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DNA Strand Exchange Promoted by RecA K72R

TWO REACTION PHASES WITH DIFFERENT Mg^{2+} REQUIREMENTS*

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Qun Shan, Michael M. Cox‡, and Ross B. Inman

From the Department of Biochemistry, University of Wisconsin-Madison, Madison, Wisconsin 53706

Replacement of lysine 72 in RecA protein with arginine produces a mutant protein that binds but does not hydrolyze ATP. The protein nevertheless promotes DNA strand exchange (Rehrauer, W. M., and Kowalczykowski, S. C. (1993) *J. Biol. Chem.* 268, 1292–1297). With RecA K72R protein, the formation of the hybrid DNA product of strand exchange is greatly affected by the concentration of Mg^{2+} in ways that reflect the concentration of a Mg·dATP complex. When Mg^{2+} is present at concentrations just sufficient to form the Mg·dATP complex, substantial generation of completed product hybrid DNAs over 7 kilobase pairs in length is observed (albeit slowly). Higher levels of Mg^{2+} are required for optimal uptake of substrate duplex DNA into the nucleoprotein filament, indicating that the formation of joint molecules is facilitated by Mg^{2+} levels that inhibit the subsequent migration of a DNA branch. We also show that the strand exchange reaction promoted by RecA K72R, regardless of the Mg^{2+} concentration, is bidirectional and incapable of bypassing structural barriers in the DNA or accommodating four DNA strands. The reaction exhibits the same limitations as that promoted by wild type RecA protein in the presence of adenosine 5′-*O*-(3-thio)triphosphate. The Mg^{2+} effects, the limitations of RecA-mediated DNA strand exchange in the absence of ATP hydrolysis, and unusual DNA structures observed by electron microscopy in some experiments, are interpreted in the context of a model in which a fast phase of DNA strand exchange produces a discontinuous three-stranded DNA pairing intermediate, followed by a slow phase in which the discontinuities are resolved. The mutant protein also facilitates the autocatalytic cleavage of the LexA repressor, but at a reduced rate.

The RecA protein of *Escherichia coli* is a 352-amino acid polypeptide chain with a predicted molecular weight of 37,842. The protein is found in all bacteria and is critical to the processes of recombinational DNA repair, homologous recombination, induction of the SOS response to DNA damage, SOS mutagenesis, and the partitioning of chromosomes at cell division (Clark and Sandler, 1994; Cox, 1994; Kowalczykowski *et al.*, 1994; Stasiak and Egelman, 1994; Livneh *et al.*, 1993; West, 1992; Roca and Cox, 1990).

In vitro, RecA protein promotes a set of DNA strand exchange reactions that mimics its presumed role in recombination and recombinational DNA repair. The reactions can involve either three or four DNA strands (Fig. 1). RecA first forms a nucleoprotein filament on the single-stranded or gapped DNA substrate. This DNA is then aligned with a homologous linear duplex DNA. A strand switch then occurs within the filament producing a nascent region of hybrid DNA, which is extended to generate the products shown. In a normal reaction, strand exchange proceeds unidirectionally, 5′ to 3′ relative to the single-stranded DNA (or the single-stranded region of the gapped DNA) to which the RecA first binds. The reaction also proceeds past a variety of structural barriers in the DNA substrates.

RecA protein is a DNA-dependent ATPase, with a monomer k_{cat} of 30 min^{-1} when bound to ssDNA.[1] ATP is hydrolyzed uniformly throughout the nucleoprotein filament (Brenner *et al.*, 1987). When a homologous duplex DNA is added to the reaction, the k_{cat} drops abruptly to about 20 min^{-1}, and remains at that level throughout the ensuing DNA strand exchange reaction as long as ATP is regenerated (Schutte and Cox, 1987; Ullsperger and Cox, 1995). The reaction is apparently quite inefficient, with about 100 ATPs hydrolyzed per base pair of hybrid DNA created. The function of this ATP hydrolysis is incompletely understood. An important clue was provided by the observation that RecA protein can promote DNA strand exchange under some conditions in the presence of ATPγS, an ATP analog that is not readily hydrolyzed by RecA (Menetski *et al.*, 1990; Rosselli and Stasiak, 1990). This observation was reinforced more recently by work on the RecA mutant K72R (Rehrauer and Kowalczykowski, 1993) and again with wild type RecA protein in the presence of ADP-AlF_4^- (Kowalczykowski and Krupp, 1995). The Lys → Arg substitution in the K72R mutant occurs in a well conserved nucleotide binding fold, and the mutant protein binds but does not hydrolyze ATP. The mutant will also promote a limited DNA strand exchange, functioning best with dATP (Rehrauer and Kowalczykowski, 1993). These results demonstrate that the RecA filament has an inherent capacity to take up at least three DNA strands and promote DNA strand exchange without ATP hydrolysis, and have been used to argue against an essential role for ATP hydrolysis in DNA strand exchange. The RecA filament tends to stabilize the hybrid DNA products of a DNA strand exchange reaction (Adzuma, 1992).

Why, then, does RecA protein hydrolyze ATP? One way to elucidate the function of RecA-mediated ATP hydrolysis is to define the limitations of reactions that occur without it. A series of studies determined that the strand exchange occurring with ATPγS is limited in extent and bidirectional (Jain *et*

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‡ To whom correspondence should be addressed: Dept. of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706. Tel.: 608-262-1181; Fax: 608-265-2603; E-mail: COXLAB@MACC.WISC.EDU.

[1] The abbreviations used are: ssDNA. single-stranded DNA; dsDNA, double-stranded DNA; wtRecA, the wild type RecA protein; DTT, dithiothreitol; AMT, 4′-aminomethyl-4,5′,8-trimethylpsoralen; bp, base pair(s); kbp, kilobase pair(s); ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; SSB, the single-stranded DNA binding protein of *E. coli*; PAGE, polyacrylamide gel electrophoresis.

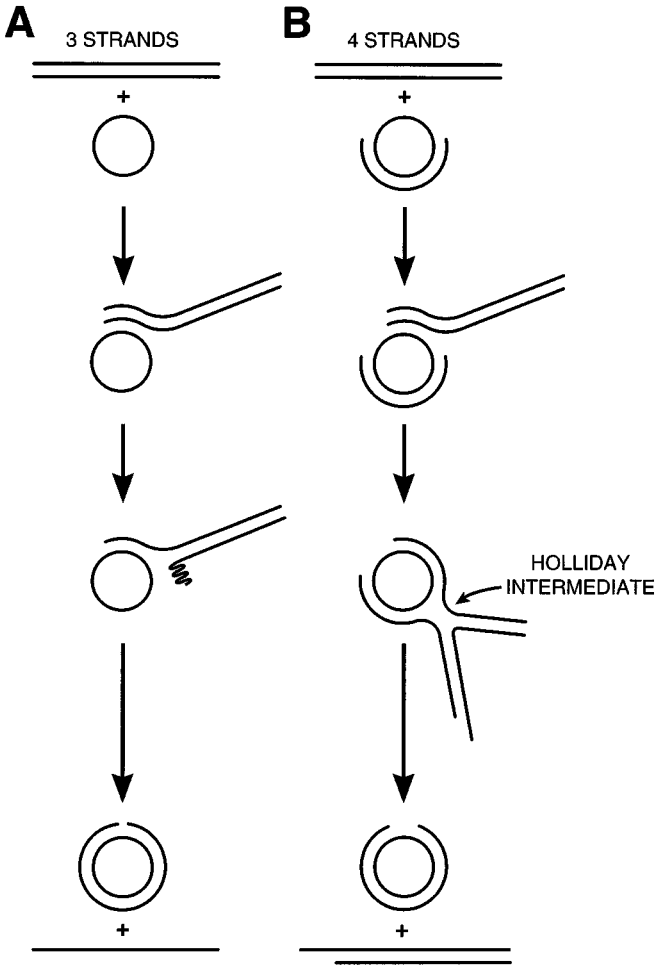


FIG. 1. **RecA protein-mediated DNA strand exchange reactions.** Typical reactions involving three and four DNA strands are shown.

al., 1994; Konforti and Davis, 1992). The ATPγS reactions also did not proceed past structural barriers in the DNA (Kim *et al.*, 1992a; Rosselli and Stasiak, 1991) and did not accommodate four DNA strands (Kim *et al.*, 1992b). ATP hydrolysis therefore appears to confer several important properties to the DNA strand exchange reaction, being required in particular for unidirectional branch movement that can bypass barriers and for four-strand exchange reactions. These observations provide some indirect mechanistic clues but have been obtained only under the conditions used with ATPγS.

The properties of RecA-mediated DNA strand exchange in the absence of ATP hydrolysis provoke questions which, if answered, might shed additional light on ATP function and strand exchange mechanism. In studies to date with three-strand exchange reactions using homologous substrates, there is a rapid formation of a limited segment of hybrid DNA product (typically 1–3 kbp). However, at this point the reaction halts or slows dramatically. Since the entire filament is capable of promoting an exchange between homologous substrates, it is surprising that the entire reaction does not proceed to completion. The slowing or cessation of strand exchange implies that discontinuities exist in some component of an early strand exchange intermediate. The one possibility presented to date involves filament discontinuities, with ATP hydrolysis needed to recycle RecA monomers and correct the discontinuities (Menetski *et al.*, 1990; Rehrauer and Kowalczykowski, 1993; Kowalczykowski and Krupp, 1995). As shown below, the only reasonable alternative involves discontinuities in a key DNA structure that serves as a strand exchange intermediate.

A similar and related unresolved question can be defined even under conditions in which ATP is being hydrolyzed. Upon addition of a homologous duplex DNA to RecA·ssDNA complexes hydrolyzing ATP, the rate of ATP hydrolysis declines abruptly by up to 30%. The observed decline is directly proportional to the length of homologous sequence in the duplex, providing evidence that the entire length of available homology is detected within a minute or two with direct DNA-DNA interactions occurring over distances of 8 kbp or more (Schutte and Cox, 1987). However, productive strand exchange detectable after RecA removal from the DNA proceeds much slower, requiring 20 min or more to encompass the same 8 kbp. There again appears to be a fast phase of strand exchange in which some short length of hybrid DNA is generated, followed by a slow phase in which the nascent hybrid DNA is extended. The fast phase is sometimes manifested as an apparent burst phase in hybrid DNA formation when ATP is hydrolyzed (Kahn and Radding, 1984; Bedale and Cox, 1996). As in the cases where ATP is not hydrolyzed, it is necessary to explain why the fast phase comes to an end before strand exchange is complete, even though the response of the filament indicates the detection of homology along the entire length of the DNA.

