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

II. FOUR-STRAND EXCHANGES*

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RecA protein promotes a substantial DNA strand exchange reaction in the presence of adenosine 5′-*O*-3-(thio)triphosphate (ATPγS) (Menetski *et al.*, 1990), calling into question the role of ATP hydrolysis in this reaction. We demonstrate here that the ATPγS-mediated process is restricted to homologous strand exchange reactions involving three strands. In four-strand exchanges between a gapped duplex circle and a second linear duplex, joint molecules are formed in the gap but are not extended into the four-strand region when ATPγS is present. This result provides evidence that one function of ATP hydrolysis in the recA system is to facilitate reciprocal DNA strand exchange involving four strands. Implications with respect to the role of four-stranded pairing intermediates and the mechanistic relationship between three- and four-strand exchange reactions are discussed.

Homologous genetic recombination is widely viewed as a reaction between two duplex DNA molecules, and models for this process generally revolve around the adventures of four DNA strands. *In vitro*, the recA protein promotes a four-strand exchange reaction that generates Holliday structures as reaction intermediates (Cassuto *et al.*, 1980; Cunningham *et al.*, 1980; DasGupta *et al.*, 1981). Although the three-strand exchange reaction (see preceding paper (Kim *et al.*, 1992)) has been studied more extensively (largely because the required substrates are much easier to prepare), the four-strand reaction is often cited as the more relevant model for homologous recombination *in vivo*.

A typical four-strand exchange reaction is illustrated in Fig. 1. The reaction follows the same general reaction pathway as the three-strand reaction (for reviews, see Roca and Cox, 1990; Radding, 1991; Kowalczykowski, 1991). A recA nucleoprotein filament forms on the gapped duplex DNA in the first step. The second duplex DNA is then aligned with the first (<1–2 min), followed by a relatively slow (~5 bp/s)[1] and unidirectional strand exchange that generates the final products. A four-strand pairing interaction has recently been detected (Conley and West, 1989, 1990; Lindsley and Cox, 1990) that appears to provide evidence for a pairing intermediate analogous to one now being characterized in the three-stranded reaction (Rao *et al.*, 1990; Hsieh *et al.*, 1990; Jain *et al.*, 1992). This has led to a picture of a recA filament with a binding groove that can bind and align two duplex DNA molecules (Howard-Flanders *et al.*, 1984; Eggleston and Kowalczykowski, 1991); products could be formed simply by rotating individual bases to form heteroduplex DNA (Fig. 2*A*). The three-strand reaction would represent a variation on the same theme in which one of the four DNA strand-binding sites was not occupied (Fig. 2*B*).

However, this picture is in conflict with a large and growing body of literature that indicates that recA is fundamentally a three-strand system, and can accommodate no more than three strands within the filament. RecA protein binds to ssDNA almost exclusively at neutral pHs and above, binding to double-stranded DNA rapidly only when a single-strand gap or unwound region is present to provide a nucleation site. The gap is not just a nucleation site for binding, it is also where the strand exchange reaction is initiated. The linear duplex must overlap the gap by 50–100 base pairs for an optimal reaction (Conley and West, 1990; Lindsley and Cox, 1990); *i.e.* four-strand exchanges begin as three-strand exchanges. More direct analyses of DNA binding by recA protein by a variety of physical techniques have shown that the recA filament can bind and coordinate three, but not four, strands in the major filament groove (Takahashi *et al.*, 1989, 1991; Kubista *et al.*, 1990; Müller *et al.*, 1990). Finally, the four-strand homologous interaction is very weak compared to pairing reactions with three strands and detected only if the second duplex is supercoiled (Lindsley and Cox, 1990). The requirement for superhelicity and the physical studies cited above suggest the possibility that the apparent four-strand pairing actually reflects a three-strand interaction in which only one strand of the supercoiled duplex (generated by transient strand separation) is incorporated into the filament.

The two competing models described above lead logically back to a consideration of the ATPγS-mediated strand exchange reaction described by Menetski *et al.* (1990). If recA protein has a "four-strand groove" as outlined in Fig. 2, then the molecular details of the strand switch that occurs during strand exchange should be essentially the same for three- and four-strand reactions, and ATPγS should permit a substantial four-strand exchange reaction much as it does with three strands. If recA protein instead has a "three-strand groove," as indicated by the work cited above, one strand of a duplex DNA occupying the groove would have to be removed before another duplex DNA could be brought in. In this case a four-strand reaction would almost certainly be blocked by ATPγS. The experiments reported here indicate that ATPγS does not support the exchange of four strands.

