RecA as a Motor Protein

TESTING MODELS FOR THE ROLE OF ATP HYDROLYSIS IN DNA STRAND EXCHANGE*

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ATP hydrolysis (by RecA protein) fundamentally alters the properties of RecA protein-mediated DNA strand exchange reactions. ATP hydrolysis renders DNA strand exchange unidirectional, greatly increases the lengths of hybrid DNA created, permits the bypass of heterologous DNA insertions in one or both DNA substrates, and is absolutely required for exchange reactions involving four DNA strands. There are at least two viable models to explain how ATP hydrolysis is coupled to DNA strand exchange so as to bring about these effects. The first couples ATP hydrolysis to a redistribution of RecA monomers within a RecA filament. The second couples ATP hydrolysis to a facilitated rotation of the DNA substrates. The RecA monomer redistribution model makes the prediction that heterology bypass should not occur if the single-stranded DNA substrate is linear. The facilitated DNA rotation model predicts that RecA protein should promote the separation of paired DNA strands within a RecA filament if one of them is contiguous with a length of DNA being rotated about the filament exterior. Here, a facile bypass of heterologous insertions with linear DNA substrates is demonstrated, providing evidence against a role for RecA monomer redistribution in heterology bypass. In addition, we demonstrate that following a four-strand DNA exchange reaction, a distal segment of DNA hundreds of base pairs in length can be unwound in a nonreciprocal phase of the reaction, consistent with the direct coupling of an ATP hydrolytic motor to the proposed DNA rotation.

RecA protein plays an essential role in recombinational repair and homologous recombination in *Escherichia coli*. A DNA strand exchange reaction that can involve either three or four DNA strands has been widely used as a model for recombinational processes *in vivo* (1–3).

RecA protein-mediated DNA strand exchange occurs in several recognized phases. First, RecA protein binds to DNA1, typically either a circular ssDNA[1] (for a three-strand reaction) or circular gapped duplex (four-strand reaction), forming a contiguous nucleoprotein filament. Next, a homologous linear duplex DNA (DNA2) is aligned with the ssDNA within the filament. Under typical conditions used *in vitro*, 1–2 kbp of DNA are then rapidly exchanged in the three-strand reaction after alignment. Finally, the nascent hybrid DNA product is extended in a slower, progressive reaction, which is able to bypass a variety of structural barriers. This leads ultimately to a nicked circular hybrid duplex DNA product along with a displaced linear single strand or partially duplex product.

The first three phases of DNA strand exchange, up to the formation of a nascent region of hybrid DNA, require ATP binding to RecA protein but not ATP hydrolysis. This has been demonstrated by the use of the ATP analog ATPγS (which is bound but not appreciably hydrolyzed by RecA) or the RecA mutant protein K72R (which binds but does not hydrolyze ATP) (4–11). In contrast, extension of the hybrid DNA in the final phase requires ATP hydrolysis (9, 11, 12). ATP hydrolysis renders this final phase of the strand exchange reaction unidirectional, with the branch moving uniquely $5' \rightarrow 3'$ relative to the single-stranded DNA in DNA1 (9, 13, 14). ATP hydrolysis is also required for the bypass of short heterologous insertions or other structural barriers in either DNA substrate (5, 6, 11) and for strand exchange reactions involving four DNA strands (7, 11). Thus, ATP hydrolysis appears to confer properties on DNA strand exchange that are important for recombinational DNA repair (3, 15–17).

The mechanism by which ATP hydrolysis influences the last phase of DNA strand exchange has not been elucidated. A number of early proposals coupled strand exchange to disassembly of RecA filaments at or near the migrating DNA branch, sometimes with reassembly on the displaced single strand (13, 18, 19). ATP hydrolysis is required for the end-dependent disassembly of RecA filaments, which has recently been observed and characterized for filaments on both single- and double-stranded DNA (20–22). The disassembly occurs from the end opposite to that at which RecA protein is added during filament assembly, proceeding $5' \rightarrow 3'$ on single-stranded DNA (22), consistent with the overall direction of the DNA strand exchange reaction. However, DNA strand exchange requires no net disassembly of RecA filaments, and RecA protein remains quantitatively bound to the hybrid DNA product of DNA strand exchange after the reaction is complete under optimal conditions *in vitro* (23, 24). In addition, the rate of end-dependent filament disassembly is highly pH-dependent, being negligible at pH 6 and relatively high above pH 8, while the rate of RecA-mediated DNA strand exchange is constant over the same pH range (20, 22). These and other results have effectively eliminated models involving net disassembly of RecA filaments as a required feature of the DNA strand exchange mechanism (3).

