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On the Role of ATP Hydrolysis in RecA Protein-mediated DNA Strand Exchange

III. UNIDIRECTIONAL BRANCH MIGRATION AND EXTENSIVE HYBRID DNA FORMATION*

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We have identified two functions for RecA-mediated ATP hydrolysis during DNA strand exchange. First, ATP hydrolysis renders RecA protein-mediated DNA strand exchange unidirectional (5′ to 3′ with respect to the single-stranded DNA). In the presence of a nonhydrolyzable analog adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), DNA strand exchange is bidirectional. Second, ATP hydrolysis is required for extensive formation of hybrid DNA. In the presence of ATP hydrolysis, the length of the exchanged region is limited only by the available homology, whereas in the absence of ATP hydrolysis, only 2 kilobase pairs or less of hybrid DNA are formed before branch migration is blocked in the majority of paired intermediates. Both of these functions of RecA protein-mediated ATP hydrolysis are crucial in ensuring the effectiveness of recombinational DNA repair, especially when the lesion to be repaired is distant from the initial crossover point.

The RecA protein is a central component in recombinational DNA repair pathways and homologous genetic recombination in *Escherichia coli*. *In vitro*, RecA protein promotes the pairing and exchange of complementary DNA strands in reactions that provide an important experimental paradigm for RecA function *in vivo*. A variety of DNA substrates are used in these reactions, including circular single-stranded DNA (ssDNA)[1] and linear duplex DNA, and gapped circular duplex DNA and linear duplex DNA. In each case, one of the DNA substrates is at least partially single-stranded. The ssDNA is the site where RecA protein filament formation nucleates and where initiation of DNA strand exchange takes place. ssDNA is highly recombinogenic *in vivo* and may be the primary signal for SOS induction of the DNA repair genes (for review, see Cox, 1993; Kowalczykowski, 1991; Radding, 1991; Roca and Cox, 1990; West, 1992).

The RecA-mediated DNA strand exchange reaction can be divided into three distinct phases (see reviews cited above). During the first phase, called presynapsis, a stoichiometric amount of RecA protein (one monomer/three nucleotides) assembles in the 5′ to 3′ direction along the phosphate backbone of the ssDNA to produce a right-handed, helical, nucleoprotein filament. In the presence of ATP or ATPγS (an ATP analog that is not readily hydrolyzed by RecA), DNA within RecA filaments is extended and, if it is duplex, underwound. There are 6 RecA monomers and 18 nucleotides or bp of DNA/helical turn of the filament, and the axial rise/bp increases form 3.4 to 5.1 Å. The nucleoprotein filament is the active species of RecA protein in the subsequent phases of the strand exchange reaction. In the second phase, called synapsis, homologous sequences in the ssDNA within the RecA filament and the duplex DNA are aligned. There is evidence for the formation of a novel triple-stranded DNA pairing intermediate in this phase (Camerini-Otero and Hsieh, 1993; Rao *et al.*, 1993; Stasiak, 1992). A strand switch or crossover occurs within the filament to form short regions of DNA in which complementary strands from the two different DNA substrates are paired.

This product DNA has often been referred to as a heteroduplex, whether or not it contains any bp mismatches or structural irregularities, to distinguish it from substrate DNA that is often identical in sequence. In this article, we use the term hybrid DNA to describe product duplexes made up of strands that are completely complementary, and we reserve the term heteroduplex to describe duplexes containing a mismatch or some other structural irregularity.

In the third and final phase of the strand exchange reaction, the region of hybrid or heteroduplex DNA is extended in a kind of facilitated branch migration. DNA pairing intermediates that have not completed DNA strand exchange are often referred to as joint molecules. 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 at a rate of 3–10 bp s^{-1}. The DNA strand exchange reaction promoted by RecA readily bypasses structural barriers in the DNA, including DNA lesions and even short (1–100 bp) heterologous inserts in one of the DNA substrates (Bianchi and Radding, 1983; Das Gupta and Radding, 1982; Jwang and Radding, 1992; Kim *et al.*, 1992a; Livneh and Lehman, 1982; Rosselli and Stasiak, 1991). This latter property is probably critical to the function of RecA in recombinational DNA repair.

RecA protein is a DNA-dependent ATPase, and ATP is hydrolyzed during the strand exchange reaction with a monomer k_{cat} of 20–24 min^{-1} (Schutte and Cox, 1987). ATP is hydrolyzed uniformly by RecA monomers throughout each nucleoprotein filament (Brenner *et al.*, 1987; Kowalczykowski and Krupp, 1987; Schutte and Cox, 1987).

The molecular function of RecA-catalyzed ATP hydrolysis is not understood and is currently a subject of controversy. At least three observations indicate that ATP hydrolysis has only a minimal, if any, function in the DNA strand exchange reaction. First, the reaction is inherently inefficient from a thermo-

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[1] The abbreviations used are: ssDNA, single-stranded DNA; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); bp, base pair(s); kbp, kilobase pair(s); SSB, *E. coli* single-stranded DNA-binding protein; AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen; dsDNA, double-stranded DNA.

dynamic viewpoint. DNA strand exchange between completely homologous DNA substrates is an isoenergetic reaction since there is no net increase or decrease in DNA bp. However, in a typical strand exchange reaction using DNA substrates derived from bacteriophage M13 or ϕX174, 100 or more ATPs are hydrolyzed per bp of hybrid DNA formed (Roca and Cox, 1990). Second, substantial DNA strand exchange can occur in the presence of ATPγS (Kim *et al.*, 1992a; Konforti and Davis, 1992; Menetski *et al.*, 1990; Rosselli and Stasiak, 1990). Under narrowly defined conditions, about 2 kbp or more of hybrid DNA is formed within 2 min of reaction (Menetski *et al.*, 1990). This result has recently been reinforced by the observation that a RecA mutant (RecA K72R) that binds but does not hydrolyze ATP can also promote a limited amount of DNA strand exchange *in vitro* (Rehrauer and Kowalczykowski, 1993). Third, eukaryotic proteins have been found which promote DNA pairing and strand exchange but do not hydrolyze ATP (Eggleston and Kowalczykowski, 1991).

However, RecA-mediated ATP hydrolysis is not readily dismissed as a molecular anomaly. The single ATP binding site in each monomer represents the most highly conserved part of the RecA amino acid sequence (Roca and Cox, 1990). When RecA protein is bound to DNA, the k_{cat} for ATP hydrolysis by each monomer within a filament generally falls into a relatively narrow range of 20–30 min^{-1}. In addition, the RecA K72R mutant exhibits a null phenotype for all RecA activities, providing evidence that ATP hydrolysis is critical to RecA function *in vivo*.[2]

One generally agreed upon function of ATP hydrolysis is the recycling of RecA monomers in nucleoprotein filaments after a DNA strand exchange reaction. Filament assembly and disassembly are end-dependent processes, with disssociation of monomers occurring predominantly at one end (the "–" end, located 5′ with respect to the ssDNA that serves as a nucleation site for filament assembly) (Lindsley and Cox, 1990) and assembly occurring at the other (the "+" end; 3′) (Register and Griffith, 1985). Assembly and disassembly exhibit different equilibria (Lindsley and Cox, 1990), implying an involvement of ATP hydrolysis in one or the other process. ATPγS, which is not hydrolyzed by RecA, blocks disassembly (Lindsley and Cox, 1990), whereas ATP hydrolysis reduces the affinity of RecA protein for DNA (Menetski *et al.*, 1988). Together, these results indicate that ATP hydrolysis by a RecA monomer at the (–) end of a nucleoprotein filament is likely to result in dissociation. Recycling of RecA monomers provides one function for ATP hydrolysis. However, ATP hydrolysis is not limited to or even enhanced in monomers at filament ends, and filament disassembly rarely accounts for more than a minute fraction of ATP hydrolytic events. Much evidence now indicates that filament disassembly does not play a mechanistic role in DNA strand exchange (Cox, 1994; Lindsley and Cox, 1990).

