro is illustrated in Fig. 7. As-yet-unpublished experiments indicate that Rad51 is activated in the presence of ATP and $Mg^{2+}$ and that this form is able to displace RPA or SSB bound to the single-stranded circular DNA to form a Rad51-ssDNA filament. Whether the complex is comparable or identical to the RAD51-ssDNA filament described by Ogawa and coworkers (34) and by West and coworkers for the human Rad51-ssDNA filament (5) is not known. We surmise, further, that the Rad51-ssDNA filament interacts transiently, and in a sequence-independent way, with dsDNA to initiate a one-dimensional search for a homologous single-strand end. Finding such a complementary 3′ or 5′ single-strand end leads to the formation of a primary short heteroduplex (2 bp) only with an overhanging end of the complementary strand; this joint molecule is readily extended either by spontaneous or by Rad51-promoted branch migration to yield the fully strand-exchanged product. Whether spontaneous or Rad51 promoted, branch migration can proceed in either of two directions depending on whether the initial pairing is with a 3′ or a 5′ end.

**Is Rad51 an incomplete version of RecA requiring association with other proteins to perform efficient, specific, and unidirectional strand exchange?** Rad51 clearly possesses functional similarity to *E. coli* RecA but lacks some of RecA's attributes that might be essential for a role in recombination. Inasmuch as Rad51 functions in recombinational repair of double-strand breaks as part of a multiprotein complex (9, 12, 15), it is reasonable to wonder whether other proteins in the complex modulate Rad51's activity: for example, to enable Rad51 to initiate joint molecule formation with linear dsDNAs with blunt or recessed 3′ ends and thereby to promote branch migration in the same direction as RecA.

To date, we have been unable to detect Rad51-mediated D-loop formation in the interaction of ssDNA and a homologous double-stranded circular DNA, an activity that RecA is known to promote (23, 33). Here, too, we are led to wonder whether Rad51, in association with one or more proteins, is able to promote the invasion of a complementary 3′ end of a single strand into duplex DNA to produce a heteroduplex and D loop. Such an activity would provide the primer template for extension of the 3′ end and elongation of the heteroduplex, both of which are implicit in most models of homologous recombination (25, 41).

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