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[1] The abbreviations used are: bp, base pair(s); ssDNA, single-stranded DNA; double-stranded DNA, double-stranded DNA; SSB, single-strand DNA binding protein of *E. coli*; SDS, sodium dodecyl sulfate; AMT, 4′-amino-4,5′,8-trimethylpsoralen.

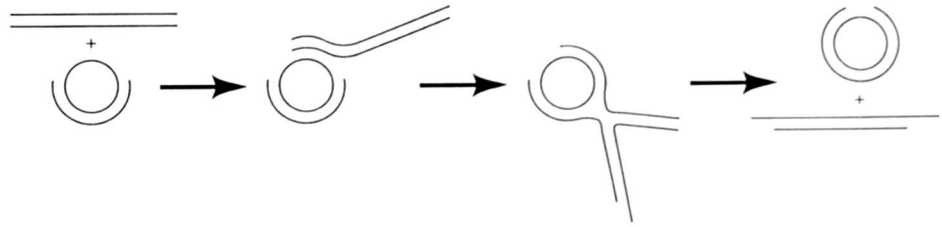


FIG. 1. **A recA protein-mediated DNA strand exchange reaction involving four DNA strands.** RecA protein binds to the gapped circular duplex before reaction begins. Note that the reaction is initiated in the gap (single-strand region) of the circular duplex.

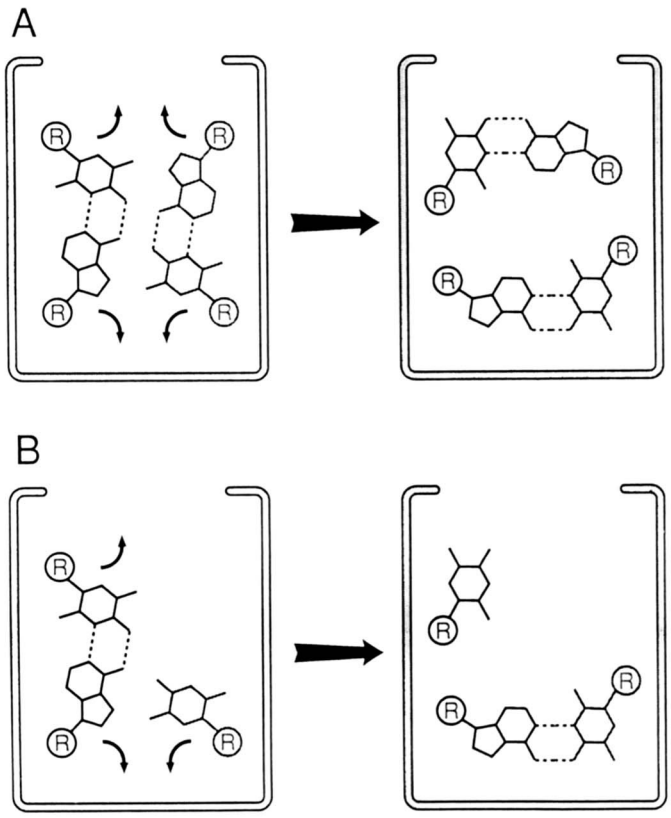


FIG. 2. **The strand switch mechanism postulated by Howard-Flanders *et al.* (1990).**

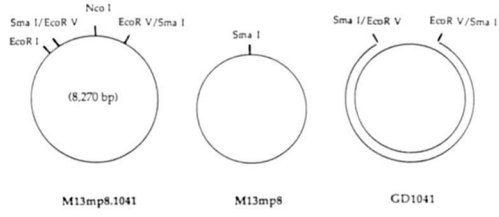


FIG. 3. **DNA substrates used in this study.**

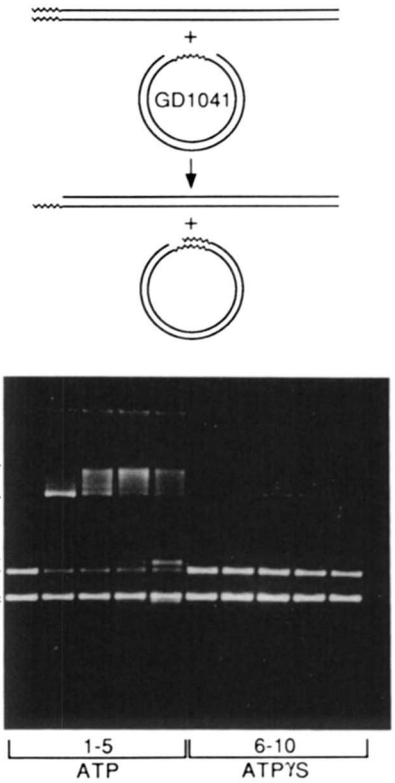


FIG. 4. **Effect of ATPγS on four-strand exchange reactions promoted by recA protein.** Reactions were carried out as described under "Experimental Procedures," with Mg^{2+} acetate concentrations of 10 or 5 mM for the ATP or ATPγS reactions, respectively. Time points for *lanes 1–5* (or *6–10*) are 0, 10, 30, 60, and 90 min from left to right. Reaction intermediates (I), substrates (GD_{1041}), and products (GD_{432}) are marked.