The DNA strand exchange reaction observed in the presence of ATPγS has provided an important new tool to explore the function of ATP hydrolysis by RecA. An examination of the limitations of the ATPγS-mediated reaction has begun to define a role for ATP hydrolysis in DNA strand exchange. These studies have shown that the DNA strand exchange reaction does not bypass structural barriers in the DNA (Kim *et al.*, 1992a; Rosselli and Stasiak, 1991) and does not accommodate four DNA strands (Kim *et al.*, 1992b) when ATPγS replaces ATP. The requirement for ATP hydrolysis in the bypass of DNA structural barriers provides an experimental link between the ATP hydrolytic activity and RecA function in DNA repair.

Another potential function of ATP hydrolysis is to render DNA strand exchange unidirectional. Two recent studies have looked for a relationship between the direction taken by the strand exchange reaction and ATP hydrolysis and have arrived at very different conclusions. Rosselli and Stasiak (1990) found that DNA strand exchange in the presence of ATPγS was unidirectional, exhibiting the same polarity as the ATP reaction. In addition, they found no difference in the efficiency with which paired DNA intermediates were formed with ATP or ATPγS. Konforti and Davis (1992) arrived at the opposite conclusion, finding no evidence for a prevalent direction in the strand exchange reaction carried out with ATPγS. The present study was carried out to resolve this discrepancy and further explore the function of ATP hydrolysis by RecA protein. We provide evidence that ATP hydrolysis is required to render the DNA strand exchange reaction unidirectional. We further demonstrate that ATP hydrolysis has a substantial effect on the lengths of hybrid DNA formed during DNA strand exchange.

MATERIALS AND METHODS

Enzymes and Reagents—*E. coli* RecA protein was purified to homogeneity and stored as described previously (Cox *et al.*, 1981). *E. coli* ssDNA-binding protein (SSB) was purified as described by Lohman *et al.* (1986), except that an additional step utilizing DEAE-Sepharose chromatography was included to ensure removal of single-strand exonucleases. The RecA protein and SSB concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\epsilon_{280} = 0.59\,A_{280}$ mg^{-1} ml (Craig and Roberts, 1981) and $\epsilon_{280} = 1.5\,A_{280}$ mg^{-1} ml (Lohman and Overman, 1985), respectively. Restriction endonucleases were purchased from New England Biolabs. Tris buffer and ATPγS were purchased from Boehringer Mannheim. DEAE-Sepharose was purchased from Pharmacia Biotech. Inc. Creatine phosphokinase, phosphocreatine, proteinase K, low melting agarose, and ATP were purchased from Sigma. 4′-Aminomethyl-4,5′,8- trimethylpsoralen (AMT) was purchased from Calbiochem. The purity of the ATP and ATPγS was assayed by thin layer chromatography, and each was shown to be at least 99 and 90% pure, respectively, with the major contaminant being ADP (Shibata *et al.*, 1981).

DNA—Circular ssDNA and supercoiled circular duplex DNA from bacteriophage M13mp8 (7,229 bp) and M13mp8.1037 (8,266 bp) were prepared using methods described previously (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). The bacteriophage M13mp8.1037 is the bacteriophage M13mp8 with a 1,037-bp sequence (*Eco*RV fragment) from the *E. coli galT* gene inserted into the *Sma*I site. The concentrations of ssDNA and dsDNA stock solutions were determined by absorbance at 260 nm, using 36 and 50 $\mu g\ ml^{-1}\ A_{260}^{-1}$, respectively, as conversion factors. DNA concentrations are reported in terms of total nucleotides. Full-length linear duplex (FIII) DNA was derived from M13mp8.1037 FI DNA by complete digestion with *Eco*RI or *Pst*I endonuclease, using conditions suggested by the enzyme supplier. Linear duplex fragments of M13mp8.1037 were prepared by digestion with two restriction endonucleases, *Eco*RI and *Bgl*II or MscI and *Bam*HI. The desired fragment was purified from low melting agarose gels by a published procedure (Sambrook *et al.*, 1989).

Strand Exchange Reaction Conditions—Reactions involving ATP were performed at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol, and an ATP regeneration system (10 units ml^{-1} creatine phosphokinase, 12 mM phosphocreatine). Reaction volumes were 130 μl, and the concentrations of DNA and proteins reported below are the final concentrations after the addition of all components. ssDNA (10 μM) was preincubated with RecA protein (6.6 μM) for 10 min before the addition of duplex DNA (11.4 μM for the 8.3-kbp duplex substrate; 2.4 μM for the 1.7-kbp substrate; 3.0 μM for the 2.2-kbp substrate). After incubation of this mixture for 10 min, ATP (3 mM) and SSB (2 μM) were added to start the reactions. The ATP regeneration system maintains the ADP concentration near zero for at least 80 min.

Reactions involving ATPγS were performed at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 4 mM magnesium acetate, and 1 mM dithiothreitol. ssDNA (10 μM) was preincubated with SSB (0.9 μM) for 10 min before the addition of RecA protein (6.0 μM) and ATPγS (1 mM). After incubation of this mixture for 10 min, the reaction was initiated with duplex DNA (11.4, 2.4, and 3.0 μM for the 8.3-, 1.7-, and 2.2-kbp substrates, respectively). Conditions for ATP and ATPγS reactions are different so that each proceeds optimally. The ssDNA molecules are in 2-fold molar excess with respect to

[2] R. Devoret, personal communication.

the dsDNA molecules in all reactions.

Agarose Gel Assays—Aliquots (15 μl) of the strand exchange reactions described above were removed at the indicated times, and the reactions were stopped by the addition of 5 μl of gel loading buffer (25% glycerol, 15 mM EDTA, 0.025% bromphenol blue, 5% sodium dodecyl sulfate). These aliquots were stored on ice until the last time point was taken. Samples were electrophoresed overnight in an 0.8% agarose gel at 2–2.5 V cm^{-1}.

Electron Microscopy—Visualization of reactions by electron microscopy was carried out by spreading the entire strand exchange reaction mixture after deproteinization and dialysis. Reactions were stopped after 60 min and incubated with proteinase K (1 mg ml^{-1} final) and sodium dodecyl sulfate (0.5% final) at 37 °C for 30 min to remove the RecA protein and SSB. All samples were dialyzed into 20 mM NaCl and 5 mM EDTA for 9 h at 25 °C before spreading as described previously (Inman and Schnös, 1970).