In this report, we further explore the properties of the fast and slow reaction phases and present a simple model that explains why the fast phase is limited in extent. The model also explains all properties of the two reaction phases and applies to reactions carried out with or without hydrolysis of ATP. The results complement and/or confirm a number of previous observations obtained with ATPγS, using RecA K72R employed under more classical reaction conditions, and further characterize the RecA K72R mutant protein. To date, many aspects of the DNA strand exchange reaction promoted by the RecA K72R mutant protein remain unexplored, but have the potential to test many of the ideas outlined above about the role of the RecA ATPase activity.

MATERIALS AND METHODS

Enzymes and Biochemicals—E. coli RecA protein was purified 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 $\epsilon_{280} = 0.59\ A_{280}\ mg^{-1}$ ml (Craig and Roberts, 1981). *E. coli* single-stranded DNA binding protein (SSB) was purified as described (Lohman *et al.*, 1986) with the minor modification that a DEAE-Sepharose column was added to ensure removal of single-stranded DNA exonucleases. The concentration of SSB protein 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). Purified LexA repressor was a generous gift from Dr. John Little (University of Arizona). Oligonucleotides were synthesized by the University of Wisconsin Biochemistry Department Synthesis Facility. The Sequenase version 2.0 sequencing kit was from U. S. Biochemical Corp. Restriction endonucleases, β-agarase, and T4 polynucleotide kinase were purchased from New England Biolabs. Terminal transferase and ATPγS were purchased from Boehringer Mannheim. Ultrapure dATP, DEAE-Sepharose resin, and a Mono Q column were from Pharmacia Biotech Inc. Amino-4,5′,8-trimethylpsoralen (AMT) was from Calbiochem. Tris buffer was from Fisher. ATP, proteinase K, lactic dehydrogenase, pyruvate kinase, phosphoenolpyruvate, and nicotinamide adenine dinucleotide (reduced form, $NADH^+$), creatine phosphokinase, phosphocreatine, and low melting agarose were purchased from Sigma. Hydroxylapatite resin was from Bio-Rad.

DNA—Duplex and ssDNA substrates were derived from bacteriophage φX174 (5386 bp) and M13mp8 (7229 bp) (Messing, 1983). φX174 supercoiled circular duplex DNA and viral circular ssDNA were purchased from New England Biolabs. Bacteriophage M13mp8.52 (7251 bp) is bacteriophage M13mp8 with a short heterologous sequence (52 bp) originally derived from the plasmid pJFS36 (Senecoff *et al.*, 1985) replacing the 30-bp *Eco*RI-*Pst*I fragment of bacteriophage M13mp8 (Kim *et al.*, 1992a). Bacteriophage M13mp8.1037 (8266 bp) is bacteriophage M13mp8 with 1037 bp (*Eco*RV fragment from the *E. coli galT* gene) inserted into the *Sma*I site (previously called M13mp8.1041)

(Lindsley and Cox, 1990b). Supercoiled circular duplex DNA and circular single-stranded DNA from bacteriophage M13mp8 and its derivatives were prepared as described previously (Davis *et al.*, 1980; Messing, 1983; Neuendorf and Cox, 1986). The concentration of dsDNA and ssDNA stock solutions were determined by absorbance at 260 nm, using 50 and 36 mg ml^{-1} $A_{260}{}^{-1}$, respectively, as conversion factors. DNA concentrations are expressed in terms of total nucleotides. Linear duplex DNA substrates were generated by complete digestion of supercoiled DNA by appropriate restriction endonucleases. The protein was removed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) followed by ethanol precipitation. Linear duplex DNA fragments were generated by digesting supercoiled DNA with appropriate restriction endonucleases and isolating from preparative low melting agarose gel using β-agarase or as described (Sambrook *et al.*, 1989). The fragments were then extracted twice with Tris-EDTA-saturated butyl alcohol, followed by 1:1 extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The fragments were finally concentrated by ethanol precipitation. Gapped duplex DNA substrates were prepared using a large scale (reaction volume = 1.2 ml) RecA reaction. The reaction contained 20 μM circular M13mp8.1037 ssDNA, 20 μM M13mp8 linear double-stranded DNA (digested with *Sma*I), 6.7 μM wtRecA protein, 2 μM SSB, and 3 mM ATP. Reactions were carried out under standard strand exchange conditions listed below, for 90 min. The reaction was stopped by adding EDTA, SDS, and proteinase K to 12 mM, 1%, and 1 mg/ml, respectively. The mixture was incubated at 37 °C for 30 min. The reaction mixture was then extracted 1:1 with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1), and the DNA was concentrated in a Microcon concentrator (Amicon). The concentrated reaction mixture was electrophoresed overnight in a 0.8% low melting agarose gel at about 2 V cm^{-1}. The gapped duplex DNA was purified from low melting agarose as described above. The resulting gapped duplex species is called GD1037 (formerly called GD1041) (Kim *et al.*, 1992b)

Buffers—P buffer contained 20 mM potassium phosphate (pH 6.8), 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol. R buffer contained 20 mM Tris-HCl, 80% cation (pH 7.5), 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol.

Cloning and Overexpressing the recA K72R Gene—The recA K72R gene was cloned by a polymerase chain reaction-based, site-directed mutagenesis procedure. Briefly, a DNA fragment containing the first 236 bp of the recA coding region was polymerase chain reaction synthesized with one primer containing the mutation code for Arg (CGA) at residue 72 and the internal *Pst*I site within the *recA* gene. This fragment was cloned into pBluescriptSK(−) (Stratagene). The identity of the mutated *recA* gene fragment was checked by sequencing, and subsequently the *recA* gene fragment containing the desired mutation was swapped with corresponding fragment from wild-type RecA gene in pUC18, to create pRecA17 (4.0 kbp). The integrity of the cloned *recA* K72R gene was confirmed by direct sequencing. To express the *recA* K72R gene, a 16-liter culture of *E. coli* strain GE643 (lexA51, ΔrecA1398) (Weisemann and Weinstock, 1985) transformed with pRecA17 was grown in LB medium for 16 h at 37 °C with aeration. Cells were collected by centrifugation at 4200 rpm at 4 °C in a Beckman JA6 rotor. The cell pellet was washed with cell harvest buffer (50 mM Tris-HCl, 80% cation, pH 7.5, 10% sucrose) and then centrifuged again. The pellet was resuspended in cell harvest buffer to give an OD_{600} reading of about 400. The cell suspension was quickly frozen in liquid N_2 and stored at −70 °C.

Purification of the RecA K72R Protein—The RecA K72R protein was purified by a modification of the procedure used for the wild type RecA protein. Briefly, 50 g of cell paste were thawed on ice overnight. Cell lysis, polymin P precipitation, and ammonium sulfate extraction were performed as described by Cox *et al.* (1981). The supernatant from the ammonium sulfate extraction was precipitated three times by adding solid ammonium sulfate to 0.28 g/ml. The pellet was finally resuspended in 10 ml of R buffer + 50 mM KCl and dialyzed overnight against 2 × 1 liter of the same buffer, giving rise to fraction II (18.8 ml, 368 mg of protein). This was loaded onto a DEAE-Sepharose column (2.5 cm × 14 cm, 70-ml bed volume). The column was washed with R + 50 mM KCl buffer, and the majority of the RecA protein appeared in the flow-through. Peak fractions were identified by SDS-PAGE. Protein in the pooled peak fractions was precipitated by adding solid ammonium sulfate to 0.28 g/ml, collected by low speed centrifugation, resuspended in 10 ml of P buffer, and dialyzed against 2 × 1 liter of P buffer overnight to generate fraction III (10 ml, 140 mg protein). Fraction III was loaded onto a hydroxylapatite column (2.5 cm × 7 cm, 35-ml bed volume). The column was washed with 40 ml of P buffer and developed with 360 ml of linear gradient from 20 to 500 mM phosphate buffer (pH 6.8). Protein-containing fractions were located by SDS-PAGE, pooled, and dialyzed against 3 × 2 liters of R + 50 mM KCl to generate fraction IV (47 ml, 74 mg of protein). Fraction IV was applied to a 1-ml Mono Q fast protein liquid chromatography column for several runs using a superloop, and the column was washed with 3 ml of R + 50 mM KCl buffer and developed with 30 ml of 0.05 to 1 M KCl linear gradient. Peak fractions were pooled and dialyzed against 2 × 3 liters of R buffer, producing fraction V (11 ml). This fraction was frozen in liquid nitrogen and stored at −70 °C. The final yield of the RecA K72R protein from this 50-g cell paste was 22 mg. The RecA K72R protein was at least 98% pure as judged by a densitometric scan of a Coomassie Blue-stained SDS-PAGE gel. The concentration of the RecA K72R protein was determined using the same extinction coefficient as wild type RecA protein. The RecA K72R protein was free of detectable endo- or exonucleases.

Strand Exchange Reaction Conditions—Unless otherwise specified, all reactions were performed at 37 °C in a standard strand exchange reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM DTT, 5% (w/v) glycerol, and an ATP (4.7 mM phosphoenolpyruvate, 5 units ml^{-1} pyruvate kinase) or a dATP (11.8 mM phosphoenolpyruvate, 10 units ml^{-1} pyruvate kinase) regeneration system. Linear duplex DNA and circular single-stranded or gapped duplex DNA were preincubated with RecA protein or RecA K72R protein for 10 min before the indicated concentrations of dATP (or ATP) and SSB protein were added to initiate the reaction. A regeneration system was omitted in some experiments as noted. The magnesium acetate and dATP concentrations, as well as the order of addition of RecA K72R and SSB, were varied in some experiments as indicated in the text and figure legends.

After the gel was stained with ethidium bromide (1 μg/ml) for at least 30 min and destained for at least 2 h, the gel was then photographed over an ultraviolet transilluminator. The intensities of DNA bands were quantified by scanning the photographic negatives using a Molecular Dynamics Personal Densitometer SI and analyzing the image with ImageQuant software (Version 4.2). In order to correct for variability in sample loading onto the agarose gel, the band corresponding to full-length products and/or the broad smear representing intermediates of the strand exchange reaction were quantified as the fraction of the total fluorescing DNA in a given gel lane.

In some experiments, the data was plotted with respect to the concentration of Mg^{2+} in excess of that involved in a complex with dATP. The concentration of "excess" Mg^{2+} was calculated based on the reported dissociation constant of 1×10^{-4} M for the Mg·ATP complex (Alberty, 1969).