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—E. coli recA protein was purified (≥98% pure by densitometric scan of polyacrylamide gels) and stored as described previously (Cox *et al.*, 1981). The recA protein concentration was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 0.58\ A_{280}\ mg^{-1}$ ml (Craig and Roberts, 1981). *Escherichia coli* SSB protein was purified as described (Lohman *et al.*, 1986) and was stored frozen at −70 °C in a buffer containing 20 mM Tris-HCl (40% cation, pH 8.4), 0.15 M NaCl, 1 mM EDTA, 1 mM β-mercaptoethanol, and 50% glycerol. The concentration of the SSB protein stock solutions was determined by absorbance at 280 nm using an extinction coefficient of $\epsilon_{280} = 1.5\ A_{280}\ mg^{-1}$ ml (Lohman and Overman, 1985). Restriction endonucleases and T4 polynucleotide kinase were purchased from New England Biolabs. Calf intestinal alkaline phosphatase, ATPγS, and Tris buffer were purchased from Boehringer Mannheim. Proteinase K, formamide, pyruvate kinase, phosphoenolpyruvate, creatine kinase, phosphocreatine, and ATP were obtained from Sigma. Radionucleotides were purchased from Amersham Corp., and 4′-amino-4,5′,8-trimethylpsoralen (AMT) was purchased from Calbiochem.

DNA—Duplex and ssDNA substrates were derived from bacteriophage M13mp8 (7229 bp, Messing and Vieira, 1982). Circular duplex and single-stranded DNA from M13mp8 and its derivatives were prepared using methods described previously (Davis *et al.*, 1980). The bacteriophage M13mp8.1041 (8270 bp) was constructed by inserting the 1041-base pair *Eco*RV fragment of the *galT* gene of *E. coli* into the *Sma*I site of bacteriophage of M13mp8 DNA (7229 bp).

Circular duplex DNA with a defined gap was prepared by a method described previously (Lindsley and Cox, 1989). In brief, the linear duplex DNA molecules of M13mp8 were completely denatured with alkaline solution. Excess of circular ssDNA of M13mp8.1041 was combined with the denatured DNA. Reannealed gapped duplex DNA molecules were purified with hydroxylapatite column chromatography, followed by the electroelution of the DNA band from an agarose gel after electrophoresis. The concentrations of double-stranded DNA stock solutions were determined by absorbance at 260 nm using 50 μg/ml A_{260} as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Complete digestion of FI M13mp8.1041 with restriction enzymes *Eco*RI and *Nco*I yielded linear duplex DNA substrates. The shorter linear duplex DNA were obtained by electroelution of DNA bands from agarose gels. Elution of DNA from agarose gels was performed by electroelution into dialysis membranes (Sambrook *et al.*, 1989).

Reaction Conditions—Unless stated elsewhere, all reactions were carried out at 37 °C in solutions containing 25 mM Tris acetate (80% cation, pH 7.5), 1 mM dithiothreitol, 5% glycerol, 3 mM potassium glutamate, 10 mM (or indicated concentration) magnesium acetate, and an ATP regenerating system (5 units/ml creatine kinase and 3 mM phosphoenolpyruvate or 10 units/ml creatine kinase and 12 mM phosphocreatine). DNA, SSB, and recA protein concentrations are indicated for each experiment. The preincubation of gapped duplex DNA with recA protein at 37 °C for 5 min was followed by addition of ATP (1 mM) and SSB. After an additional 5-min incubation, linear duplex DNA was added to start the four-strand exchange reactions. The standard reaction employed the order of addition as mentioned above unless indicated in the text for each experiment. SSB protein was preincubated with DNA substrates for 5 min before addition of recA protein when ATPγS (1 mM) was used. ATPγS was added with recA protein.