To block spontaneous branch migration, some strand exchange reaction mixtures were cross-linked with AMT prior to deproteinization. Aliquots (20 μl) of the strand exchange reaction at 60 min (or shorter periods of time for the experiment in Fig. 7) were immediately mixed with AMT (30 μg ml^{-1}, final concentration), incubated at 25 °C for 3 min, and irradiated with long wave UV light for 4 min at 25 °C (Umlauf *et al.*, 1990). The UV light was generated with two 15-watt fluorescent black light tubes. Samples were placed 8 cm below the light source. Intensity of UV light ranged between 2 and 4 milliwatts/cm^2 at 365 nm (as measured with a Blak-Ray J-221 long wave UV monitor). A glass microscope slide was placed over the samples to filter out UV radiation below 320 nm. RecA protein and SSB were removed and the samples dialyzed as described above.

Treatment of Electron Microscopic Data—There are three types of information gained from the electron microscopic experiments.

1. We wanted to confirm that deproteinized reaction mixtures contained recombinational intermediates of the expected type. For the substrates shown in Fig. 1, we would expect intermediates to consist of a circular region (containing ssDNA and dsDNA segments) with separate dsDNA and ssDNA tails. The dsDNA segment within the circular region represents the DNA that has undergone exchange, whereas the dsDNA tail is that part of the dsDNA substrate which has not or cannot be exchanged. For examples, see Fig. 5.

2. We wished to estimate the proportion of the duplex linear substrates which was involved in DNA strand exchange reactions leading to intermediates. This was determined by counting the intermediates and unreacted linear duplex substrate molecules found in a representative sample derived from a reaction. Complex recombinational events involving more than two DNA substrate molecules and events that were interpreted to arise from broken or nicked substrates (the latter produce low levels of gapped circular and more complex products in the reactions) were ignored in these estimates. In some experiments, the grids from different reactions were assigned an undescriptive identification code by S. K. J. and provided in a random sequence to R. B. I. without further identification. The molecules on them were subsequently counted "blind" by R. B. I.

3. Finally, it was important to estimate the extent of DNA strand exchange (the length of hybrid DNA generated) for a representative sample of the intermediates. Because of the large number of samples, obtaining an accurate measurement of significant numbers of intermediates in all of the samples was impractical. Therefore, the length of the exchanged region was estimated by eye in many experiments. As an example, in Fig. 5*B*, the ratio of the exchanged region (length of the double-stranded segment within the circle) to the unexchanged region (length of the double-stranded tail) was judged to be about 1:4.5. For a duplex linear DNA substrate of 8.3 kbp, this would mean that DNA strand exchange had progressed about 1.5 kbp. For convenience, each linear duplex was divided into eight segments; judgments about the length of the exchanged region are given in 1-kbp segments for the 8.3-kbp molecules shown in Figs. 4 and 7, and in 0.21- or 0.27-kbp segments for the 1.7- and 2.2-kbp molecules shown in Fig. 6. Because of the way in which these data were accumulated, the first segment (starting at 0 kbp) is 50% wider than all of the others for all of the duplex substrates shown in Figs. 4, 6, and 7; the counts in the first segment have therefore been reduced proportionally so that the height of the bar reflects a constant percent of intermediates per incremental length (*e.g.* in Fig. 4, the height of the bars reflect the percent of intermediates/kbp). The areas underneath the bars are an accurate reflection of the number of intermediates observed in each segment.

These judgments were checked in two ways. First, two grids each from two samples (corresponding to those shown in Fig. 4, *B* and *D*) were counted blind as described above. The four sets of data were then compared by the χ^2 two-way contingency test at the 95% confidence level. The two data sets corresponding to sample *B* were not significantly different ($p = 0.53$), and the same was true for the data sets corresponding to sample *D* ($p = 0.38$). On the other hand, when the two data sets corresponding to *B* were compared with those from *D*, there was a difference ($p = 0.0002$). The numbers of molecules counted in these data sets were 32 and 20 for the samples of Fig. 4*B*, and 29 and 30 for the samples of Fig. 4*D*. Second, measurements were made in two samples corresponding to Fig. 4, *C* and *D*, and these more accurate data were converted to the less accurate judgment scale shown in Fig. 4 and 6. χ^2 two-way contingency tests of data in Fig. 4 showed that there was no significant difference ($p = 0.27$ (*C*) and 0.22 (*D*)). Measurements were made on 34 and 32 molecules, respectively. Photography and computer-assisted length measurements of DNA molecules were performed as described previously (Littlewood and Inman, 1982).

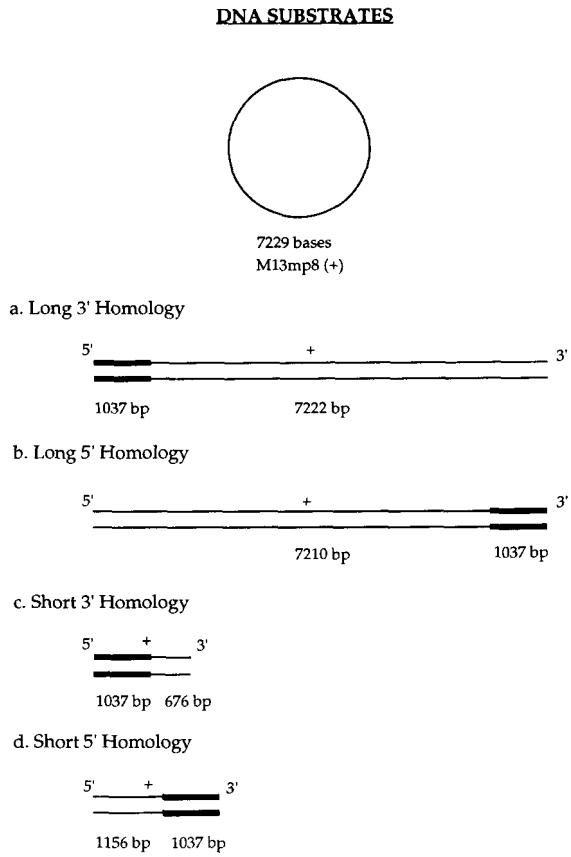


FIG. 1. **DNA substrates used in this study.** ssDNA and duplex DNA with partial homology were reacted with RecA protein to form complexes, which will be referred to as DNA pairing intermediates or joint molecules. The numbers denote the length of homology (*thin lines*) and heterology (*thick lines*) in the DNA substrates. At the "heterologous" end of each molecule there are very short sequences with homology to M13mp8 (not shown) which total 7 (*a*), 19 (*b*), 7 (*c*), and 3 (*d*) bp. None of these regions is long enough to take part in DNA pairing reactions under the conditions used for these experiments. *M13mp8(+)*, circular ssDNA genome of bacteriophage M13mp8.