Two additional models have been proposed that are generally consistent with observations published to date. The first,

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

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proposed by Kowalczykowski and colleagues (4, 8, 10), is based on the premise that RecA-mediated DNA strand exchange in the absence of ATP hydrolysis is limited only by discontinuities in the RecA filament. ATP hydrolysis would therefore be needed to redistribute RecA monomers to eliminate the discontinuities. An exchange of RecA monomers in the filament interior between free and bound forms has been observed when RecA protein is bound to duplex DNA (25) and also occurs during DNA strand exchange reactions (26). This fulfills a key requirement of the RecA monomer redistribution idea.

The second model involves a facilitated rotation of segments of the DNA substrates about each other, coupled to ATP hydrolysis (3, 11, 12, 16). This model neither requires nor precludes a limited exchange of RecA monomers between free and bound forms. Recent measurements of the rates of ATP hydrolysis and DNA branch movement during DNA strand exchange conform closely to predictions of the facilitated DNA rotation model (12).

These models make a number of additional and testable predictions. To be successful, both models must explain the requirement for ATP hydrolysis both in the bypass of heterologous DNA insertions and for four-strand exchange reactions. As shown by Jwang and Radding (27), the bypass of heterologous insertions involves the application of torsional stress to unwind any heterologous insert in the duplex DNA. In the facilitated DNA rotation model, the required unwinding is a direct and predictable result of the rotation of one DNA substrate about the other, coupled to an ATP-driven motor (3, 16, 28). In contrast, RecA redistribution could in principle bring about unwinding of the heterologous insertion indirectly (Fig. 1). The DNA within a RecA filament is underwound by about 40% (29, 30). Upon dissociation of a segment of RecA filament, the underwinding could be translated into strand separation in the insertion, with up to eight bp separated for every six RecA monomers dissociated, provided that free rotation of the filament was constrained to prevent spontaneous relaxation of the freed DNA (Fig. 1). This condition might be met in a RecA filament formed on circular DNA where there were no free nucleoprotein filament ends. However, if the RecA filament were formed on linear ssDNA, the RecA redistribution model predicts that insertion bypass should not occur. This is because any DNA underwinding liberated by RecA dissociation would be immediately relaxed by simple and unconstrained rotation about the long axis of the filament. Insertion bypass by facilitated DNA rotation, in contrast, should occur whether the ssDNA substrate is linear or circular.

DNA strand exchange involving four strands presents both models with a different set of challenges. Under conditions in which ATP is not hydrolyzed, strand exchange can be initiated by pairing DNA2 within the single strand gap of DNA1, where the reaction involves only three of the four strands. However, the reaction does not proceed beyond the gap unless ATP is hydrolyzed (7, 11). In any strand exchange reaction, the ends of DNA2 are often referred to as proximal and distal, reflecting the ends at which a unidirectional strand exchange begins and ends, respectively; we use this terminology to facilitate the continuing discussion. The proximal end of linear duplex DNA2 generally overlaps the ssDNA gap of DNA1, and at the distal end it is either coincident with or overlaps the gap of DNA1 (Fig. 2A). At the proximal end of DNA2, the overlap reflects the requirement to initiate even a four-strand reaction in a three-stranded region (31, 32). The distal end of DNA2 must also be at least coincident with the end of the duplex in DNA1 so as to permit a reciprocal exchange throughout the four-stranded part of the reaction (Fig. 2A). If the duplex portion of DNA1 extends beyond the distal end of DNA2, the strand exchange
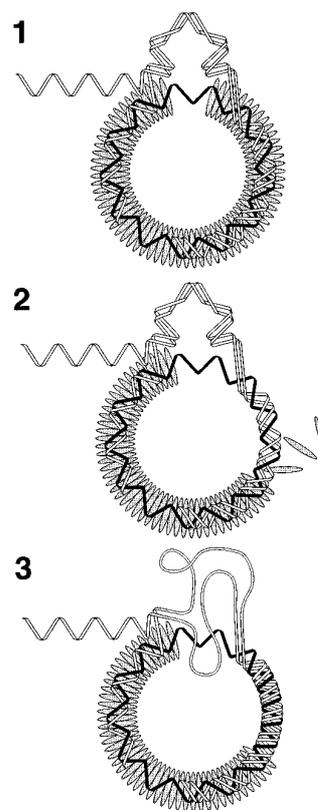