Agarose Gel Assays—Aliquots (10 μl) of the reactions were removed at each indicated time point, and the reactions were stopped by the addition of 0.25 volume of gel loading buffer (60 mM EDTA, 5% SDS, 25% (w/v) glycerol, 0.2% bromphenol blue). Samples were electrophoresed overnight in a 0.8% agarose gel at 2 V cm^{-1}. In some experiments, aliquots of the reactions were cross-linked with AMT before gel electrophoresis as described below.

DNA-dependent ATPase and dATPase Assays—The ssDNA-dependent ATPase or dATPase activity of the RecA K72R and the wild type RecA protein was measured by a coupled enzyme assay (Lindsley and Cox, 1990a; Morrical *et al.*, 1986). In addition to the appropriate phosphoenolpyruvate/pyruvate kinase regeneration system described above, reactions contained 3 mM NADH and 4.5 units ml^{-1} lactic dehydrogenase. Absorbances were measured at 380 nm, rather than 340 nm (the absorbance maximum for NADH), to remain within the linear range of the spectrophotometer. An NADH extinction coefficient of ϵ_{380} = 1.21 mM^{-1} cm^{-1} was used to calculate the rate of ATP or dATP hydrolysis. Reaction mixtures (400 μl) also contained 8 μM circular M13mp8 ssDNA and RecA protein as noted, in standard strand exchange reaction buffer. Reactions were started by the addition of SSB protein and ATP or dATP to final concentrations of 0.8 μM and 3 mM, respectively.

Electron Microscopy—Samples for electron microscopy were obtained by spreading the entire strand exchange reaction mixture. Reaction mixtures were cross-linked with AMT prior to examination by electron microscopy to prevent spontaneous branch migration during sample preparation. Samples were cross-linked by addition of AMT to a final concentration of 30 μg ml^{-1}, incubated at room temperature for 3 min and irradiated with long wave UV light (Umlauf *et al.*, 1990) for 4 min at room temperature. The UV light was generated with two 15-watt fluorescent black light tubes. Samples were placed 8 cm below the UV light source. The cross-linked samples were incubated with proteinase K (1 mg ml^{-1} final) and SDS (1% final) for 30 min at 37 °C. The samples were dialyzed into 20 mM NaCl and 5 mM EDTA for 5 h at room

temperature on Millipore type VM (0.05 mm) filters (Jain *et al.*, 1994) and then spread as described previously (Inman and Schnös, 1970). Photography and measurements of the DNA molecules were performed as described previously (Littlewood and Inman, 1982).

Three types of information were derived from the electron microscopy experiments. First we wished to confirm that the reaction intermediates observed on gels had the anticipated structure. Representative molecules are shown for some experiments and results described in the text. Second, we wished to determine the proportion of the duplex linear substrates that was involved in DNA strand exchange reactions leading to intermediates. This was done by counting the intermediates and unreacted linear duplex DNA molecules found in a representative sample from each experiment. 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 complex reaction products in the reactions) were ignored in these estimates. In some experiments, the grids from different reactions were assigned an undescriptive identification code by Q. S., and were subsequently counted in a random sequence by R. B. I. Third, it was important to estimate the length of hybrid DNA generated for a representative sample of intermediates in some experiments. Because of the large numbers of samples, obtaining accurate measurements of significant numbers of intermediates in all of them was impractical; we have therefore estimated the degree of exchange as described earlier (Jain *et al.*, 1994). Briefly, the ratio of unexchanged to exchanged duplex DNA was judged. These ratios were then converted into base pairs of exchanged DNA using the known length of the linear dsDNA substrate. The data were sorted into 8–10 degrees of exchange and plotted as histograms. Degrees of exchange with the 1.3-kbp DNA fragment used as a substrate in some experiments was divided in this manner into eight equal segments of 165 bp; linear M13mp8.1037 dsDNA was divided into 10 segments of 830 bp.

These judgments were checked in two ways. First, two grids each from two different samples were counted and judged "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. In both cases, the data sets for the two samples were not significantly different. The judgments have also been checked directly by comparing the data obtained to data from more detailed measurements done on the same samples (Jain *et al*., 1994). Statistical analysis again showed that there is no significant difference in the result obtained with the two methods.

LexA Repressor Cleavage Assay—Reactions (100 μl) were carried out at 37 °C and contained 20 mM Tris-HCl (80% cation, pH 7.5), 50 mM NaCl, 4 mM $MgCl_2$, 1 mM ATP, an ATP regeneration system (12 mM phosphocreatine, 10 units ml^{-1} creatine phosphokinase), 40 μM circular M13mp8 ssDNA, 8.9 μM LexA repressor, and 1.33 μM wild type RecA or RecA K72R protein. The reactions did not contain SSB (Little, 1984). Additional LexA repressor cleavage assays were performed using dATP (with the dATP regeneration system described above) or ATPγS (1 mM) as a nucleotide cofactor. Aliquots (10 μl) were removed at different time points, followed by addition of 5 μl of SDS-PAGE loading buffer (250 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 100 mM 2-mercaptoethanol, 0.1% bromphenol blue). Samples were heated at 95 °C for 3 min before they were analyzed by 13% SDS-PAGE. The destained gels were scanned and quantified using the NIH Image Analysis Program (Version 1.44).

Copies of the electron micrographs discussed in this publication are available for viewing or downloading using a World-Wide Web client at the URL: http://phage.bocklabs.wisc.edu/.

RESULTS

Experimental Design—The goal of these experiments is a more complete characterization of the DNA strand exchange reaction promoted by RecA K72R as a means to address ATPase function. A few experiments were done to measure basic ATPase and strand exchange activities to ensure that our mutant protein exhibited the properties reported by Rehrauer and Kowalczykowski (1993). Proceeding from this base line, we then characterized the magnesium requirements for RecA K72R-mediated DNA strand exchange in detail as a means to distinguish two phases in the reaction. Finally, we tested the capacity of the K72R mutant protein to promote unidirectional strand exchange, bypass of structural barriers in the DNA during strand exchange, and DNA strand exchange with four DNA strands, using strategies similar to those employed in several recent studies (Jain *et al.*, 1994; Kim *et al.*, 1992a, 1992b). We also explored the capacity of the mutant protein to facilitate the cleavage of LexA protein.

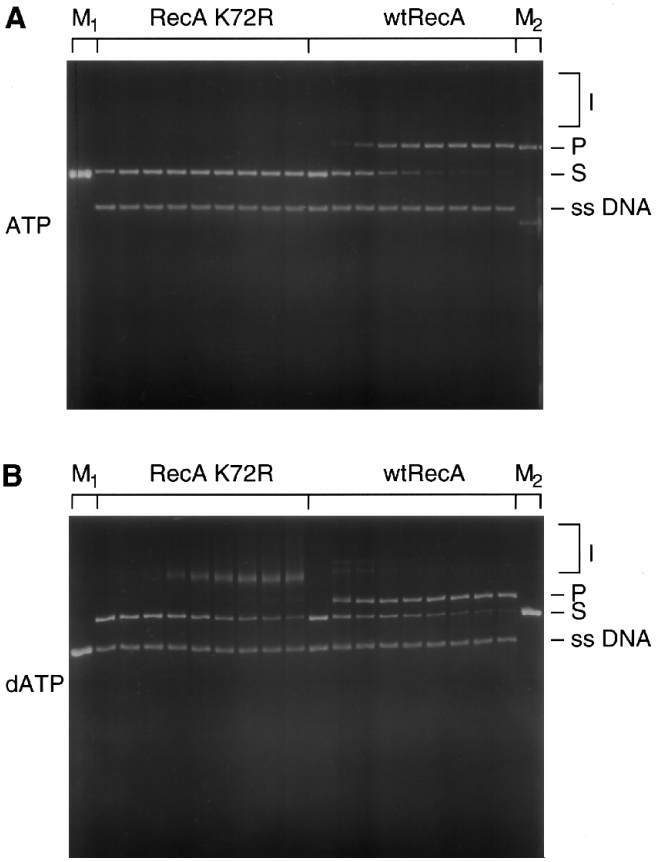


FIG. 2. **DNA strand exchange reactions promoted by RecA K72R.** Reactions were carried out as described under "Materials and Methods," with 6.7 μM wtRecA or RecA K72R proteins, 2 μM SSB, 20 μM ϕX174 circular ssDNA, and 20 μM linear ϕX174 dsDNA (cleaved with *Pst*I). Reactions also contained 3 mM ATP (*A*) or dATP (*B*). In *Panel A*, lane M_1 contains ϕX174 linear duplex DNA as a marker, and M_2 contains supercoiled and nicked circular ϕX174 DNA. In *Panel B*, the markers are ϕX174 ssDNA (M_1) and linear duplex DNA (M_2). In the reactions themselves, time points are 0, 10, 20, 40, 60, 90, 120, 150, and 180 min, respectively, left to right. Labels are: *P*, the nicked circular duplex product of DNA strand exchange; *S*, the linear duplex substrate; *ss*, the circular ssDNA substrate; and *I*, reaction intermediates.

The RecA K72R Mutant Protein Does Not Hydrolyze ATP or dATP—Using a coupled spectrophotometric assay, we measured the ssDNA-dependent ATPase activity of both the mutant and wild type proteins with ATP and dATP. The mutant did not produce a rate of ATP or dATP hydrolysis detectable above background. Based on an evaluation of assay sensitivity, we estimated the upper limit for ATP and dATP hydrolysis (k_{cat}) to be 0.12 min^{-1} and 0.24 min^{-1}, respectively. The actual rate is probably much lower, and the results coincide well with the 600–850-fold reduction in NTP hydrolysis observed by Rehrauer and Kowalczykowski (1993). RecA K72R protein was also tested for dATP hydrolytic activities at 2, 4, 6, and 8 mM Mg^{2+}, with no hydrolysis observed in any case. The measured k_{cat} for the wtRecA protein bound to ssDNA in the presence of SSB was 29 min^{-1} and 36 min^{-1} for ATP and dATP hydrolysis, respectively, consistent with published findings.