RESULTS

Experimental Design—In the following discussion, the ends of the duplex DNA substrate are identified as 5′ or 3′ with respect to the strand that is identical to the circular ssDNA substrate. To provide information on the directionality of branch migration, duplex DNA substrates in which homology was restricted to the 5′ or 3′ end were used in strand exchange reactions with circular ssDNA (Fig. 1). A heterologous region (about 1 kbp) at one end of the duplex prevents the complete exchange of strands. Two sets of duplex substrates were used which differed in the length of the segment that was homologous to the ssDNA. Formation of joint molecules preferentially

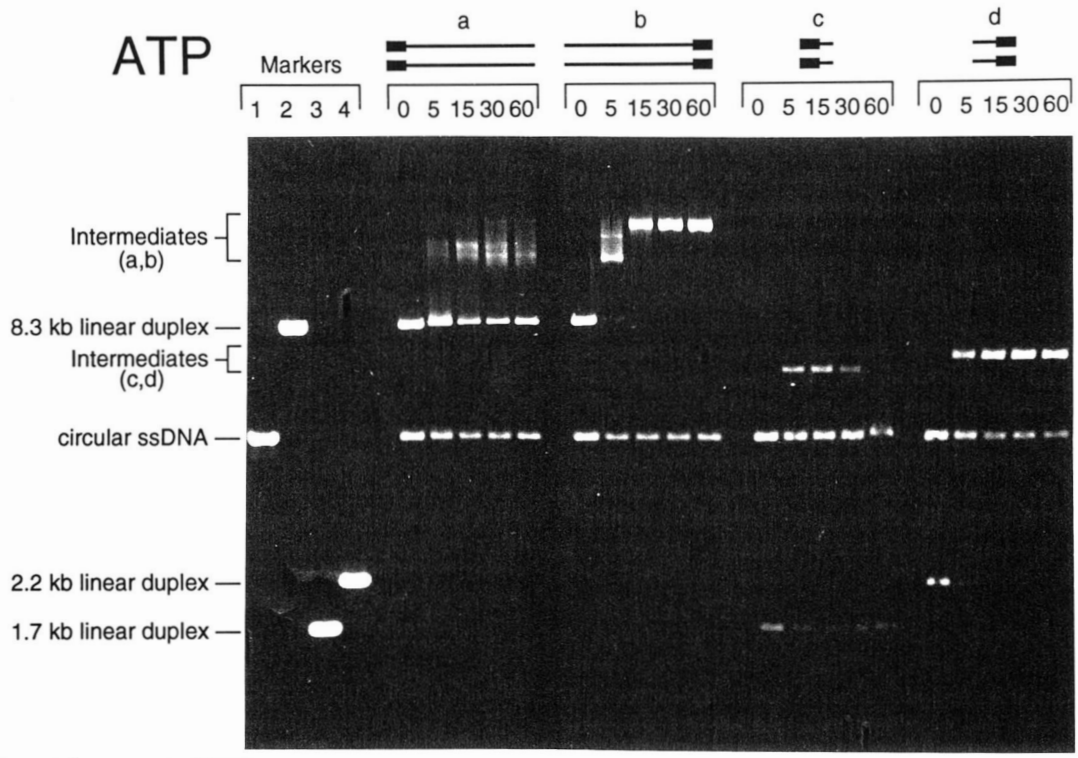


FIG. 2. **RecA protein-promoted DNA strand exchange with ATP hydrolysis.** DNA substrates in which homology was restricted to the 5′ or 3′ end were used in strand exchange reactions with circular ssDNA (illustrated in Fig. 1). Reaction conditions are described under "Materials and Methods" and the legend to Table I. Time points in each set of reactions were 0, 5, 15, 30, and 60 min, respectively, as indicated by the numbers above the gel lanes in reactions *a–d*.

at one end reflects both the polarity of branch migration and the intrinsic stability of the joint molecules after RecA protein is removed. The strand exchange reactions were analyzed by agarose gel electrophoresis and by electron microscopy. Utilization of AMT cross-linking in some experiments permitted us to correct for loss of joint molecules due to spontaneous branch migration after removal of RecA.

In the Presence of ATP, DNA Pairing Intermediates Are Extended and Preferentially Stabilized at the 5′ End of the Duplex DNA—Although pairing occurs at either end of the duplex DNA, the number of joint molecules observed when homology is restricted to the 5′ end of the dsDNA is much greater than that observed with homology restricted to the 3′ end. This is shown in Fig. 2, reactions a and c (3′ homology) as compared with reactions b and d (5′ homology). The bias for intermediate formation at the 5′ end was similar for reactions involving long or short regions of homology in the duplex and is consistent with the established 5′ to 3′ direction of strand exchange in the presence of ATP hydrolysis. The intermediates formed on the 5′ end migrate in a discrete band, as opposed to a diffuse set of bands observed for intermediates at the 3′ end. As shown below, the discrete band in the joint molecules formed with homology restricted to the 5′ end is a direct result of unidirectional DNA strand exchange, which extends the hybrid DNA to the end of the homologous region.

The results obtained by agarose gel electrophoresis were confirmed by direct observation of the pairing intermediates by electron microscopy (Table I). In the presence of ATP, intermediates were observed preferentially at the 5′ end with both sets of DNA substrates.

There Is an Apparent Substrate-dependent End Preference for Joint Molecule Formation in the Absence of ATP Hydrolysis—Strand exchange reactions involving ATPγS are shown in Fig. 3. In reactions involving long (7 kbp) regions of homology, pairing intermediates appeared to form more efficiently at the 5′ end as compared with the 3′ end (Fig. 3, reactions *b versus a*). However, the observed bias for intermediate formation was reversed in strand exchange reactions involving duplex DNA molecules with short (about 1 kbp) regions of homology (Fig. 3, reactions *c versus d*).

The results were again reinforced by electron microscopy. In the presence of ATPγS, joint molecules appeared to form more efficiently at the 5′ end of the duplex than at the 3′ end when long (7 kbp) regions of homology were present; the apparent end bias was reversed with short homologous regions in the duplex DNA (Table I).

AMT Cross-linking of the Paired Intermediates Reveals That the Apparent End Bias Observed in the ATPγS Reactions Is Artifactual—We carried out additional reactions in the presence of ATPγS, with the same substrates and under conditions identical to those described above. Just prior to termination of the reaction and removal of RecA protein and SSB, the DNA in these reactions was cross-linked with a photoreactive reagent (AMT) that cross-links pyrimidines in opposite DNA strands (Table I). The cross-linking conditions used result in a cross-linking density of approximately one AMT cross-link/220 bp of DNA (Bedale *et al.*, 1991). This treatment prevented branch migration during the deproteinization and dialysis steps that precede spreading the DNA for electron microscopy.

AMT cross-linking increased the fraction of the DNA substrates found in joint molecules for both long and short regions of homology whether it was positioned at the 5′ or 3′ end of the linear duplex. More significantly, the cross-linking abolished the end bias observed in the ATPγS reactions (Table I). Therefore, the apparent bias in favor of one end or the other in joint molecule formation with ATPγS is an artifact brought about by spontaneous branch migration following deproteinization. Since the bias is distinctly different with two different sets of duplex DNA substrates, the branch migration appears to be

TABLE I

Quantitation of DNA strand exchange intermediates by electron microscopy

Reactions were performed and prepared for electron microscopy as described under "Materials and Methods." ATP-containing reactions had 6.6 μM RecA protein and 2.0 μM SSB. ATPγS reactions had 6.0 μM RecA protein and 0.9 μM SSB. All reactions contained 10 μM ssDNA, and 11.4, 2.4, and 3.0 μM dsDNA for the 8.3-, 1.7-, and 2.2-kbp duplex DNA substrates, respectively. Other reaction conditions are described under "Materials and Methods." Molecules were counted, and the numbers of joint molecules are expressed as the percentage of the total duplex DNA substrates associated with a ssDNA molecule. The error limits shown reflect statistical counting errors and are not meant to indicate estimated error arising from repeated experiments. *N* is the number of DNA molecules containing duplex DNA counted in each experiment and represents the sum of the duplex DNA substrate molecules and the intermediates in which the duplex is paired with a ssDNA. Uncross-linked samples are those that were deproteinized, dialyzed, and spread as described under "Materials and Methods." The cross-linked samples were treated with AMT as described prior to deproteinization. DNA substrates are labeled as in Fig. 1. ND denotes a single reaction for which we were unable to generate meaningful data. AMT treatment of this particular sample resulted in unexpected multistranded DNA species in more than five separate experiments. This phenomenon is under investigation.