FIG. 1. **Model for the bypass of heterologous DNA insertions by RecA monomer redistribution during RecA-mediated DNA strand exchange.** The DNA within a RecA filament is underwound by about 40%. The duplex DNA substrate could be aligned to the ssDNA in the filament on both sides of the heterologous insertion, with net DNA strand exchange occurring up to the point of the insertion without ATP hydrolysis (*1*). Partial disassembly of the RecA filament (*2*) would release underwound DNA as RecA monomers (*ovals*) were removed. If there were no free ends to allow rotation about the long axis of the filament (as in a contiguous filament formed on circular ssDNA), the released underwinding could be translated into strand separation in the region of the heterologous insertion (*3*), and DNA strand exchange could then continue without ATP hydrolysis. Note that the displaced strand in *panel 1* is shown as being free of the filament only to indicate that a strand switch has taken place in this region; in fact, the model works only if this strand is constrained as the others are. Also, although three strands are shown to be interwound within the filament, this state need not constitute a DNA triplex. The model requires only that the three strands be bound and constrained in an underwound state (relative to free duplex DNA) within the filament. This model is based in part on ideas communicated to us by S. Kowalczykowski.

reaction might be expected to halt where the reciprocal reaction ends. Completion of the reaction would require a helicase-like separation of the strands in the distal duplex extension in DNA1 (Fig. 2B), and RecA has no significant intrinsic helicase activity (33). However, if RecA promotes an ATP-dependent DNA rotation, this activity might produce an unraveling of the duplex extension by means of the torsional stress generated in the DNA by the ATPase motor.

This report describes our tests of the predictions presented above.