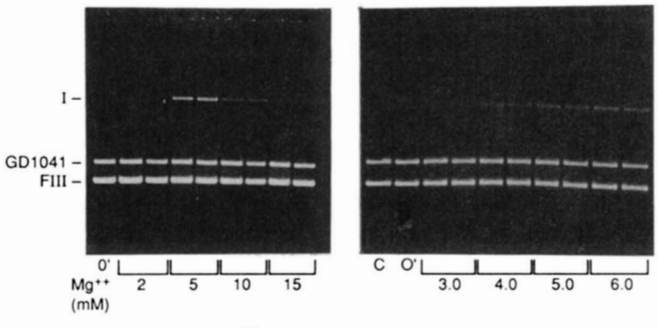
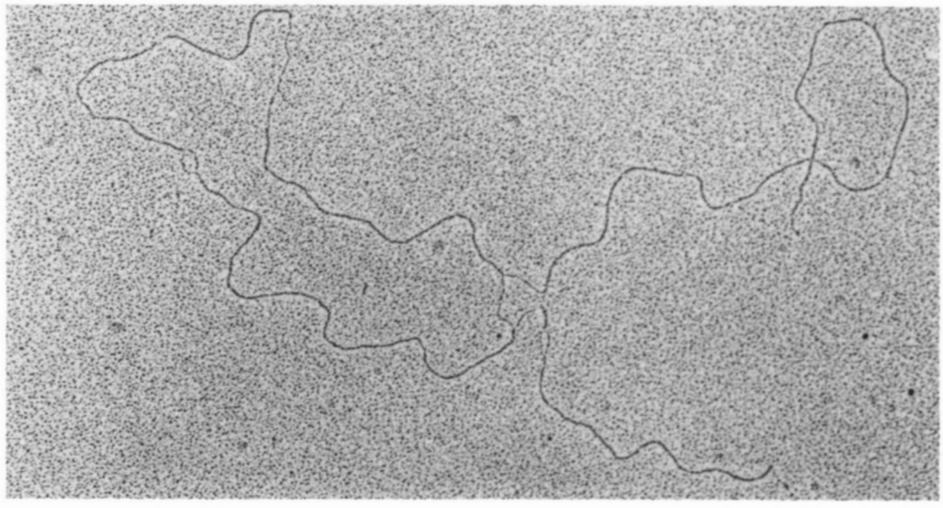


FIG. 5. **Effect of Mg^{2+} concentrations on recA protein-mediated four-strand exchange reactions in the presence of ATPγS.** Reactions were carried out as for those in Fig. 4, *lanes 6–10*, except that the magnesium acetate concentration was varied as indicated in *numbers* below *brackets*. *C* is a control with no recA protein (DNAs incubated with 5 mM magnesium acetate for 120 min). *O'* is a zero time control. The two lanes in each reaction are 60- and 120-min time points, left to right.

Agarose Gel Assays—Aliquots (20 μl) of the strand exchange reactions described above were removed at each time point, and the reactions were stopped by the addition of 5 μl of gel-loading buffer (0.125% bromphenol blue, 25 mM EDTA, 25% glycerol, and 5% SDS). These aliquots were stored on ice until after the last time point was taken. Samples were electrophoresed overnight in a 0.8% agarose gel. Where indicated, some samples were cross-linked with AMT before loading onto agarose gel electrophoresis.

Cross-linking Condition—Strand exchange reaction mixtures were cross-linked with AMT prior to deproteinization in order to prevent spontaneous branch migration. The psoralen derivative (AMT) was added to a final concentration of 30 μg/ml, and the samples were incubated for 3 min at room temperature followed by a 5-min exposure to long-wave UV light on ice. Intensity of UV light ranged between 2 and 4 milliwatts/cm^2 at 365 nm. This AMT cross-linking treatment resulted in at least one cross-link/220 bp (Bedale *et al.*, 1991).

Electron Microscopy—Samples for electron microscopy were obtained by spreading the entire strand exchange reaction mixture as described in the text. RecA and SSB proteins were removed after cross-linking by treatment with 1% SDS and 1 mg/ml (final concentration) proteinase K for 30 min at 37 °C. All cross-linked samples were either dialyzed against 20 mM NaCl and 5 mM EDTA overnight at room temperature, or loaded onto small spun columns (Umlauf *et*