Duplex DNA substrate	Nucleotide	Uncross-linked		Cross-linked	
		Joint molecules (%)	*N*	Joint molecules (%)	*N*
Long 3′ homology (7.2 kbp)	ATP	28 ± 5	313	ND	ND
Long 5′ homology (7.2 kbp)	ATP	90 ± 7	93	95 ± 6	65
Short 3′ homology (0.7 kbp)	ATP	9 ± 4	287	16 ± 5	257
Short 5′ homology (1.2 kbp)	ATP	77 ± 6	192	98 ± 5	50
Long 3′ homology (7.2 kbp)	ATPγS	31 ± 5	379	69 ± 9	108
Long 5′ homology (7.2 kbp)	ATPγS	59 ± 7	203	76 ± 8	125
Short 3′ homology (0.7 kbp)	ATPγS	52 ± 7	242	56 ± 10	113
Short 5′ homology (1.2 kbp)	ATPγS	29 ± 4	469	55 ± 12	80

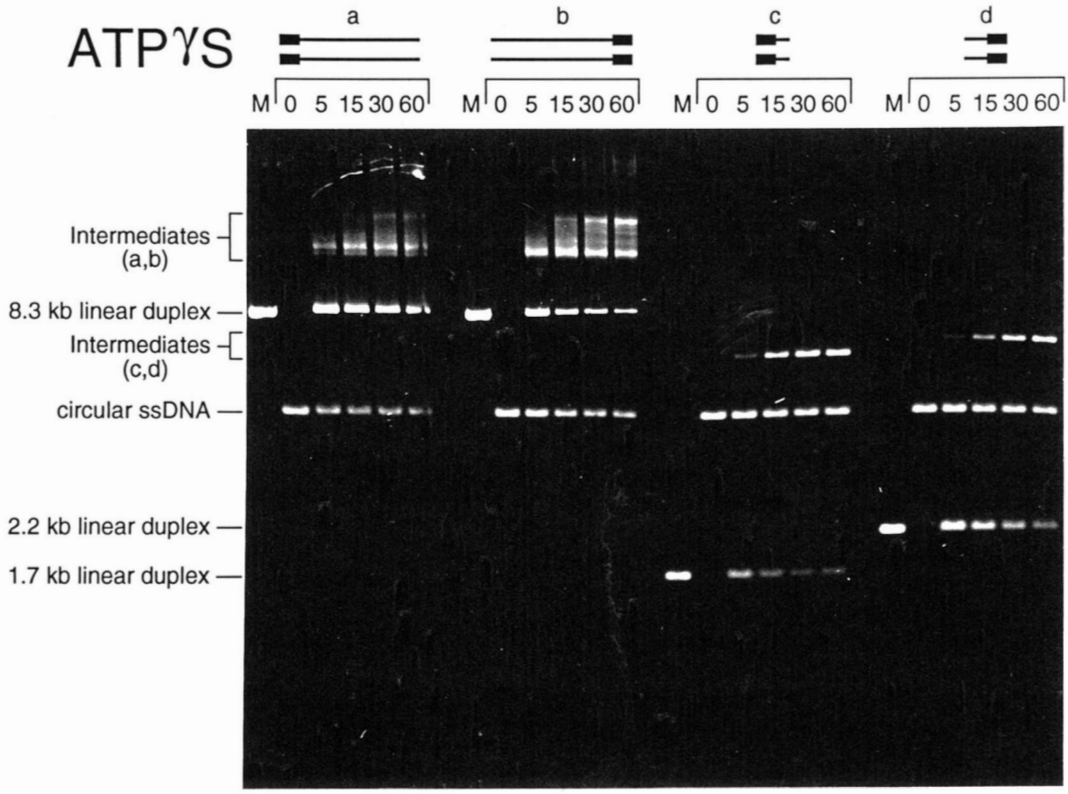


FIG. 3. **RecA protein-promoted DNA strand exchange in the presence of ATPγS.** DNA substrates and time points are as described in the legend to Fig. 2. Reaction conditions were optimized for the ATPγS reactions as described under "Materials and Methods." Protein and DNA concentrations are described in the legend to Table I.

selective. We do not understand the bias differences for the two substrate sets.

The results obtained with ATP were essentially unaffected by AMT cross-linking. There was still a strong bias for joint molecule formation at the 5′ end with either a long or short homologous region in the duplex substrate (Table I).

ATP Hydrolysis Facilitates Branch Migration and Permits Extensive DNA Strand Exchange—Even when ATP is hydrolyzed, DNA pairing can occur anywhere within the duplex DNA substrate, forming an intermediate that can contain thousands of bp of paired or hybrid DNA. With the subsequent strand exchange proceeding 5′ to 3′, ATP hydrolysis will extend the hybrid region when the joint is formed on the 5′ end of the duplex and eliminate it when it is formed on the 3′ end (Cox and Lehman, 1981; Dutreix *et al.*, 1991). This would not only result in a selective accumulation of joint molecules on the 5′ end, it should also be evident in the length of the hybrid DNA in the joints that are formed. Joints formed on the 5′ end should be extended to the boundary of the heterology; 3′ joints should be fewer in number, vary in length, and be much shorter (on average) than 5′ joints.

The effect of ATP hydrolysis on the extent of strand exchange was quantified by reacting dsDNA, with a long (7 kbp) region of homology limited to one end of the duplex, with circular ssDNA. The extent of DNA strand exchange was determined by visualizing pairing intermediates by electron microscopy and esti-

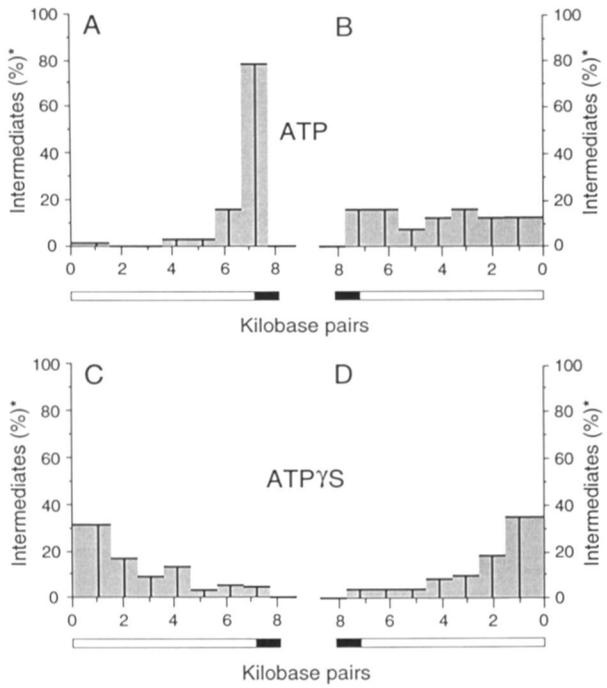


FIG. 4. **ATP hydrolysis is required for extensive and unidirectional DNA strand exchange.** Reactions were performed under conditions optimized for the ATP and ATPγS reactions as described under "Materials and Methods." Uncross-linked joint molecules formed with DNA substrates involving long regions of homology were visualized by electron microscopy. The length of hybrid DNA formed in the strand exchange reactions was estimated as described under "Materials and Methods." The homologous (7.2 kbp) and heterologous (1.0 kbp) regions within each dsDNA substrate are depicted by the *open* and *filled boxes* below each graph. The *left* and *right* ends of the dsDNA refer to the 5′ end and the 3′ end, respectively. *A* and *B*, ATP-mediated strand exchange reactions with 5′ or 3′ homology, respectively. *C* and *D*, ATPγS-mediated reactions with 5′ or 3′ homology, respectively. The data in *panels C* and *D* were confirmed by accurate measurements of the hybrid DNA formed in a sample of 34 and 32 molecules, respectively. *The data are plotted in terms of the percent of total intermediates counted per 1-kbp increment. See "Materials and Methods" for details.