<center>MATERIALS AND METHODS</center>

Enzymes and Reagents—E. coli RecA protein was purified to homogeneity and stored as described previously (34). *E. coli* ssDNA-binding protein (SSB) was purified to homogeneity as described (35), except that an additional step utilizing DEAE-Sepharose chromatography was included to ensure removal of single-stranded exonucleases. The RecA protein and SSB concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\epsilon_{280} = 0.59\ A_{280}\ \text{mg}^{-1}$ ml (36) and $\epsilon_{280} = 1.5\ A_{280}\ \text{mg}^{-1}$ ml (35), respectively. RecA and SSB preparations were free of detectable endo- and exonuclease activities on double- or
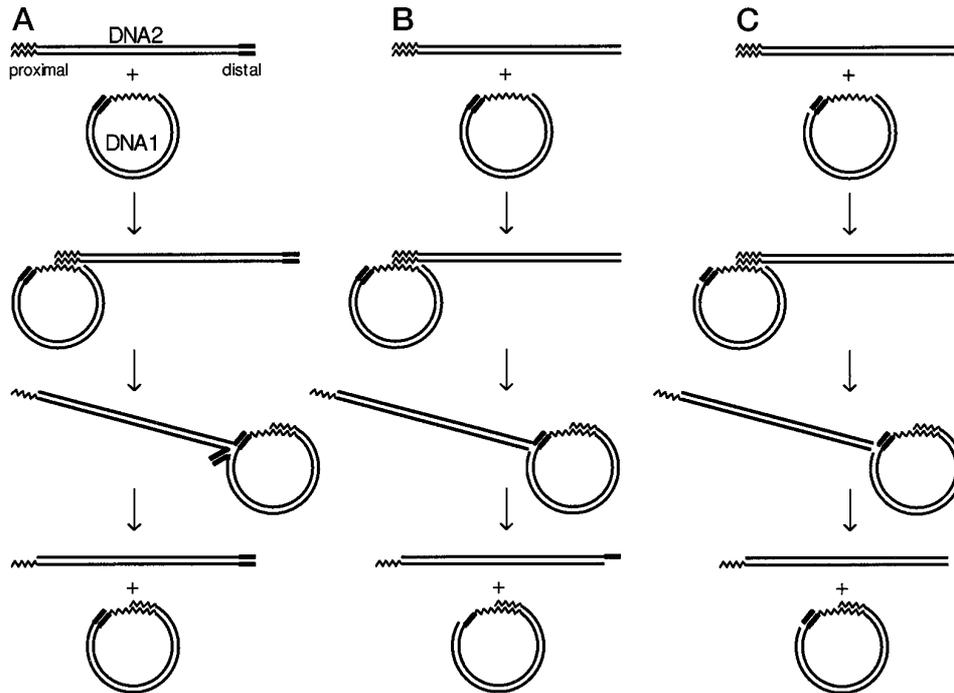
FIG. 2. **RecA protein-mediated strand exchange reactions involving four DNA strands.** *A*, generally, *in vitro* RecA protein-mediated four-strand exchange reactions utilize linear duplex substrates in which the distal end is either identical to or overlaps the distal end of the duplex region in the gapped circular substrate. After the reaction is initiated in the gap, strand exchange proceeds 5′ to 3′ relative to the single-stranded DNA in the gap and is fully reciprocal. The *jagged lines* denote sequences appearing in the single strand gap region of the gapped circular duplex. *B*, this study investigates unwinding of a region at the distal end of the duplex region of the gapped circular substrate that lacks a homologous partner in the linear duplex substrate. We refer to this as a distal extension (region denoted with *thickened lines*). Completion of strand exchange to yield two discrete products requires unwinding of this region without reciprocal strand exchange. *C*, in some experiments, the distal extension in the gapped strand is separated from the remainder of the strand by a nick.

single-stranded DNA. Restriction endonucleases, Vent polymerase, T4 polynucleotide kinase, and β-agarase I were purchased from New England Biolabs. Tris buffer was purchased from Fisher. DEAE-Sepharose was purchased from Pharmacia Biotech Inc. Pyruvate kinase, phosphoenolpyruvate, creatine phosphokinase, phosphocreatine, and ATP were purchased from Sigma. Ultrapure dATP was from Pharmacia. Low melting agarose was purchased from FMC and Sigma. Microcon-10 microconcentrators were purchased from Amicon. 4′-amino-4,5′,8-trimethyl-psoralen (AMT) was purchased from Calbiochem.

DNA—Duplex and ssDNA substrates were derived from bacteriophage M13mp8 (37). Bacteriophages M13mp8.52 and M13mp8.121 were constructed by replacing the 30-bp *Eco*RI-*Hin*dIII fragment of bacteriophage M13mp8 with 52- or 121-bp heterologous DNA fragments as described (38). Bacteriophage M13mp8.198 is bacteriophage M13mp8 with 198 bp (*Rsa*I-*Rsa*I fragment from the *E. coli gal*T gene) inserted into the *Sma*I site (21). Bacteriophage M13mp8.1037 is bacteriophage M13mp8 with 1037 bp (*Eco*RV-*Eco*RV fragment from the *E. coli gal*T gene) inserted into the *Sma*I site (32). Supercoiled circular duplex DNA and circular single-stranded DNA from bacteriophage M13mp8 and its derivatives were prepared using methods described previously (37, 39, 40). The concentrations of dsDNA and ssDNA stock solutions were determined by absorbance at 260 nm, using 50 and 36 μg ml^{-1} $A_{260}$$^{-1}$, respectively, as conversion factors. DNA concentrations are expressed in terms of total nucleotides.