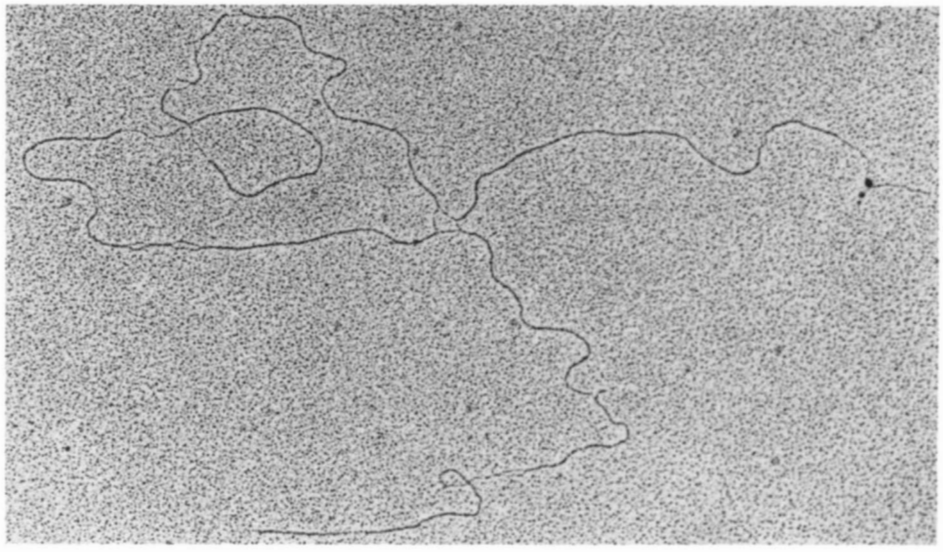


FIG. 6. **Electron microscopy of reaction intermediates generated during recA protein-mediated four-strand exchange reactions with ATP.** Reactions were carried out and samples treated and spread as described under "Experimental Procedures." These intermediates were found in a 60-min reaction. The Holliday junction, with all four strands intact, is evident in both cases.

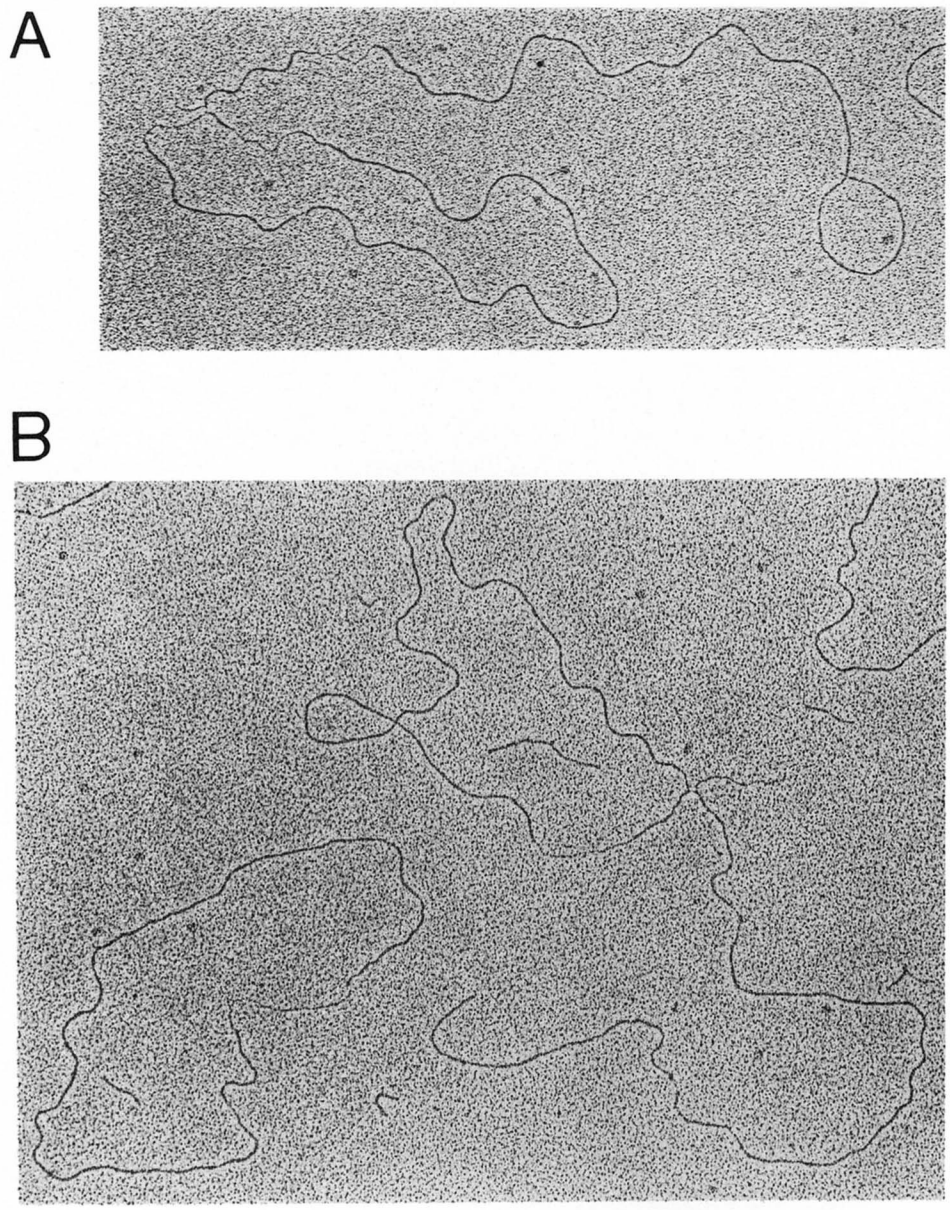


FIG. 7. **Electron microscopy of reaction intermediates generated during recA protein-mediated four-strand exchange reactions with ATPγS.** Reactions were carried out as in Fig. 4, *lanes 6–10*. These molecules were observed in a 60-min reaction sample. Short, displaced single strands are evident at the junction in both intermediate molecules. The second molecule (lower left) in *B* is an unreacted molecule of GD_{1041}.