mating the length of the hybrid DNA as described under "Materials and Methods" (Fig. 4). When ATP was hydrolyzed, DNA strand exchange proceeded to the homology/heterology boundary (7 kbp) in the majority of joint molecules formed on the 5′ end (Fig. 4*A*). This is in contrast to the random distribution of the length of hybrid regions seen with long homology at the 3′ end of the duplex (Fig. 4*B*). The data in Fig. 4 were obtained for joint molecules that were not cross-linked. When AMT was used to cross-link the joint molecules prior to deproteinization, virtually all of the joints formed at the 5′ end had hybrid DNA extending to the homology/heterology boundary. The small number of molecules with shorter hybrid regions seen in Fig. 4*A* was eliminated (data not shown).

In ATPγS-mediated reactions, joint molecules form efficiently at either end of the duplex, but only 2 kbp or less of hybrid DNA is formed at either end of the duplex DNA before branch migration is blocked in the majority of pairing intermediates (Fig. 4, *C* and *D*). The results were the same when the reactions were extended to 90 min or when the paired intermediates were cross-linked (data not shown).

Two representative pairing intermediates formed in ATP-mediated reactions with long (7.2 kbp) homology restricted to the 5′ end are shown in Fig. 5*A*. The extensive region of duplex DNA encompassing the circles and the long single-stranded tails reflect a DNA strand exchange reaction that has proceeded to the homology/heterology boundary. Two typical joint molecules formed in the presence of ATPγS are shown in Fig. 5, *B* and *C*. The short region of dsDNA within the circle represents the exchanged DNA, and the long duplex tail depicts the unreacted DNA within the duplex substrate.

A similar analysis was carried out for strand exchange reactions involving duplex DNA with short regions of homology in the presence of ATP or ATPγS, using electron microscopy (Fig. 6). These reactions were cross-linked with AMT prior to removal of the RecA protein and SSB. The paired intermediates were visualized by electron microscopy, and the length of the exchanged regions was estimated as before. In ATP-mediated reactions, intermediates were still found preferentially at the 5′ end of the duplex DNA. More than 98% of the duplex DNA substrates were found in joint molecules when homology was limited to the 5′ end; only about 16% of the substrate duplexes were paired when the homology was at the 3′ end (Table I). The effect of ATP hydrolysis on directional DNA strand exchange and the extent of hybrid DNA formation is less evident when a short region of homology is used, however. The length of hybrid regions in the joint molecules ranged up to the homology/heterology boundary in both 5′ or 3′ pairing intermediates (Fig. 6, *A* and *B*), although a sharper peak at the homology/heterology boundary is seen on the 5′ end.

In the presence of ATPγS, there was no preference for joint formation at either end of the duplex DNAs with short regions of homology (Table I), and DNA strand exchange produced hybrid regions ranging up to the homology/heterology boundary (Fig. 6, *C* and *D*). Therefore, formation of short regions of hybrid DNA proceeds efficiently and without a directional bias in the absence of ATP hydrolysis.

RecA-mediated DNA Strand Exchange in the Presence of ATPγS Is Bidirectional—The data in Fig. 4 and Table I suggest that the strand exchange reaction is bidirectional when ATPγS replaces ATP. However, the 60-min time points used in these experiments could obscure a substantial kinetic bias in the reaction. In addition, the conclusion of bidirectionality depends on the assumption that joint molecules are initiated at a free end and that strand exchange then proceeds toward the middle of the duplex DNA. We carried out experiments to quantify more closely the early stages of the reaction with electron microscopy. The work of Menetski *et al.* (1990) indicates that the DNA strand exchange reaction reaches equilibrium within a few minutes in the presence of ATPγS. We concentrated our efforts on the first minutes of reaction to capture preequilibrium conditions and identify the initiation point of DNA strand exchange.

DNA strand exchange reactions were again carried out in the presence of ATPγS with substrates *a* and *b* of Fig. 1, except that aliquots were removed and cross-linked with AMT at 1, 2, 3, 5, 10, and 20 min. The samples were visualized by electron microscopy, and a subset of these was examined in detail as noted below. We detected no bias in the numbers of joint molecules formed at either end of the duplex DNA, even after 1 min of reaction (data not shown); in this respect, the results are similar to those presented in Table I.

The data described below focus only on intermediates in which one single-stranded circle is paired with one linear duplex DNA. Three types of paired complexes were observed. In the first type, the two DNA substrates were apparently linked at one point, which could be at any point along the linear duplex. These complexes probably reflect the initial pairing interaction between the DNA substrates prior to net strand exchange, as documented in numerous studies. The point contacts were not observed in the absence of cross-linking, and displacement of a strand in the duplex substrate was not observed in any of them at any time point. The second type of complex was the branched intermediate illustrated in Fig. 5.