The RecA K72R Protein Promotes Limited DNA Strand Exchange in the Presence of dATP—DNA strand exchange reactions between homologous linear double-stranded DNA and circular ssDNA substrates derived from ϕX174 phage in the presence of ATP and dATP under standard reaction conditions (including 10 mM magnesium acetate) were monitored by aga-

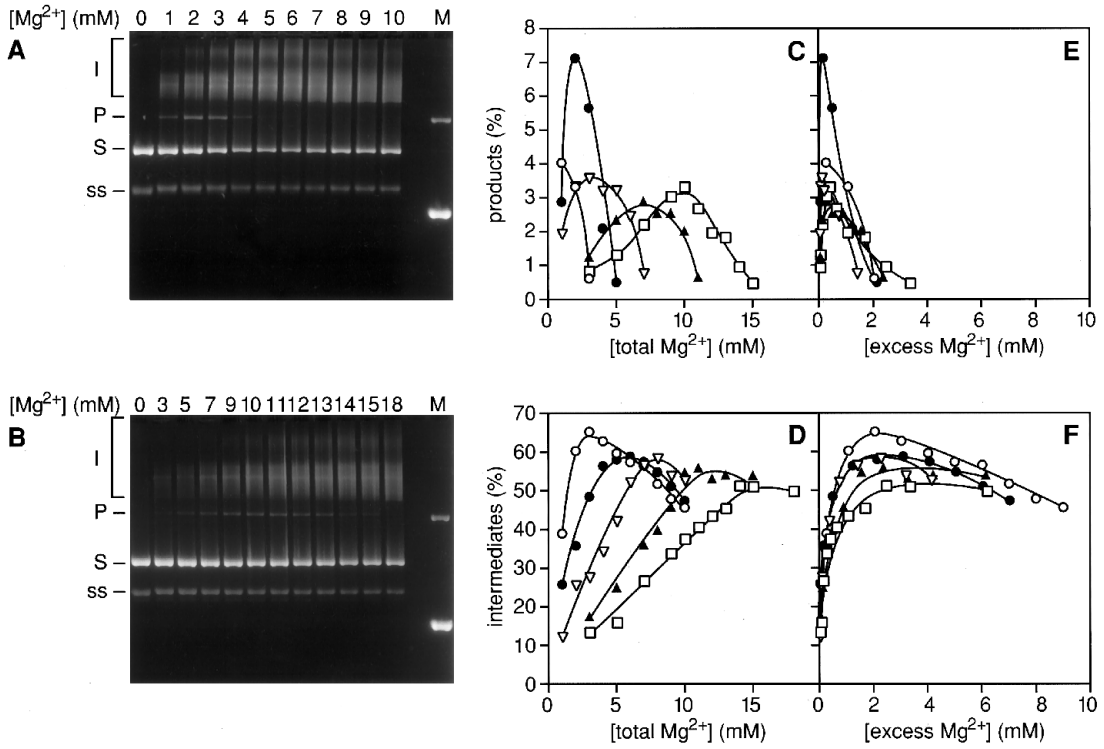


FIG. 3. **RecA K72R-mediated DNA strand exchange is affected by the concentration of magnesium ion.** Reactions (20 μl) were carried out for 3 h as described under "Materials and Methods" and contained 6.7 μM RecA K72R protein, 2 μM SSB, 20 μM M13mp8 circular ssDNA, and 20 μM linear M13mp8 dsDNA (cleaved with *Sma*I). The intermediates and products were quantified as described under "Materials and Methods." *Panels A* and *B* show reactions with dATP concentrations at 3 and 12 mM, respectively, and Mg^{2+} concentrations indicated at the top of the gels. Labels are as described in Fig. 2. *Panels C* and *D* show the quantified results of several experiments plotted as a function of total Mg^{2+} concentration, with full-length hybrid DNA products shown in *C* and intermediates plotted in *D*. In *Panels E* and *F*, the same results are plotted as a function of excess Mg^{2+} concentration (relative to that involved in a Mg·dATP complex), calculated as described under "Materials and Methods." The dATP concentrations in *Panels C–F* are: (○), 1 mM; (●), 3 mM; (▽), 6 mM; (▲), 9 mM; (□) 12 mM. The marker lane (*M*) contains supercoiled and nicked circular M13mp8 DNA, providing a marker for the full-length nicked circular products of DNA strand exchange.

rose gel electrophoresis (Fig. 2). In the presence of ATP, no DNA strand exchange reaction was observed with RecA K72R (Fig. 2*A*). Under the same reaction conditions, the wild type RecA protein promoted an efficient reaction (Fig. 2*A*). When dATP was used as the nucleotide cofactor, the mutant protein converted much of the substrate DNA to strand exchange intermediates (joint molecules). Very little complete strand exchange was observed, although a very weak product band was evident in some experiments with the ϕX174 DNA substrates at late times. The reaction of the mutant protein shown in Fig. 2*B* was the best reaction observed in several trials. In the presence of dATP, the wild type RecA reaction converted most of the double-stranded DNA substrates into completed hybrid DNA products (Fig. 2*B*). The overall results are again in agreement with the observations reported by Rehrauer and Kowalczykowski (1993). Reactions described below with the RecA K72R mutant employed dATP unless indicated.

The Lengths of Hybrid DNA Produced by RecA K72R Mutant-mediated DNA Strand Exchange Are Affected by the Magnesium Ion Concentration—The magnesium ion concentration was varied systematically in DNA strand exchange experiments, with the results shown in Fig. 3. In the presence of 3 mM dATP, a high yield of joint molecules is observed with 10 mM Mg^{2+} as described above. At lower Mg^{2+} concentrations the yield of joint molecules was reduced, but surprisingly, a band corresponding to strand exchange products appeared, nicked circular duplex DNA molecules with over 7 kbp of hybrid DNA (Fig. 3*A*). The products appeared to peak at Mg^{2+} concentrations approximately equivalent to the dATP concentration, where most of the Mg^{2+} would be bound in a complex with the dATP nucleotide. To determine if the peak product formation was related to dATP concentration, the experiment was repeated at a number of different dATP concentrations. The result obtained with 12 mM dATP is shown in Fig. 3*B*. Full-length hybrid DNA products were again generated, but a much higher concentration of Mg^{2+} was required. Results of many such trials are plotted against total Mg^{2+} in Fig. 3, *Panels C* and *D*. At every dATP concentration tested, a peak of full-length hybrid DNA products was observed, although the concentration of Mg^{2+} required to produce it shifted in concert with the dATP concentration (Fig. 3*C*). The production of joint molecule strand exchange intermediates also shifted with dATP concentration, although higher levels of Mg^{2+} were required for optimal yield of intermediates (Fig. 3*D*). The results were also plotted as a function of the concentration of Mg^{2+} in excess of that in the Mg·dATP complex (Fig. 3, *E* and *F*). In all cases, the yield of full-length hybrid DNA products is at a maximum when excess Mg^{2+} is very low. The yield of strand exchange intermediates reaches a broader maximum when several mM of excess Mg^{2+} is present.

The results indicate that the K72R mutant protein can promote a complete strand exchange reaction generating over 7 kbp of hybrid DNA at significant levels. Excess Mg^{2+} beyond that in the Mg·dATP complex is required for optimal uptake of substrate duplex DNA into the nucleoprotein filament formed on ssDNA to form strand exchange intermediates, but the same excess Mg^{2+} inhibits the formation of the extensive lengths of hybrid DNA needed to generate the completed products. The weak uptake of substrate duplex DNA when excess Mg^{2+} concentrations are low appears to be one factor limiting the yield of full-length products under conditions otherwise optimal for their generation.

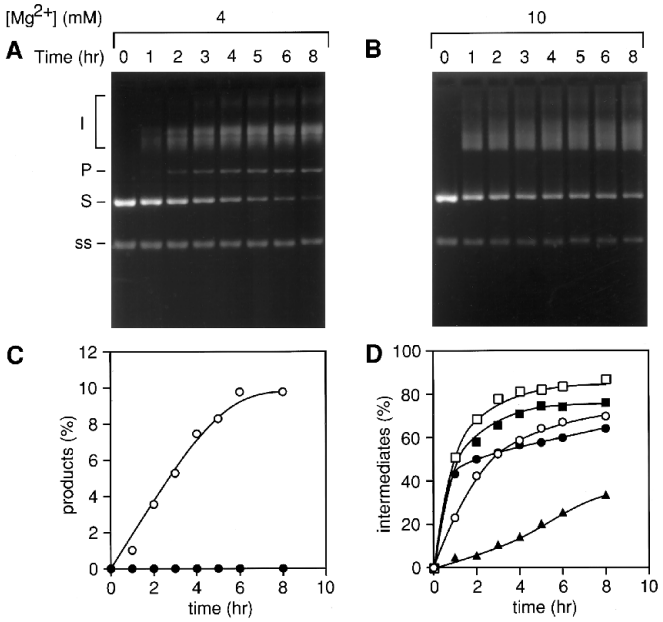


FIG. 4. **Generation of full-length hybrid DNA products by RecA K72R is slow.** Reactions (100 μl) were carried out as described in Methods, with 6.7 μM RecA K72R proteins, 2 μM SSB, 20 μM M13mp8 circular ssDNA, and 20 μM linear M13mp8 dsDNA (cleaved with *Sma*I). Reactions also contained 3 mM dATP. *Panels A* and *B* show the reactions with 4 and 10 mM Mg^{2+}, respectively. Time points are 0, 1, 2, 3, 4, 5, 6, and 8 h, respectively, left to right. Labels are as described in Fig. 2. The marker lanes (*M*) contain supercoiled and nicked circular M13mp8 DNA as in Fig. 3. *Panel C* is a plot of the quantified full-length product formation for the reactions in *Panels A* and *B*. *Panel D* shows the quantified formation of joint molecule intermediates in an expanded set of reactions including those in *Panels A* and *B*. Symbols for Mg^{2+} concentrations in *Panels C* and *D* are: (▲), 2 mM; (○), 4 mM; (□), 6 mM; (■), 8 mM; (●), 10 mM.