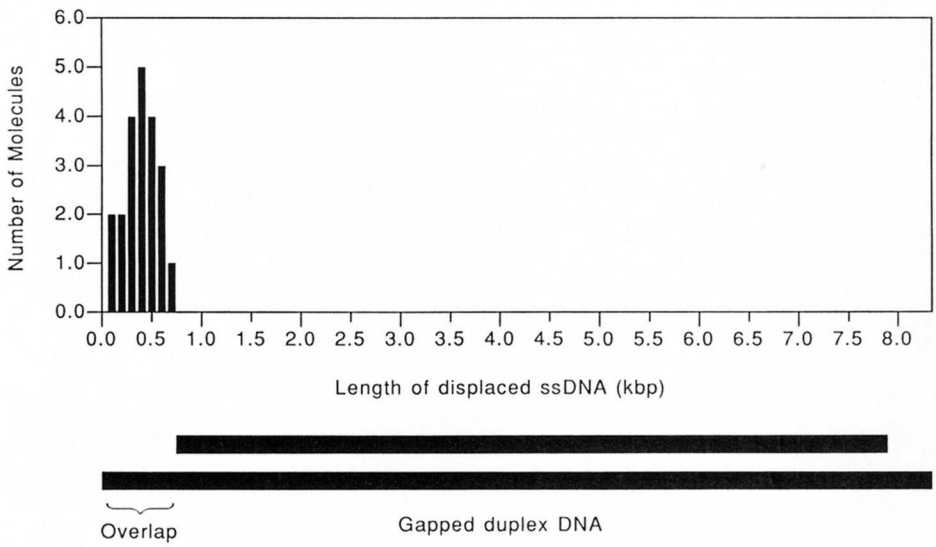


FIG. 8. **Distribution of the lengths of heteroduplex DNA (generated in the reaction of Fig. 7).** The displaced single strand was measured in 25 molecules as described in the text. The schematic illustration at the bottom indicates the length of the single-strand gap overlapped by the linear duplex substrate.

al., 1990), and the column flow-through was dialyzed against 20 mM NaCl and 5 mM EDTA. The structures of intermediates formed in the strand exchange reactions were verified by partially denaturing the samples (Inman and Schnös, 1970). Low degrees of denaturation allowed the examination of strand continuity at junctions. Photography and computer-assisted length measurements of DNA molecules

were performed as described previously (Littlewood and Inman, 1982).

RESULTS

Experimental Design—The purpose of the study was to determine if ATP hydrolysis is required for recA-mediated four-strand exchange reactions. As in the previous paper, the criterion used is based on the ATPγS-mediated three-strand exchange reaction observed by Menetski *et al.* (1990) as follows. If ATPγS also supports a four-strand exchange, then ATP hydrolysis is *not* required for four-strand exchanges. The substrates used in this study are illustrated in Fig. 3. Four-strand exchange reactions were carried out with ATP or ATPγS under conditions separately optimized for each as described under "Experimental Procedures" and in the preceding paper. The homologous sequences in the linear duplex overlap the single strand gap in the gapped duplex ($GD_{1,041}$) by 609 bp. This extensive overlap was designed to permit formation and detection of three-stranded joint molecules within the gap whether or not a four-strand exchange took place. ATPγS permits efficient and extensive strand exchange with three strands, and the presence of three-strand joint molecules in the reaction provides an important positive control. Electron microscopy was used to complement agarose gel electophoresis assays. DNAs were cross-linked before recA removal to prevent spontaneous branch migration, and the heteroduplex DNA formed in individual joint molecules could then be measured directly.

ATPγS Does Not Support Four-strand Exchange Reactions—Reactions carried out with ATP or ATPγS are compared in Fig. 4. Reaction conditions differ primarily in the inclusion of an ATP regenerating system in the ATP reaction, and the use of lower (5 mM) magnesium acetate concentrations in the ATPγS reaction as required for an optimal reaction in each case. An efficient strand exchange is observed in the ATP reaction, with a substantial yield of product (a gapped duplex with a 432-nucleotide gap, GD_{432}) seen after 60 min. At earlier times, much of the DNA is found in bands that correspond to strand exchange intermediates (joint molecules).