Complete digestion of supercoiled M13mp8 derivatives with appropriate restriction enzymes resulted in linear duplex DNA substrates. After digestion, residual protein was removed by sequential 1:1 extractions with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) followed by ethanol precipitation. When necessary, linear duplex DNA fragments were purified by separation in low melting agarose gels and removed from the gels with β-agarase according to manufacturer's instructions.

Full-length linear ssDNA substrates were generated by digestion with *Alw*NI after 18 base oligonucleotides complementary to the *Alw*NI site were annealed to the ssDNA. After digestion, residual protein was removed by 1:1 extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). Excess oligonucleotides were removed by washing the solution through a microconcentrator. The substrates were concentrated by ethanol precipitation. After cleav-

age, the annealed oligonucleotide remnants paired to the very ends of the linearized ssDNA are quite short and generally lost during the subsequent procedures. We did not confirm their loss in the current study, since none of the experiments involved pairing or strand exchange at any location near the ends where these oligonucleotides may have been paired. Linear ssDNA fragments were similarly generated by digesting ssDNA, annealed to appropriate 18-base oligonucleotides, with *Pac*I and *Bgl*II. The cleaved DNA was isolated from preparative low melting agarose gel using β-agarase, according to the manufacturer's instructions. The fragments were extracted 1:1 with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The fragments were concentrated by isopropyl alcohol precipitation.

Preparation of Gapped Duplex DNA—Gapped duplex DNA molecules were prepared by large scale RecA protein-mediated strand exchange reactions between circular ssDNA and linear duplex DNA molecules.

Circular duplex DNA with a defined gap, GD_{2038}, was prepared from circular M13mp8.1037 ssDNA and *Ava*II-*Bgl*II-cut duplex DNA molecules of M13.mp8. The *Ava*II-*Bgl*II-cut duplex molecules were purified from an agarose gel with β-agarase, according to the manufacturer's instructions.

Other gapped duplexes, GS_1, GS_2, GS_3, and GS_4, were prepared from circular M13mp8.198 ssDNA and linear, PCR-generated DNA molecules. Linear duplex DNA molecules were prepared by PCR using a Perkin-Elmer DNA thermal cycler. All PCR reactions were performed in a standard reaction buffer containing 10 mM KCl, 20 mM Tris-Cl (pH 8.8 at 25 °C) 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 200 μM concentration of each nucleotide (dATP, dTTP, dCTP, and dGTP), 250 nM concentration (in total oligonucleotides) of each primer, 20 units ml^{-1} Vent DNA polymerase, and 2 μg ml^{-1} M13mp8.198 DNA (linearized by *Bam*HI). The total reaction volume was 100 μl. Denaturation, annealing, and polymerization temperatures were 94, 55, and 72 °C, respectively, and amplification was carried out for 25 cycles. The PCR products were separated on a 1% agarose gel, and the amplified fragment was excised and purified from low melting agarose by a published procedure (41). The DNA was ethanol-precipitated and resuspended in Tris/Cl-EDTA. Some PCR products were 5′-end-labeled as described (41).

The primers used for the 1947-bp fragment 1 were 5′-GAAAT-GAATAATTCGCCTCTGCG-3′ and 5′-GGGAATTCGTAATCATGGT-

CAT-3′, yielding a fragment corresponding to positions 4292–6238 on the M13mp8.198 map. The G + C content of the 1947-bp fragment was 45%. The 183-bp fragment 2 used primers 5′-ACCCATGGGTGCAG-GTTTTTGAA-3′ and 5′-GCTCGCGCTGAACATAATCCAC-3′, yielding a fragment corresponding to positions 6239–6421 on the M13mp8.198 map (G + C content = 53%). The 2130-bp fragment 3 used the first and last of these four primers, yielding a fragment corresponding to positions 4292–6421 on the M13mp8.198 map (G + C = 45%).