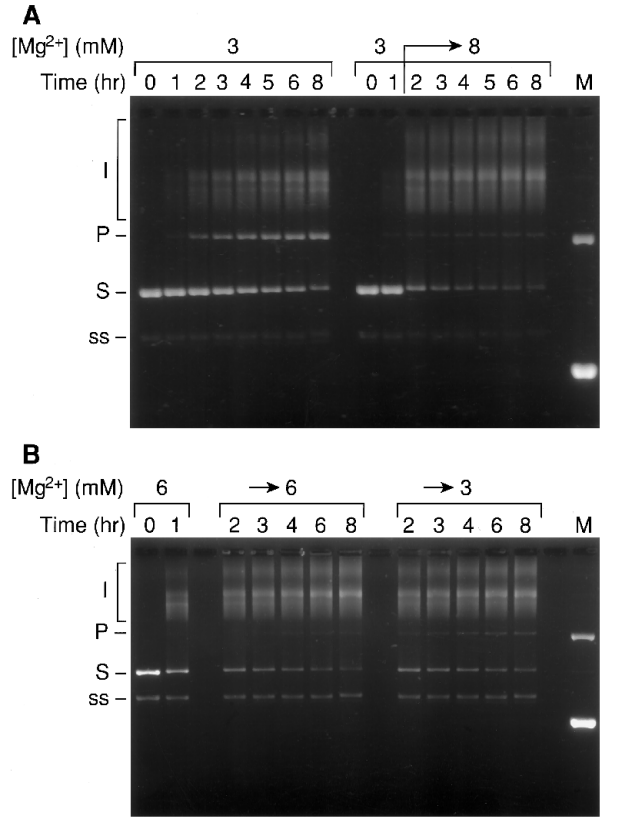


FIG. 5. **Generation of full-length hybrid DNA products can be stimulated or blocked by adjusting magnesium ion concentration.** Reactions were carried out as described under "Materials and Methods." Reactions contained 6.7 μM RecA K72R protein, 2 μM SSB, 20 μM M13mp8 circular ssDNA, and 20 μM linear M13mp8 dsDNA (cleaved with *Sma*I). Reactions also contained 3 mM dATP. In *Panel A*, two reactions (100 μl) are shown containing 3 mM magnesium acetate. One hour after the reactions were initiated, 2 μl of 10 mM Tris acetate (80% cation, pH 7.5) or concentrated magnesium acetate was added to the reactions at left and right, respectively, bringing the final Mg^{2+} concentration in the reaction on the right to 8 mM. The reaction time points are 0, 1, 2, 3, 4, 5, 6, and 8 h, respectively, left to right, and the additions were made immediately after the 1-h time point shown. In *Panel B*, a single reaction (120 μl) was started in the standard reaction buffer containing 6 mM magnesium acetate. After taking the 0- and 1-h time points (*lanes 1* and *2*), the reaction was divided into two 40-μl aliquots. Each aliquot was diluted 1:1 into a buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 3 mM potassium glutamate, 1 mM DTT, 5% (w/v) glycerol, 3 mM dATP, and a dATP regeneration system (11.8 mM phosphoenolpyruvate, 20 units ml^{-1} pyruvate kinase), and either 6 mM magnesium acetate (reaction → *6*) or no magnesium acetate (reaction → *3*). RecA K72R, SSB, and DNA substrates in these reactions were diluted 2-fold. The reactions proceeded for additional 7 h, with the five gel lanes in each set representing 2-, 3-, 4-, 6-, and 8-h time points, respectively, left to right. Labels are as described in Fig. 2. The marker lane contains supercoiled and nicked circular M13mp8 DNA as in Fig. 3.

As shown in Fig. 4, the generation of full-length strand exchange products by the K72R mutant is unsynchronized (in different nucleoprotein filaments) and very slow. Products appear slowly over an 8-h time course. Under optimal Mg^{2+} conditions (where the concentrations of dATP and Mg^{2+} are approximately equal), 10–20% of the input duplex DNA was readily converted to full-length products in 8 h. The yield of products is still increasing at 8 h and may be limited only by time and the stability of the nucleoprotein filaments. When significant levels of free Mg^{2+} were present, the generation of full-length products was greatly reduced even over a long time course. In the presence of 3 mM dATP, products were generated at 4 mM Mg^{2+}, but not at 6 mM or above with substrates derived from M13mp8. The generation of intermediates peaked at 6 mM Mg^{2+}, and appeared to decrease somewhat at higher Mg^{2+} concentrations, although the yield was still considerable at 10 mM.

The limits to the length of hybrid DNA that can be formed by the RecA K72R mutant protein in the presence of 10 mM magnesium acetate was explored further (data not shown). The RecA K72R mediated-reaction generated completely exchanged products with a 1.3-kbp duplex substrate. A similar reaction with a 2.9 kbp DNA fragment derived from M13mp8 (the small fragment from *Cla*I digestion) exhibited some product formation even in the 10 min time point, but the reaction was much weaker than that promoted by the wtRecA protein. When the 4.3-kbp *Cla*I fragment of M13mp8 was used as the duplex substrate, no product generation was detected (data not shown). As noted in Fig. 2, limited product formation was sometimes promoted by RecA K72R with somewhat longer DNAs when the substrates were derived from ϕX174. We concluded that the mutant protein could promote the rapid generation of over 1 kbp of hybrid DNA, with extended hybrid DNA regions observed at efficiencies declining rapidly as a function of length, when the dATP and Mg^{2+} concentrations were 3 and 10 mM, respectively.

Binding of SSB to ssDNA prior to addition of the K72R mutant protein eliminated the production of strand exchange products under all conditions, and also inhibited the formation of strand exchange intermediates (data not shown). Optimal reactions with the mutant protein are observed only when it is added prior to the SSB. The generation of full-length strand exchange products under optimal conditions also required a stoichiometric concentration of the RecA K72R mutant protein relative to the ssDNA. The generation of full-length products did not increase at all when the concentration of mutant pro-

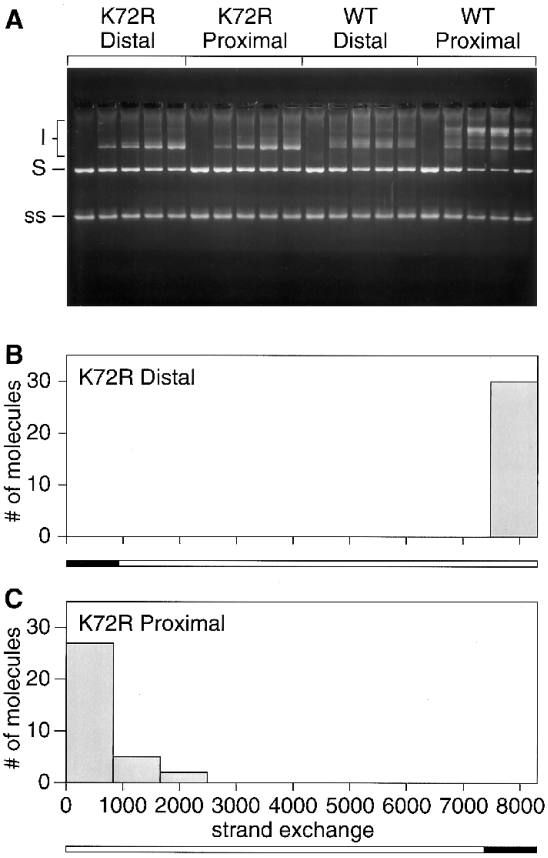


FIG. 6. **DNA strand exchange is bidirectional with RecA K72R.** Reactions were carried out as described under "Materials and Methods," with 6.7 μM wtRecA or RecA K72R proteins, 2 μM SSB, 20 μM M13mp8 circular ssDNA, and 20 μM linear M13mp8.1037 dsDNA. The M13mp8.1037 DNA was cleaved with either *Eco*RI (distal homology) or *Bam*HI (proximal homology). Reactions also contained 3 mM dATP. For each reaction, time points are 0, 10, 30, 60, and 90 min, respectively. Labels are as in Fig. 2, with *ss* designating the circular ssDNA substrate. *Panels B* and *C* are the RecA K72R-mediated reactions with distal and proximal homology, respectively. An aliquot from a 60-min reaction mixture was removed, cross-linked, deproteinized, and analyzed by electron microscopy as described under "Materials and Methods." Lengths of DNA exchanged in intermediates produced by RecA K72R, using a judgment procedure described under "Materials and Methods."

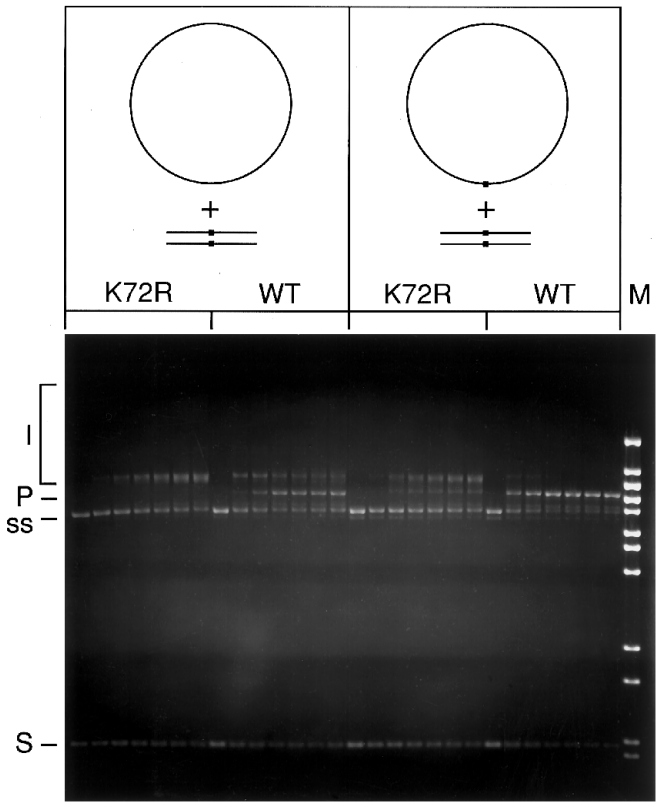


FIG. 7. **RecA K72R-mediated DNA strand exchange does not bypass structural barriers in the duplex DNA substrate.** Agarose gel assays were carried out as described under "Materials and Methods." Reactions contained 6.7 μM wtRecA or RecA K72R proteins, 2 μM SSB, 3 mM dATP, 20 μM M13mp8 (*left*) or M13mp8.52 (*right*) circular ssDNA, and 10 μM of the 1323-bp linear duplex substrate derived from M13mp8.52. In the reaction illustrated on the right, both substrates contain the 52-bp insert, and thus they are homologous throughout their length. DNA markers in *lane M* are λ DNA digested with *Bst*EII. The reaction time points for all four reactions are 0, 10, 20, 40, 60, 90, and 180 min, from left to right. The labels are: *I*, reaction intermediates; *P*, gapped circular DNA molecules produced by a complete strand exchange; *ss*, circular ssDNA substrate; *S*, 1323-bp linear duplex fragment used as a substrate.

tein exceeded the stoichiometric level of one monomer per three nucleotides of ssDNA (data not shown). Excess protein, which might fill or eliminate discontinuities in the filaments, does not improve the reaction.