In contrast, the ATPγS reaction is much less efficient. Some joint molecules are formed, but these are much less abundant and more homogeneous than those seen in the ATP reaction.

The inefficient reaction mediated by ATPγS suggested that the conditions used for three-strand exchange with ATPγS might not be optimal for four-strand exchange reactions. We therefore varied reaction conditions to determine if a more efficient four-strand exchange could be generated with ATPγS. The results in Fig. 5 (in which Mg^{2+} concentration is varied) show that altering the reaction conditions does not improve the reaction. As with the three-strand reaction with ATPγS (Menetski *et al.*, 1990), a sharp reaction optimum is seen with 4–5 mM Mg^{2+} acetate. We found no set of conditions that improved the result shown in Fig. 4.

Joint molecules generated in both the ATP and ATPγS reactions were characterized by electron microscopy, as shown in Figs. 6 through 8. For these experiments, the entire reaction mixture was cross-linked, deproteinized, and spread as described under "Experimental Procedures." At early times the ATP reaction generates substantial amounts of reaction intermediates (34% of total DNA molecules observed) such as those illustrated in Fig. 6. In most cases the exchange proceeds well past the gapped region and leads to Holliday junctions formed by four-strand exchanges. At later times, many of these intermediates are converted to reaction products, as seen in Fig. 4 and in many earlier studies. Intermediates generated in the ATPγS reaction involved only 5% of the DNA substrates present. Typical examples are shown in Fig. 7. In every case, joint molecules contained only short regions of heteroduplex DNA. The short arm in these molecules was single-stranded, and only three strands were evident at the junctions, indicating that strand exchange had not proceeded beyond the gap and into the four-strand region. Twenty-one of these molecules were chosen at random and the lengths of the circle and the long and short arms were measured. The lengths of the short single-strand arms, which correspond to the lengths of heteroduplex DNA generated in each molecule, are presented in Fig. 8. The distribution halts abruptly at the boundary of the gap, confirming that ATPγS does not support strand exchange into the four-strand region.

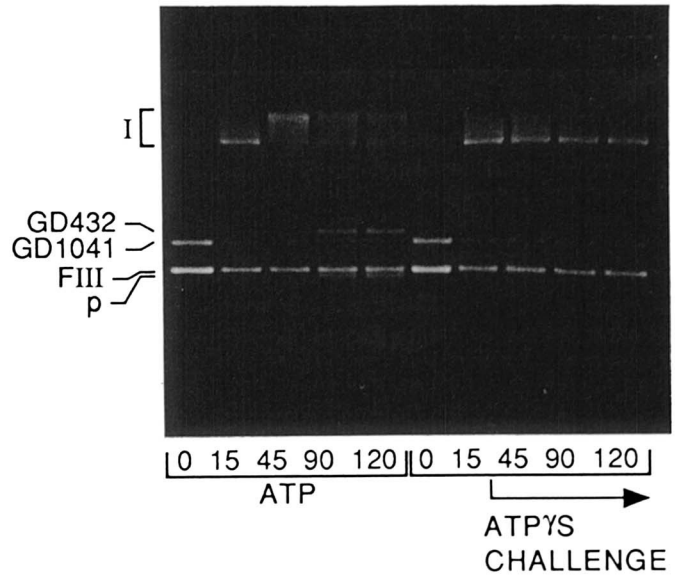


FIG. 9. **Addition of ATPγS halts an ongoing four-strand exchange reaction.** Reactions were carried out as in Fig. 4, except that 5 mM Mg^{2+} acetate was used throughout. Numbers below the gel lanes denote time in minutes. The reaction at right was challenged just after the $t = 15$ min time point with the addition of ATPγS to 1 mM final concentration. The same result was obtained when 10 mM Mg^{2+} acetate was used in both reactions (not shown).

We reasoned that this barrier might reflect some critical step in the transition from three- to four-strand reaction at the gap boundary and that ATPγS might support four-strand exchanges once they were initiated. We therefore tried several experiments in which four-strand exchanges were initiated with ATP, then challenged with ATPγS at an early point in the reaction. As shown in Fig. 9, the challenge with ATPγS effectively "freezes" the reaction. The migration of reaction intermediates in an agarose gel has been characterized in some detail.[2] Early (and late) intermediates with one short arm and one long arm migrate faster than "half-way" intermediates with two arms of equal length. Hence, the intermediates seen early in the reaction generally migrate faster in the gel than those observed later, as seen in the ATP reaction in Fig. 9. The challenge with ATPγS not only prevented the formation of reaction products, but also froze the distribution of reaction intermediates at the point at which it was added. The result was the same with either 5 mM (shown in Fig. 9) or 10 mM magnesium acetate (data not shown), except that the ATP-mediated strand exchange was somewhat more efficient in the latter case.