The linear duplex and circular ssDNA molecules were combined in large scale RecA-mediated three-strand exchange reactions to generate gapped duplex DNA molecules. Reactions contained 7 μM RecA protein, 2 μM SSB, 3 mM ATP, and 21 μM circular single-stranded DNA. The reactions to generate gapped duplex substrates also contained one or more linear duplex DNA molecules: GD_{2038}, 16 μM M13mp8 DNA cut with *Ava*II and *Bgl*II; GS_1, 5.5 μM PCR fragment 1; GS_2, 6.0 μM PCR fragment 3; GS_3, 11 μM PCR fragment 1 plus 2.1 μM 5′-end-labeled PCR fragment 2; GS_4, 2.1 μM 5′ end-labeled PCR fragment 2. The reaction conditions were otherwise as described below, except that reaction volumes ranged from 0.6 to 1.4 ml. After 2 h of incubation, the DNAs were concentrated in Microcon-10 microconcentrators, and the products were separated on a 1.0% agarose gel. Gapped duplex DNA molecules were purified from the gel with β-agarase, according to the manufacturer's instructions.

Strand Exchange Reaction Conditions—Reactions between linear ssDNA and linear dsDNA were carried out at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol (w/v), and a dATP-regenerating system (20 units ml^{-1} pyruvate kinase and 10 mM phosphoenolpyruvate). The pH of the reaction mixture was 7.0 after the addition of all components except proteins and DNA (storage buffers were substituted for protein and DNA solutions in the pH measurement). Reaction volumes were 55 μl, and concentrations of DNA and proteins reported below are the final concentrations after the addition of all components. Single-stranded DNA (20 μM) and duplex DNA were incubated with RecA protein (10 μM) for 10 min before dATP (3 mM) and SSB (2 μM) were added as a mixture to start the reactions. Duplex DNA and ssDNA molecules were present in a 1:1 ratio.

Reactions between gapped circular duplex DNA and linear dsDNA were carried at 37 °C in a standard reaction buffer containing 25 mM Tris acetate (80% cation, pH 7.5), 10 mM magnesium acetate, 3 mM potassium glutamate, 1 mM dithiothreitol, 5% glycerol, and an ATP-regenerating system (10 units ml^{-1} creatine phosphokinase and 12 mM phosphocreatine). The concentrations of DNA and proteins reported here are the final concentrations after the addition of all components. ATP and SSB were added to start strand exchange reactions after the incubation of linear duplex and gapped duplex DNA with RecA protein at 37 °C for 10 min.

Monitoring DNA Strand Exchange with Agarose Gel Electrophoresis—Aliquots (12 μl) of strand exchange reactions described above were removed at 0, 20, 40, and 90 min, and the reactions were stopped by the addition of 6 μl of gel-loading buffer (25% glycerol, 15 mM EDTA, 0.025% bromphenol blue, 5% sodium dodecyl sulfate). These aliquots were stored on ice until the last time point was taken. Samples were electrophoresed overnight in an 0.8% agarose gel at 2–2.5 V cm^{-1}. In some cases, gels were dried and bands were visualized by autoradiography. To prevent the loss of small DNA fragments during gel drying, gels were dried on DEAE-cellulose paper. The DEAE-cellulose paper was prepared by soaking for 5 min in 10 mM EDTA (pH 8.0), soaking for 5 min in 1 N NaOH, and washing six times with double distilled H_2O (41).