To further test the idea that free Mg^{2+} stimulates formation of intermediates but inhibits subsequent branch migration needed to generate products, experiments were carried out in which strand exchange was initiated at one Mg^{2+} concentration, and then shifted by the addition of more Mg^{2+} or dilution. The dATP concentration was set at 3 mM. As shown in Fig. 5, addition of Mg^{2+} to reactions initiated under conditions optimal for product formation (3 mM Mg^{2+}) resulted in strong inhibition. In this case, the added free Mg^{2+} would be expected to block the branch migration needed to generate products. Contrasting to a degree, the complementary dilution experiment did not always produce the anticipated restoration of product formation. When the reaction was initiated at 6 mM Mg^{2+}, then diluted 1 h later (after intermediates had formed) so as to make the Mg^{2+} concentration equal to that of the dATP, product formation was stimulated only slightly. The reaction shown after the dilution in Fig. 5*B* was not nearly as strong as that observed in reactions initiated with 3 mM Mg^{2+}, indicating that the use of excess Mg^{2+} produces a degree of hysteresis. The same result was observed when EDTA was used to remove excess Mg^{2+} (data not shown). The hysteretic effect of excess Mg^{2+} was time-dependent, since a dilution at only 5 min after the reaction was initiated fully restored product formation comparable to that observed in reactions without excess Mg^{2+} (data not shown). The results suggest that when the concentration of Mg^{2+} exceeds that of dATP, intermediates are formed that slowly take on a structure that is not easily resolved when Mg^{2+} concentrations are lowered.

RecA K72R-mediated DNA Strand Exchange Is Bidirectional—Linear duplex DNA substrates, with about 1 kbp of heterologous sequences on one end or the other to block strand exchange, were used to determine if there was a directional bias to the strand exchange reaction promoted by RecA K72R. These substrates include 7.2 kbp of DNA that are homologous to the M13mp8 circular ssDNA (Jain *et al.*, 1994). When ATP is hydrolyzed, wtRecA protein promotes DNA strand exchange 5′ to 3′ relative to the strand of the duplex that is identical to the ssDNA circle. In the continuing discussion, we refer to the ends of the duplex DNA substrate as proximal and distal, reflecting the ends where a productive DNA strand exchange reaction normally begins and ends, respectively. Reactions were carried out with 3 mM dATP and 10 mM Mg^{2+}.

With wtRecA protein, the duplex DNA with proximal homol-

ogy was converted efficiently into a slowly migrating product, previously identified as a branched molecule in which strand exchange has proceeded to the homology/heterology junction, creating 7.2 kbp of hybrid DNA (Jain *et al*., 1994). When homology is restricted to the distal end, the reaction is weaker (Fig. 6*A*), and lengths of hybrid DNA produced are much shorter (Jain *et al*., 1994). RecA K72R protein-mediated strand exchange produced intermediates whether the homology was located on the proximal or distal ends, with little evident bias (Fig. 6*A*). There was no significant change in these results when the reactions were cross-linked with AMT prior to electrophoresis to eliminate spontaneous branch migration, or when the Mg^{2+} concentration was lowered to 3 or 6 mM, although the yield of intermediates declined with the lower Mg^{2+} concentrations (data not shown).

The branched DNA intermediates formed in these reactions were examined by electron microscopy, and the approximate lengths of hybrid DNA in each molecule determined. The results (Fig. 6, *B* and *C*) confirm that the reaction with RecA K72R proceeded with no substantial bias on either end of the duplex substrate, and produced only limited lengths of hybrid DNA. We conclude that strand exchange mediated by the K72R mutant is bidirectional.

A Short Heterologous DNA Insert Blocks RecA K72R-mediated DNA Strand Exchange—Since formation of hybrid DNA by the mutant appears to be limited to about 1.5 kbp (Rehrauer and Kowalczykowski, 1993), testing its capacity to bypass a heterologous barrier during strand exchange requires a suitably short substrate. The duplex DNA substrate (Fig. 7) was a 1.3-kbp DNA fragment with a 52-bp heterologous insertion near the center and about 600 bp of homologous sequences on either end (Kim *et al*., 1992a). The ssDNA substrate was either circular M13mp8 ssDNA, which does not have the insert, or circular M13mp8.52 ssDNA, which has the 52-bp sequence and is homologous to the duplex substrate throughout its length. The product of a complete strand exchange reaction is a circular DNA molecule with a 1.3-kbp duplex region and a single-stranded region extending over 5900 nucleotides.

In the RecA K72R reactions (Fig. 7), the reaction of the 1.3-kbp duplex DNA fragment with circular M13mp8 ssDNA produced reaction intermediates that accumulated with time, but no complete products. In contrast, significant product formation was observed for the completely homologous reaction using M13mp8.52 ssDNA. Therefore, a 52-bp heterologous insert in the duplex DNA blocked RecA K72R-mediated DNA strand exchange. Even in the completely homologous reaction, the generation of completely exchanged products was weak with the K72R mutant, and reaction intermediates were still the predominant species at the end of the reaction. Both of the reactions proceeded much better in the presence of wtRecA protein and dATP. Substantial amounts of the completely exchanged product were produced even when the duplex contained the heterologous insertion. The Mg^{2+} concentration had no effect on the capacity of the mutant protein to bypass the barrier (data not shown). In a series of reactions carried out with Mg^{2+} concentrations ranging from 2 to 10 mM, intermediates were produced in significant quantities but no completed products were seen with the mutant protein under any conditions.

These reactions were examined by electron microscopy at the 40-min time point (Fig. 8). In the RecA K72R-mediated reaction, 121 intermediates but no completed products were found in 362 randomly chosen duplex or partial duplex molecules. Several different types of intermediates were found (Fig. 8, *A–D*), with 101 (84%) identified as the standard type (Fig. 8, *A* and *B*), and 20 (16%) falling into a more complex class in which

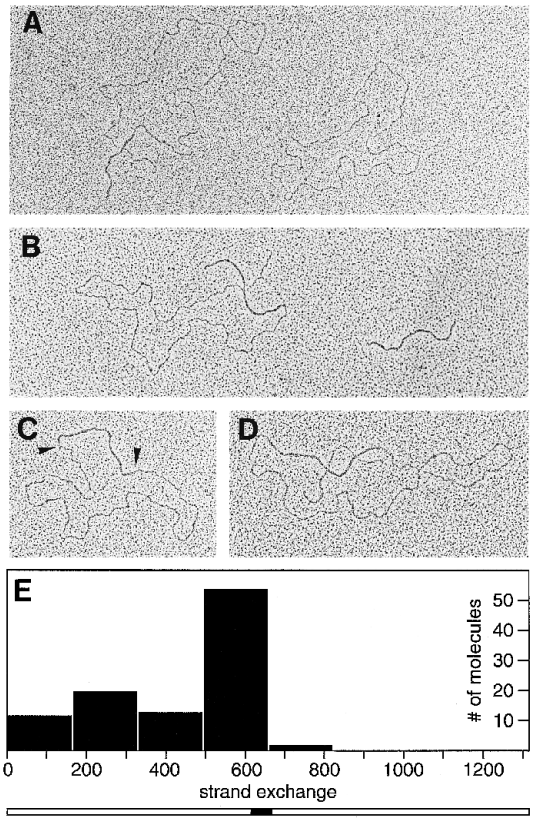


FIG. 8. **Electron microscopy of DNA species found in reactions involving a structural barrier in the duplex substrate.** Samples taken 40 min into reactions such as those in Fig. 10 (carried out under identical reaction conditions) were cross-linked with AMT, deproteinized, and spread as described under "Materials and Methods." *Panels A* and *B* show typical reaction intermediates found in the reaction with RecA K72R. At the right of these panels are shown examples of the circular ssDNA and linear dsDNA substrates, respectively. *Panel C* shows a reaction product found in the reaction with wtRecA. *Panel D* shows a class of reaction intermediate described in the text, in which strand exchange appears to have progressed from both ends of the linear duplex substrate. Such molecules were observed in both the wtRecA and RecA K72R reactions (this one is from the RecA K72R sample). *Panel E* gives lengths of DNA exchanged in intermediates produced by RecA K72R, using a judgment procedure described under "Materials and Methods."

strand exchange appeared to have progressed from both ends without unwinding the 52-bp insert (Fig. 8*D*). The molecules in the latter class were observed at similar levels in every repetition of this experiment. Their probable origin is described under "Discussion" in the context of a broader model for DNA pairing. In the wtRecA-mediated reaction, 29 (9%) of 327 randomly chosen duplex or partial duplex molecules were in the product form (Fig. 8*C*), 91 (28%) were standard intermediates, and 7 (2%) were intermediates with more complex structures.

The extent of strand exchange was also quantified for the reaction by RecA K72R (Fig. 8*E*). Of 101 randomly chosen standard intermediates (Fig. 8, *A* and *B*), 52% had halted in the middle of the linear duplex DNA, and the remainder had shorter regions of hybrid DNA. One molecule was found in which strand exchange appeared to have bypassed the insert (we attribute an incidence this low to an artifact produced by the low level of nicked or broken DNA molecules present in every DNA preparation). We conclude that RecA K72R protein will not promote bypass of heterologous insertions during DNA strand exchange.

The RecA K72R Mutant Protein Will Not Promote a Four-strand Exchange between Two Duplex DNAs—Four strand exchange reactions were carried out with the K72R mutant pro-