[2] R. B. Inman, unpublished data.

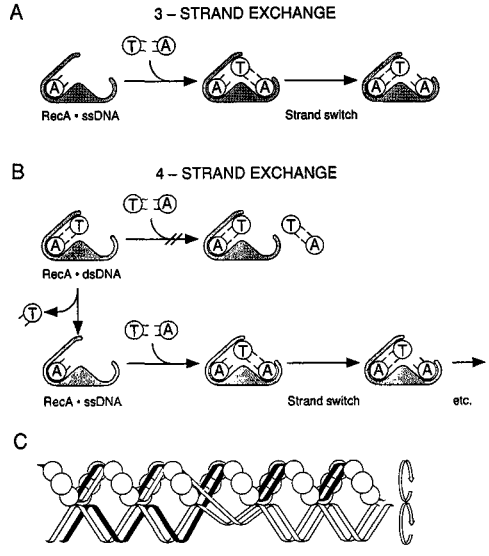


FIG. 10. **Mechanism for the DNA strand switch in recA protein-mediated DNA strand exchange.** This model is based on the observations of Takahashi *et al.* (1989, 1991) and Kubista *et al.* (1990) that document binding sites for three DNA strands in the filament groove. *A*, a three-strand exchange occurs readily within the groove, with one strand of the duplex switching to pair with the single strand in the site at lower left. The switch would be facilitated by the extension of the DNA bound to recA (which disrupts stacking interactions) and binding energy that could favor product formation. In this arrangement, homologous alignment may involve a triple-helical intermediate or it could simply involve a rapid sampling mechanism in which the strand to be switched moves back and forth between alternate pairing partners. Note that, in this mechanism, the ssDNA to which recA protein is first bound remains in one location and orientation throughout the reaction. *B*, in the case of a four-strand exchange, one strand of the duplex DNA in the filament must be removed before the second duplex DNA can enter the groove. *C*, a facilitated rotation of the two DNAs as shown will bring about strand separation of the duplex DNA in the groove prior to introduction of a new DNA strand.

DISCUSSION

Our primary conclusion is that ATP hydrolysis is required for four-strand exchange reactions mediated by recA protein. ATPγS permits the formation of three-strand joint molecules within the single-stranded region of a gapped duplex DNA, as expected from the results of Menetski *et al.* (1990) and Rosselli and Stasiak (1990). However, strand exchange does not proceed beyond the gap into the four-strand region.

The accompanying paper provides evidence that ATP hydrolysis is required for strand exchange past a structural barrier in one DNA substrate. Previous work (Lindsley and Cox, 1990) has shown that ATP hydrolysis is also required for the dissociation of recA protein from one end of a filament. The present results suggest an important third function for ATP hydrolysis in this system.

The results also bear upon current models for recA protein-mediated strand exchange, since they provide one of the few clear experimental distinctions between the three- and four-strand reactions. If recA protein carried out strand exchange by aligning three or four DNA strands in the groove and facilitating a strand switch as illustrated in Fig. 2, it would be reasonable to expect that ATPγS would promote four-strand exchange reactions as well as three-strand exchanges. However, the four-strand reaction is completely blocked with ATPγS. This result, when combined with other experimental results outlined in the Introduction, tends to support the idea that recA protein can coordinate no more than three strands in the groove. As shown in Fig. 10, this presents an interesting problem: if a duplex substrate is bound in the groove, one strand must be removed before the second duplex can enter. A simple solution to this problem can be seen if strand exchange is envisioned as an ordered rotation of the two DNA duplexes so that the exchanging strands spool from one to the other as strand exchange proceeds (Fig. 10*C*). The rotation itself would lead to strand separation and displacement, with strand replacement following close behind. Once strand exchange was initiated, there would be no further need for the concurrent pairing of three or four strands as long as both DNAs remained in homologous register. It is clear from model building that recA protein-mediated DNA strand exchange involves rotation of the two DNA substrates, and direct experimental tests (Honigberg and Radding, 1988) indicate a rotation around the longitudinal axes of the DNAs, as shown in Fig. 10*C*, must take place. We have suggested elsewhere that ATP hydrolysis in the recA system is directly coupled to an ordered rotation of DNAs of the kind illustrated in Fig. 10*C* and proposed a mechanism by which this coupling might occur (Cox *et al.*, 1987; Cox, 1989, 1990; Roca and Cox, 1990).

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