Electron Microscopy—Samples for electron microscopy were obtained by either elution of DNA product bands from agarose gels or by spreading the entire strand exchange reaction mixture as noted. Elution of DNA from 0.7% agarose gels was carried out by adding 90 mM Tris borate and 2 mM EDTA to excised and chopped gel slices, followed by incubation for 10 min at 37 °C and overnight at 4 °C. The samples were spun for 10 min in a microcentrifuge, and the supernatants were collected. The centrifugation was repeated. Samples from entire strand exchange reaction mixtures were cross-linked with AMT prior to spreading. Aliquots (15 μl) of the strand exchange reactions at the 120-min time point were mixed with AMT (30 mg ml^{-1}, final concentration), incubated at 25 °C for 3 min, and irradiated with long wave UV light for 4 min at 25 °C (42). Cross-linked samples were incubated with proteinase K (1 mg ml^{-1} final concentration) and sodium dodecyl sulfate (0.9% final) for 60 min at 37 °C. All samples were dialyzed into 20 mM NaCl and 5 mM EDTA overnight at 25 °C on Millipore type VM
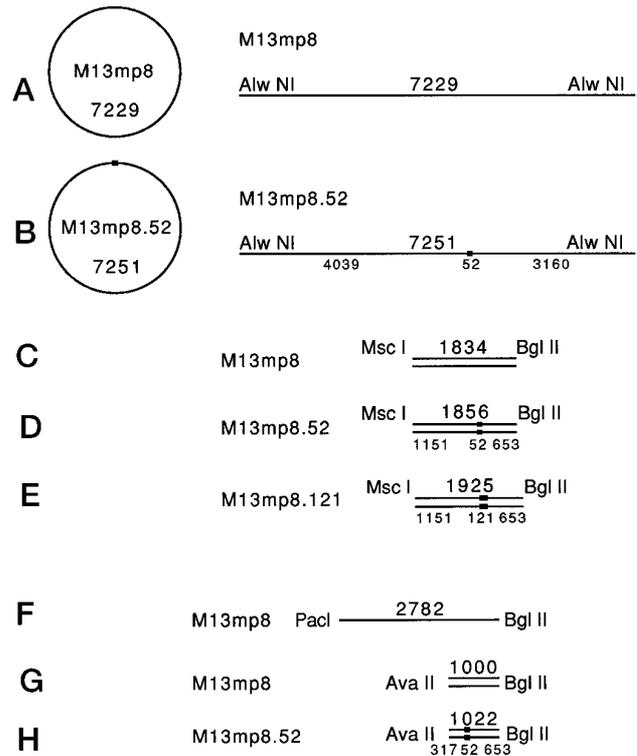

FIG. 3. **DNA substrates used in this study.** *Thickened portions* of the *lines* indicate locations of short heterologous insertions in the DNA relative to the sequence of M13mp8. *Numbers above* the *lines* denoting DNA indicate the total length (in bases or base pairs as appropriate) of the applicable DNA molecule. *Numbers below* the *lines*, where present, denote the lengths of segments within the overall DNA molecule, with segment ends defined by the boundaries of the heterologous insertions.

(0.05-mm) filters and were then spread as described previously (43). Photography and measurements of the DNA molecules were performed as described (44).

RESULTS

Experimental Design—There were two sets of experiments. In the first we compared DNA strand exchange reactions between either linear or circular ssDNA and linear duplex DNA to test a mechanism for the bypass of heterologous DNA insertions outlined in Fig. 1. The second was an attempt to test the facilitated DNA rotation model using four-strand DNA exchange reactions outlined in Fig. 2.

Testing RecA Monomer Redistribution: Heterologous DNA Insertions Were Bypassed Even When the ssDNA Substrate Was Linear—A key issue in the experimental design was the stability of the filaments. At least one report (45) and our own experience have indicated that DNA strand exchange reactions involving linear ssDNA substrates are less efficient than those with circular ssDNA. This reflects filament instability in the form of a recently characterized end-dependent disassembly of RecA filaments from ssDNA in the presence of SSB that proceeds 5′ → 3′ (22). End-dependent RecA filament disassembly is blocked at pH values below 6.5 or if dATP replaces ATP as a nucleotide cofactor or if the RecO and RecR proteins are present in the reaction mixture (22). From an experimental standpoint, the simplest way to stabilize the RecA filament on linear ssDNA is to use dATP instead of ATP. The nucleotide dATP is hydrolyzed by RecA protein at rates about 20% higher than observed with ATP yet has a general stabilizing effect on RecA filaments and supports DNA strand exchange reactions somewhat better than ATP (22, 46). The *in vivo* significance of the effects of dATP are not clear.