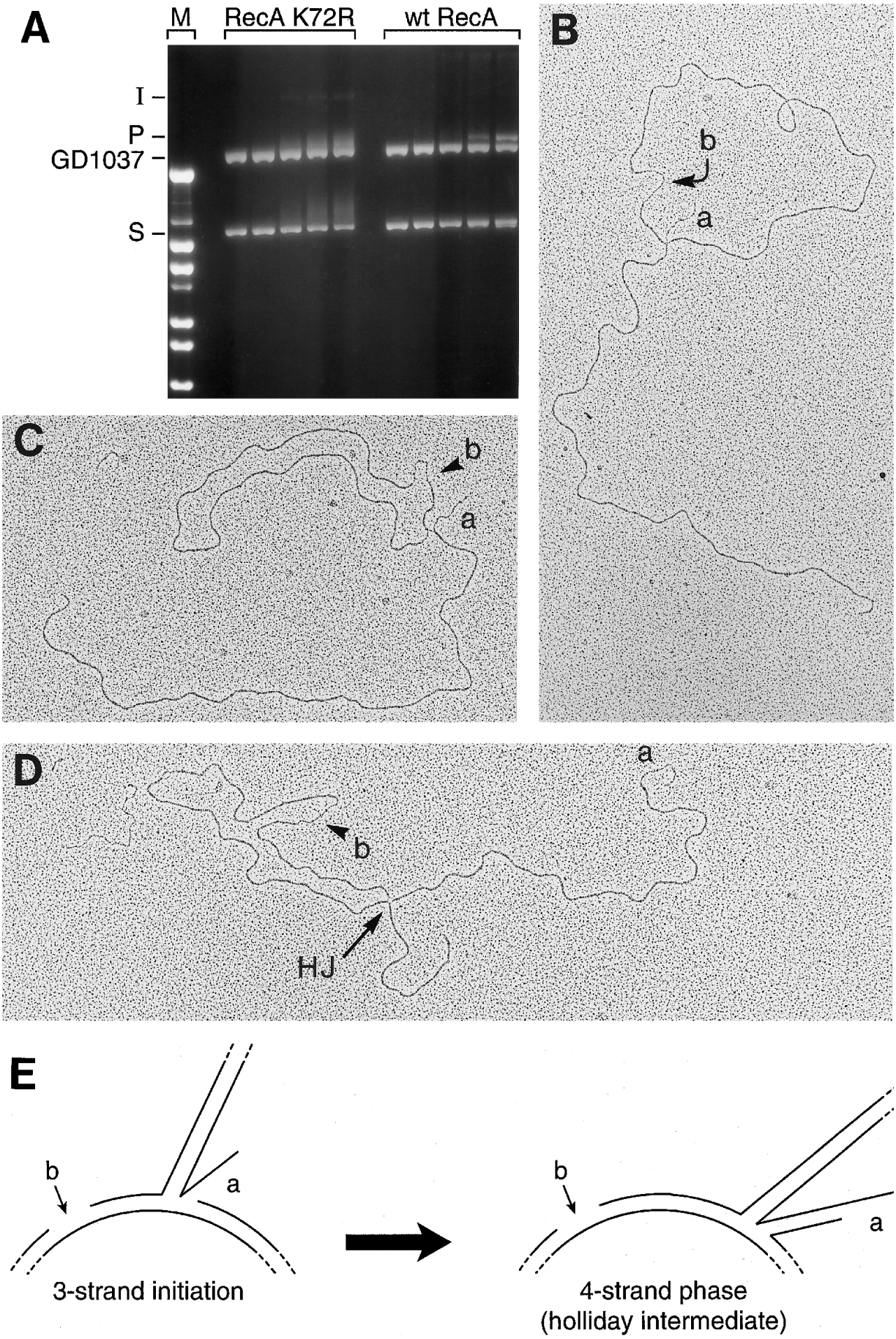

A
M
RecA K72R
wt RecA
I
P
GD1037
S
B
b
a
C
b
a
D
a
b
HJ
E
b
a
3-strand initiation
b
a
4-strand phase
(holliday intermediate)

FIG. 9.

tein. At concentrations of magnesium acetate equivalent to the dATP concentration (3 mM), the production of joint molecules bordered on undetectable and no complete four-strand exchange occurred (data not shown). The yield of joint molecules was greater with 10 mM magnesium acetate (Fig. 9). The wtRecA protein promoted a complete strand exchange under these conditions with these substrates, while the mutant protein promoted the generation of a modest level of reaction intermediates (Fig. 9*A*). The samples shown in Fig. 9*A* were not cross-linked, and the GD1037 band becomes somewhat diffuse in the RecA K72R reaction with time. This slight smearing disappeared and a sharper band at the position labeled *I* appeared when the samples were cross-linked prior to RecA removal; cross-linking had no effect on the results observed for the reaction with wtRecA in the gel assay (data not shown). The intermediates produced by wtRecA and RecA K72R were examined by electron microscopy (Fig. 9). The DNA samples used for electron microscopy were cross-linked with AMT prior to removing the RecA protein to prevent spontaneous branch migration. In a sample taken from the RecA K72R reaction 60 min after the reaction was initiated, there were no Holliday intermediates found among 269 molecules examined at random. There were 21 strand exchange intermediates in this sample (8% of the molecules examined), all with the structures exemplified in Fig. 9, *B* and *C*. In none of these molecules had strand exchange proceeded beyond the single strand gap in the gapped duplex substrate, as determined by the complete absence of duplex regions in the short tail of the branched molecules (the displaced strand in the three-strand reaction, labeled *a* in Fig. 9, *B* and *C*). Odd types representing broken DNA molecules represented less than 5% of the molecules in this sample. None of the odd types contained Holliday junctions. The remaining molecules were reaction substrates. In a sample taken at the same time point, there were 13 Holliday intermediates (Fig. 9*D*) in the wtRecA-mediated reaction, representing 9% of 154 molecules examined at random. Another 2 molecules (1%) had initiated a strand exchange reaction that had not proceeded into the four-strand region. The remaining molecules were linear or gapped duplex molecules of the types representative of the expected products and known substrates. Odd types reflecting broken molecules were 7% of this sample. We conclude that the RecA K72R mutant protein will not promote a four-strand exchange reaction.

The RecA K72R Mutant Protein Facilitates LexA Repressor Cleavage at Slower Rates—In vitro LexA repressor cleavage reactions were carried out using ATP as cofactor along with an ATP regeneration system, and $t_{1/2}$ measurements for LexA cleavage are summarized in Table I. While the mutant protein facilitated repressor cleavage, the rate was much reduced. The $t_{1/2}$ for LexA repressor cleavage was 4 min and 28 min for the wild type RecA and the RecA K72R mutant protein, respectively. A similar kinetic defect was also observed in the presence of dATP and a dATP regeneration system. In the presence of ATPγS, reactions with wild type RecA protein and the K72R mutant exhibited similar kinetics.

TABLE I

Measured $t_{1/2}$ for LexA repressor cleavage reactions

Reactions were carried out and analyzed as described under "Materials and Methods." The bands for either wtRecA protein or RecA K72R protein, along with those for creatine phosphokinase or pyruvate kinase (depending on the ATP regeneration system in a given experiment), were used as internal controls to normalize the data. All LexA cleavage reactions proceeded to completion.

Nucleotide	$t_{1/2}$	
	RecA K72R	wtRecA
	min	
ATP	28	4
dATP	22	4
ATPγS	16	17

DISCUSSION

Our primary conclusions are: (*a*) that there are two phases to a RecA-mediated DNA strand exchange without ATP hydrolysis, with different Mg^{2+} requirements, and (*b*) that the RecA protein-mediated DNA strand exchange is severely constrained when ATP is not hydrolyzed. The RecA K72R protein, which does not hydrolyze dATP or ATP at detectable levels, will promote the generation of DNA products with over 7 kbp of hybrid DNA. However, the reaction is slow and greatly affected by the concentration of Mg^{2+}. Under all conditions the reaction is also bidirectional, will not bypass heterologous insertions in the duplex substrate, and will not accommodate four DNA strands. This last set of limitations are seen with wild type RecA protein in the presence of ATPγS (Rosselli and Stasiak, 1991; Kim *et al*., 1992a, 1992b; Konforti and Davis, 1992; Jain *et al*., 1994). Many of the results with the mutant protein were obtained under conditions typical of reactions with wild type protein and ATP.

A mechanistic context for further discussion of the results is provided by the model in Fig. 10. The model is designed to explain the observed limitations to the lengths of hybrid DNA generated during RecA-mediated DNA strand exchange when ATP is not hydrolyzed. As an alternative to the discontinuous RecA filaments proposed by Kowalczykowski and colleagues (Menetski *et al*., 1990; Rehrauer and Kowalczykowski, 1993; Kowalczykowski and Krupp, 1995), we suggest that the discontinuity is instead found in a key DNA pairing intermediate. In any DNA strand exchange reaction with RecA protein (with or without ATP hydrolysis), initiation is presumed to occur via the alignment of a ssDNA within the filament with a homologous duplex to form a pairing intermediate with all three strands interwound (structure unspecified for purposes of this discussion). This must involve a spooling of the duplex into the filament groove, with both the filament and DNA rotating in

FIG. 9. **Four-strand exchange reactions are not promoted by RecA K72R.** Reactions were carried out as described under "Materials and Methods," and contained 3 μM wtRecA or RecA K72R proteins, 0.6 μM SSB, 3 mM dATP, 12 μM gapped duplex DNA substrate (*GD1037*), and 10 μM of the 7834-bp linear duplex substrate, generated by *Nco*I and *Eco*RI cleavage of M13mp8.1037 (8226 bp). The linear duplex overlaps the single strand gap in the gapped duplex by 605 bp. *Panel A* shows the reactions monitored with a agarose gel. Markers (*M*) are bacteriophage λ DNA digested by *Bst*EII. The time points for both reactions are 0, 10, 30, 60, and 90 min, respectively, left to right. Labels are: *GD1037*, the gapped duplex substrate; *S*, the 7834-bp linear duplex substrate; *P*, the GD432 gapped duplex product generated by a complete strand exchange reaction and linear duplex DNA with 7229-bp duplex region and a 605-base single-stranded tail; *I*, reaction intermediates. *Panels B–D*, samples taken at 60 min into the reaction were cross-linked with AMT, deproteinized, spread, and examined by electron microscopy. The labels *a* and *b* are explained in the legend for *Panel E*. *B* and *C*, typical reaction intermediates generated in the reaction with RecA K72R. *D*, a Holliday intermediate generated in the reaction with wtRecA protein. The Holliday junction, slightly denatured to display the individual strands, is labeled *HJ*. *Panel E* gives a schematic focusing on two stages of the strand exchange reaction. First, the reaction is initiated as a three-strand reaction in the single strand gap, producing a branched molecule with a short displaced single strand labeled *a*. In the substrates used, the linear duplex overlaps the gap by 605 bp, leaving a 432-bp region of ssDNA that is not included in the region undergoing exchange (labeled *b*). In the wtRecA-mediated reaction, the branch moves into the neighboring duplex region of the gapped duplex, producing a Holliday intermediate as shown, and ultimately a complete strand exchange. The *a* and *b* labels remain the same.