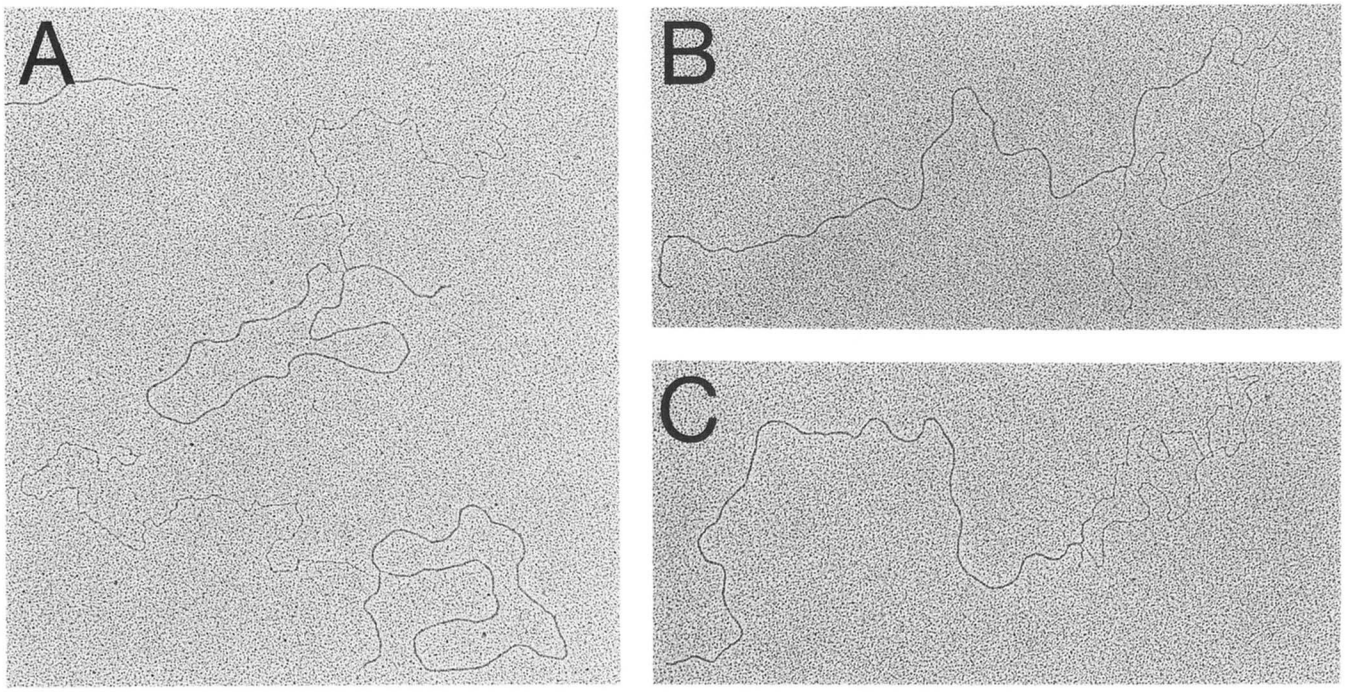


FIG. 5. **Electron micrographs of paired DNA reaction intermediates: ATP *versus* ATPγS.** RecA-mediated DNA strand exchange reactions were carried out in the presence of ATP or ATPγS, using linear duplex substrates with a long homologous region at the 5′ end (Fig. 1*b*). Samples were prepared for electron microscopy as described under "Materials and Methods." The samples shown were not cross-linked. *Panel A*, two representative pairing intermediates formed in ATP-mediated reactions. The circular dsDNA corresponds to the hybrid DNA formed in the strand exchange reaction and the long single-strand represents the displaced homologous strand. *Panels B* and *C*, two typical intermediates formed in ATPγS-mediated reactions. The short region of dsDNA within the *circle* represents the exchanged DNA and the long duplex tail depict the unreacted DNA within the duplex substrate.

The observed strand displacement extended to one end of the duplex substrate in *all* of these molecules. The third category of paired complexes consisted of more complicated species, which we presume arose from broken DNA substrates. These represented, on average, 6% of the paired complexes in the samples (2–3% in many samples) and were ignored in our analysis.

The progression of the strand exchange reaction was similar when the homology was restricted to either the 5′ or the 3′ end of the dsDNA. Within the 1st min of all reactions, the predominant DNA complexes (73 and 64% of the total paired molecules, on the 5′ and 3′ ends, respectively) were point contacts. The intermediates in which strand exchange had commenced (25 and 34% of the total on the 5′ and 3′ ends, respectively) exhibited a short region (less than 1 kbp) of exchanged DNA which was limited to the end of the duplex DNA molecule. There were no unambiguous instances in which DNA strand exchange had initiated from an unnicked internal site within the dsDNA since no displaced single-stranded loops (D-loops) were observed within the duplex DNA.

As the reaction progressed, the number of complexes with point contacts declined in number, whereas the numbers of joint molecules increased. At 20 min, fewer of the complexed molecules (18% on the 5′ end and 28% on the 3′ end) were point contacts, and more (75 and 70% on the 5′ and 3′ ends, respectively) were joint molecules. The absence of internal D-loops strongly indicates that net DNA strand exchange initiates only from a free end of the duplex DNA.

As the ATPγS reactions progressed, the length of hybrid DNA within the intermediates increased as shown in Fig. 7. Between 1 and 20 min of reaction, the fraction of joint molecules in which DNA strand exchange had progressed more than 1.5 kbp from an end increased from 12 to 52% with homology on the 5′ end and from 10 to 27% on the 3′ end. As determined by a χ^2 two-way contingency test at the 95% confidence level, the increase in the length of exchanged DNA was significant for both ends ($p = 1.0 \times 10^{-6}$ and 1.0×10^{-3} for the 5′ and 3′ ends, respectively). The apparently larger increase observed on the 5′ end does not necessarily reflect a bias favoring 5′ to 3′ branch migration, since the average increase in the length of exchanged DNA was modest in both cases. Much of the change occurred within the first 3 min. The lack of a more dramatic change is consistent with the rapid rate at which these reactions come to equilibrium in the presence of ATPγS. When pairing occurs near a free DNA end, there appears to be a "burst" of DNA strand exchange which can result in rapid formation of an exchanged region of 1–2 kbp. Further branch migration is very slow when ATPγS replaces ATP. Nevertheless, the increase in the length of exchanged DNA as the ATPγS reaction progressed indicates that a branch point in a joint molecule can migrate toward the middle of the dsDNA molecule regardless from which end it initiates and confirms that DNA strand exchange in the presence of ATPγS is bidirectional.

DISCUSSION

ATP hydrolysis is required to render the DNA strand exchange reaction unidirectional. When ATP is hydrolyzed, many more joint molecules are observed on the 5′ end of the duplex substrate, where they are stabilized by unidirectional strand exchange, than on the 3′ end. When ATPγS is present, the end bias is no longer observed. Careful analysis of the reaction indicates that DNA strand exchange with ATPγS is bidirectional. ATP hydrolysis also permits the formation of much more extended regions of hybrid DNA than are formed with ATPγS.

Gels such as those in Figs. 2 and 3 have been used to determine the polarity of reported DNA strand exchange reactions promoted by a wide range of prokaryotic and eukaryotic proteins. An analysis utilizing agarose gels alone can clearly be misleading, however. The effect of ATP hydrolysis in rendering the DNA strand exchange reaction unidirectional is most evident only when (*a*) a long (several kbp) region of homologous

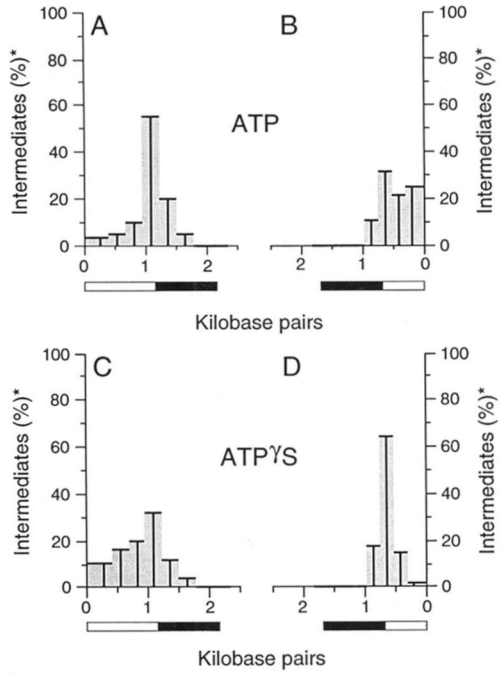


FIG. 6. **Hybrid DNA formed in the absence of ATP hydrolysis.** Strand exchange reactions involving circular ssDNA and duplex DNA containing a short region of homology were carried out under conditions optimized for the ATP and ATPγS reactions as described under "Materials and Methods." Reaction mixtures were cross-linked with AMT and visualized by electron microscopy. The length of heteroduplex DNA formed in the strand exchange reactions was measured. The homologous (1.2 kbp or 0.7 kbp) and heterologous (1.0 kbp) regions within each dsDNA substrate are depicted by the *open* and *filled boxes* below each graph, respectively. The left and right ends of the dsDNA refer to the 5′ end and the 3′ end, respectively. *Panels A* and *B*, ATP-mediated strand exchange reactions with 5′ or 3′ homology, respectively. *Panels C* and *D*, ATPγS-mediated reactions with 5′ or 3′ homology, respectively. *The data are plotted in terms of the percent of total intermediates counted per 0.27-kbp (*panels A* and *C*) or 0.21-kbp (*panels B* and *D*) increment. See "Materials and Methods" for details.

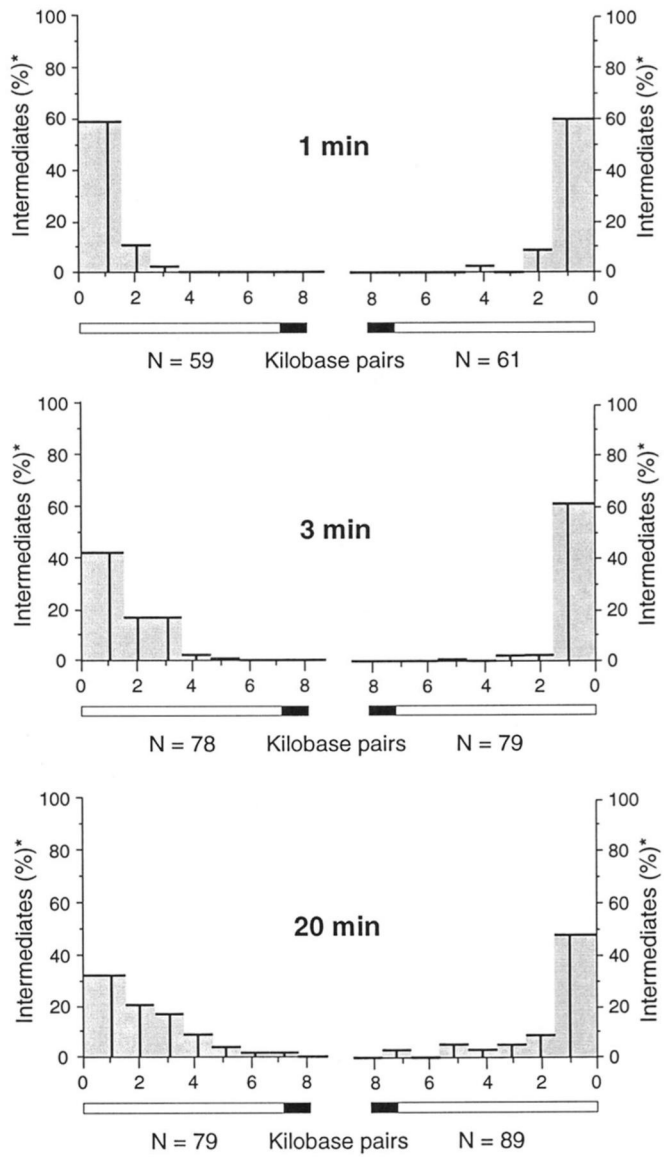


FIG. 7. **RecA-mediated DNA strand exchange in the presence of ATPγS is bidirectional.** Reactions were performed under conditions optimized for the ATPγS reactions as described under "Materials and Methods." Cross-linked joint molecules formed with DNA substrates involving long regions of homology were visualized by electron microscopy. The length of hybrid DNA formed in the strand exchange reactions was estimated as described under "Materials and Methods." Substrates, symbols, and the treatment of data are the same as in Fig. 4. Reactions involving duplex DNA substrates with 5′ or 3′ homology are shown on the *left* and *right*, respectively. *N* denotes the number of molecules analyzed. *Data are presented as the percent of intermediates counted per 1-kbp increment. See "Materials and Methods" for details.

DNA is present in the duplex DNA substrate; (*b*) spontaneous branch migration in joint molecules is blocked by cross-linking; and (*c*) the length of hybrid or heteroduplex DNA formed is considered and is part of the analysis. As noted previously by Radding and colleagues (Dutreix *et al.*, 1991), it can be especially important to include an assay to assess directly the length or extent of hybrid DNA formation. Our results indicate that another factor, a spontaneous branch migration process that may affect some deproteinized joint molecules more than others, can also produce artifacts in the standard gel assays. We do not yet know why some joint molecules formed with ATPγS are selectively destabilized when they are not cross-linked prior to deproteinization.

One earlier study (Rosselli and Stasiak, 1990) found that DNA strand exchange with ATPγS was unidirectional, whereas another did not (Konforti and Davis, 1992). Both studies based their conclusions on the results of agarose gel assays for joint molecule formation; spontaneous branch migration was not controlled, and the extent of hybrid DNA formation was not measured directly. Our results support the conclusion of the latter study. A corollary is that evidence derived from agarose gel assays alone should be regarded as insufficient to establish the polarity of a DNA strand exchange reaction.

Another aspect of the RecA-mediated DNA strand exchange reaction in the presence of ATPγS, its kinetics, deserves some additional comment. There appears to be a rapid, or burst phase of this reaction, during which most of the net strand exchange occurs. After the first 2 or 3 min, additional DNA strand exchange occurs very slowly, consistent with the results reported by Menetski *et al.* (1990). The only explanation for this phenomenon suggested to date is that continued strand exchange after the burst may be blocked by discontinuities in the RecA filament (Menetski *et al.*, 1990). An alternative explanation can be found in a reexamination of the ATP-mediated process. Kahn and Radding (1984) reported previously a burst phase in the ATP-mediated DNA strand exchange reaction. We have recently confirmed this result and found that 1 kbp or more of hybrid DNA may be formed by RecA nucleoprotein filaments with ATP during the burst phase.[3] The early steps of DNA strand exchange may therefore be very similar with ATP

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

or ATPγS. Two DNA molecules are aligned, and if a free end is available, a burst of strand exchange occurs in which 1–2 kbp of hybrid DNA are formed. Extension of the exchanged hybrid DNA beyond the initial 1–2 kbp, however, is highly dependent on ATP hydrolysis.

Some DNA strand exchange occurs without ATP hydrolysis, and the simple conclusion often drawn is that ATP hydrolysis and DNA strand exchange are unlinked. A RecA nucleoprotein filament has an intrinsic capacity to take up a homologous duplex DNA and promote DNA strand exchange. In the absence of ATP hydrolysis, however, DNA strand exchange is bidirectional, limited in extent, does not bypass structural barriers in the DNA substrates, and does not accommodate four DNA strands. Although not required for DNA strand exchange, ATP hydrolysis clearly confers new properties on the reaction, some of which are likely to be critical for RecA function in DNA repair. When ATP is hydrolyzed, DNA strand exchange: (*a*) is unidirectional; (*b*) generates much more extensive regions of hybrid DNA; (*c*) readily bypasses substantial structural barriers in the DNA (Kim *et al.*, 1992a; Rosselli and Stasiak, 1991); and (*d*) accommodates four DNA strands (Kim *et al.*, 1992b). The overall conclusion we draw from these observations is that a major function of RecA-mediated ATP hydrolysis is to direct and transform the intrinsic DNA strand exchange activity of RecA filaments. ATP hydrolysis is coupled to DNA strand exchange, not as a requirement for the basic strand exchange process, but as an augmentation that greatly enhances the effectiveness of the reaction in recombinational DNA repair.

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