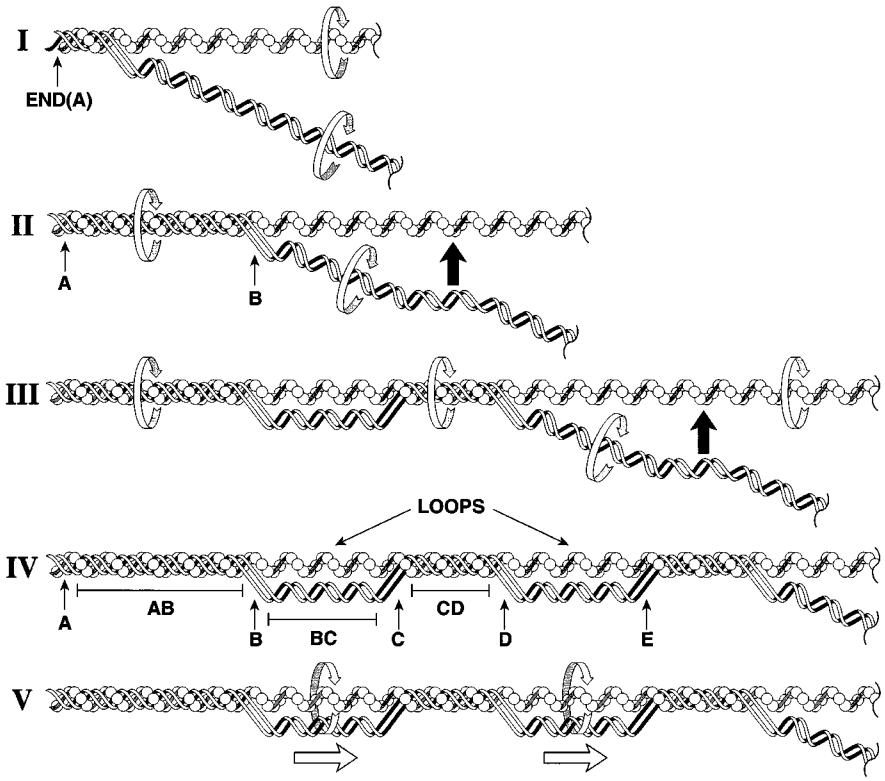


FIG. 10. **A model explaining the cessation of the rapid phase of strand exchange in the context of a discontinuous DNA pairing intermediate.** Formation of the hypothetical DNA pairing intermediate shown in *Panel V* is illustrated in five steps. *I*, pairing is initiated at one end of a duplex DNA substrate. Extension of the paired region requires the rotation of both the filament and the duplex DNA, as shown by *circular arrows. II*, as the paired region lengthens, some probability exists for an intramolecular pairing interaction elsewhere in the filament (*black arrow*). *III*, pairing at the new location creates a new point for continued spooling of the duplex DNA into the filament. However, a segment of DNA is left outside of the filament as an external loop by the second pairing initiation. *IV*, multiple loops can form (*e.g.* segments *B–C* and *D–E*), with paired segments (*e.g. C–D*) between them. *V*, resolution of the loops requires their rotation about the axis of the RecA nucleoprotein filament.

solution as shown in Fig. 10. If the rotation and accompanying spooling proceed uninterrupted, a uniform DNA pairing intermediate would be created throughout the length of the DNA substrates. Since the filament stabilizes the hybrid DNA products of strand exchange, this intermediate would be rapidly converted to hybrid DNA throughout its length. However, the entire RecA filament is set up to initiate DNA pairing. Once pairing is initiated at one location along the filament, a pairing interaction at another location in the same filament becomes intramolecular and much more likely. As spooling lengthens the initial pairing interaction to some point *B* (Fig. 10), pairing at another point *C* will initiate another segment of DNA pairing intermediate that can be lengthened by spooling as was the first. Further spooling at point *B* will then be blocked, because the duplex DNA between points *B* and *C* has been constrained at point *C* by the new pairing interaction. We define the segment between points *B* and *C* as an external loop. The pairing process could generate any number of such loops along the length of a paired duplex DNA. The loops may be long or very short, and the average distance between them would reflect the efficiency of intramolecular DNA pairing under a given set of reaction conditions. We propose that formation of a pairing intermediate with alternating loops and paired regions along the entire length of available homology defines the rapid phase of DNA strand exchange under conditions generally used for RecA reactions. Because of topological constraints, the only stable and productive strand exchange in such an intermediate (where one strand of the duplex substrate can be displaced) would occur between an end of the duplex and the beginning of the first loop, such as the *A* to *B* segment in Fig. 10. If the reaction shown was terminated at *Panel V*, the *A–B* segment contains the only stable hybrid DNA that would remain after protein removal.

In this model, the *A–B* segment defines the extent of hybrid DNA formation in the rapid phase of strand exchange. Extension of the *A–B* segment requires the rotation of the loop around the filament axis, with the loop DNA axis more or less parallel to the filament axis, so that DNA is wound into the filament groove at one end of the loop and out of the filament at the other end. Since a given paired region is lengthened only at the expense of another paired region (*e.g.* the *A–B* segment can lengthen at the expense of the *C–D* segment), this process is inevitably much slower than the rotary diffusion/spooling process that generates the various paired regions in the first place. In the bottom panel of Fig. 10, *V*, if the viewer looks down the filament axis from the left side, and rotates the loop clockwise about the axis as shown, the loop will migrate away from the viewer (or to the right as it is drawn). Counterclockwise rotation will move the loop in the opposite direction. The rate of any migration that occurred would be limited or blocked altogether by steric interference, the stability of neighboring paired segments, and other factors. *In vitro*, some of the "loops" would inevitably be intermolecular, spanning different filaments and creating the aggregate networks first described by Radding and colleagues (Tsang *et al.*, 1985).

This scenario is consistent with the observed effects of Mg^{2+} on the reaction promoted by RecA K72R. Whereas increased Mg^{2+} concentrations have a destabilizing effect on protein-DNA interactions, they can have a stabilizing effect on the pairing interactions between DNA strands (Record and Spolar, 1990; Record, 1975), including triplex DNA structures (Kohwi and Kohwi, 1988; Wells *et al.*, 1988; Malkov *et al.*, 1992; Shchyolkina *et al.*, 1994). Concentrations of Mg^{2+} in excess of that required to form Mg·dATP complex should therefore facilitate the initial formation of pairing intermediate in the rapid phase (and the formation of additional paired regions to generate loops), leading to an enhancement of joint molecule formation. However, since extension of the stable hybrid DNA in the joint molecule must come at the expense of other paired segments, stabilization of the other paired segments by the excess Mg^{2+} will tend to block extension and the formation of completely exchanged products. The formation of complex structures with multiple external loops might block resolution of the intermediates to products even when the Mg^{2+} concen-

tration was subsequently reduced, leading to the observed hysteresis in reactions initiated with excess Mg^{2+} and then diluted (Fig. 5). When the Mg^{2+} concentration is just sufficient to form the Mg·dATP complex, the decreased pairing efficiency could reduce the number of external loops and allow better production of completed strand exchange products over time. All of these effects are observed. The effects of Mg^{2+} concentration on loop migration are analogous in many respects to the effects of Mg^{2+} on spontaneous DNA branch migration in solution. DNA branch movement requires the formation of base pairs on one side of the branch at the expense of base pairs on the other side, and the rate of this process is reduced by up to 3 orders of magnitude by added Mg^{2+} (Panyutin and Hsieh, 1994).

We also routinely observe molecules by electron microscopy that must be formed by a process like that illustrated in Fig. 10. If the homologous duplex DNA substrate is sufficiently short, a limiting case might be observed where pairing was initiated at one end, and then a single external loop was sometimes formed followed by extension of the three-stranded DNA pairing intermediate out to the opposite end of the duplex. Stable strand displacement could then be seen after protein removal that appears to proceed from both ends, held together by an unexchanged loop of substrate duplex as in the molecule shown in Fig. 8*D*. Note that this type of molecule *cannot* form by independent pairing initiation at the two ends, since simultaneous pairing at either end and extension of both paired segments toward the center is topologically forbidden (the duplex DNA would have to rotate in opposite directions to extend each paired region). If one paired segment is initiated at the left end of the duplex and extended to the right, the second paired segment must be initiated away from the right end and extended to the end from left to right.

When ATP is hydrolyzed, the nascent hybrid duplex DNA is extended unidirectionally. The external loops would have to be rotated uniquely in one direction to bring this about. Elsewhere, we have proposed a model for how ATP hydrolysis might be coupled to such a rotation of external DNA relative to the filament axis (Cox, 1994). ATP hydrolysis also permits the bypass of barriers. A four-strand exchange reaction will not occur at all unless ATP is hydrolyzed. These properties can best be rationalized in the context of RecA's function in recombinational DNA repair (Clark and Sandler, 1994; Cox, 1993).

We note that even if filament discontinuities occur and help to limit DNA pairing in the absence of ATP hydrolysis, the DNA loops we describe above can still be formed when duplex DNA is paired at two separated filament segments (and may be inevitable). These loops would have to be resolved irrespective of any redistribution of RecA protein monomers, and their resolution may require ATP hydrolysis.

The observed effects of Mg^{2+} suggest that the reactions with the K72R mutant are not seriously limited by filament discontinuities. Full-length hybrid DNA products are generated, albeit slowly, at appropriate Mg^{2+} concentrations. Excess mutant protein, which might plug any gaps in a discontinuous filament, has no effect on the reaction.

The absolute requirement for ATP hydrolysis in the four-strand exchange reaction is also potentially instructive in discriminating between mechanistic alternatives for the slower hybrid DNA extension phase. The segments of RecA filament present when ATP is not hydrolyzed in a hypothetical discontinuous filament cannot promote a four-strand exchange under any conditions, and a simple redistribution of RecA monomers to create a contiguous but otherwise identical filament at other locations should not change this result. Many lines of evidence indicate that the RecA filament can only assimilate three DNA strands (Cox, 1993, 1995). A four-strand exchange reaction therefore requires a contribution from ATP hydrolysis that goes beyond the turnover of RecA filament complexes already bound to hybrid DNA product. The proposal that RecA-mediated ATP hydrolysis is coupled to a coordinated rotation of DNA molecules to bring about branch movement during strand exchange provides a mechanism to explain the promotion of four-strand exchanges by a RecA filament that can only assimilate three DNA strands (Kim *et al.*, 1992b; Cox, 1994).

The RecA K72R mutant protein is surprisingly competent in the promotion of DNA strand exchange reactions *in vitro*. It fulfills the requirements of a DNA pairing activity, which in some scenarios would generate branched recombination intermediates before yielding to specialized branch migration activities such as RuvAB or RecG (West, 1992; Kowalczykowski *et al.*, 1994). However, cells in which the wild type recA gene is replaced by a recA K72R gene display a $recA^-$ phenotype. They are as deficient in homologous recombination, as sensitive to UV radiation, and as unable to induce the SOS response as a recA null mutant (Konola *et al.*, 1994).[2] These results indicate that the ATPase activity of RecA is important *in vivo*. The point in recombinational processes where RecA is replaced by RuvAB or RecG is currently undefined.

The RecA K72R mutant protein has the capacity to facilitate the cleavage of LexA repressor *in vitro*, especially in the presence of ATPγS. The defect that the K72R mutation confers on cells in SOS induction can be explained by the slower kinetics of LexA autocatalytic cleavage with the K72R mutant in the presence of ATP and/or dATP.

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