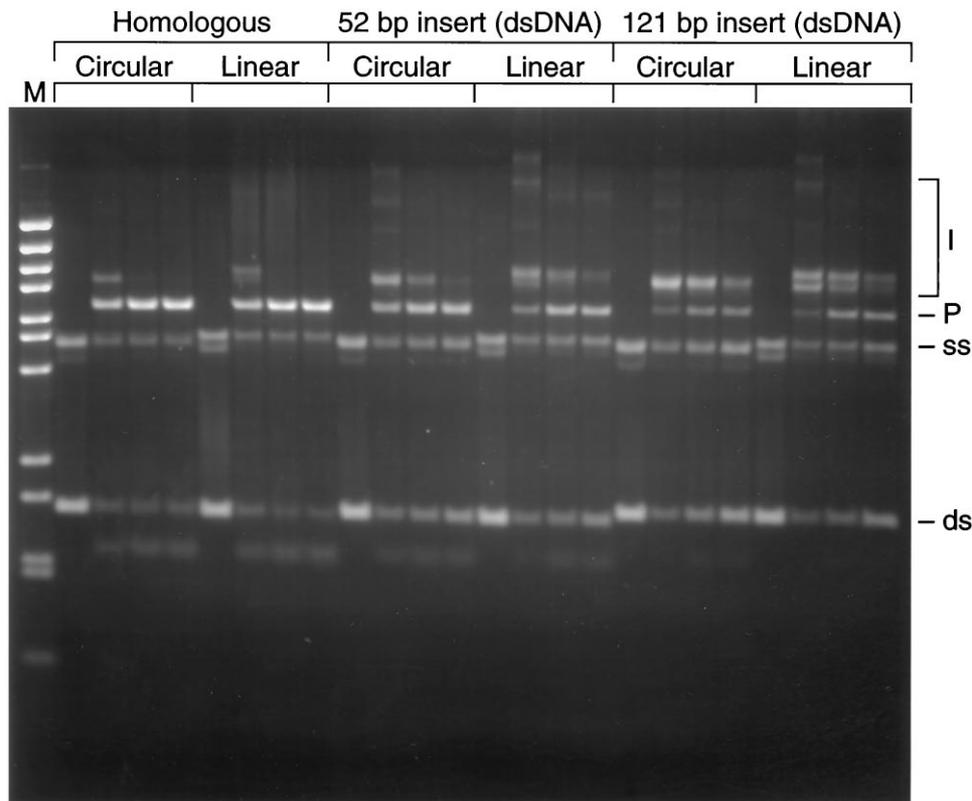The reactions of circular or linear ssDNA with 1.8-kbp du-

FIG. 4. **RecA protein-mediated DNA strand exchange with a medial 52- or 121-bp heterologous insert in the duplex DNA substrate.** Reactions were carried out as described under "Materials and Methods." Reaction time points for each reaction were 0, 20, 40, and 90 min. The ssDNA (20 μM) in each reaction is M13mp8, circular or linearized as indicated above the six individual reactions. The duplex DNA substrates for the homologous reactions (the first two reactions on the *left*) and for those involving 52 and 121 bp inserts are substrates A, B, and C, respectively, in Fig. 2. Duplex DNA concentrations for these reactions were 10.2, 10.3, and 10.7 μM, respectively, for substrates A, B, and C to account for small changes in the DNA lengths and maintain a 1:1 ratio in molecules between ssDNA and dsDNA substrates. *ss*, single-stranded DNA substrate; *ds*, linear double-stranded DNA substrate; *P*, partially double-stranded linear DNA product; *I*, reaction intermediates.

plex DNA fragments, some containing a 52- or 121-bp heterologous insert (Fig. 3, *substrates A plus C–E*), were monitored on an agarose gel (Fig. 4). The expected product of complete strand exchange contains 1.8 kbp of hybrid duplex DNA (with a bubble corresponding to the insert sequences) and 5.4 kbp of ssDNA. Where linear ssDNA was used, the product has 2.9 and 2.5 kb of ssDNA, respectively, on the proximal and distal sides of the 1.8-kbp region of hybrid duplex DNA. In all six reactions, production of hybrid duplex product was readily observed. In general, the reactions with the linear ssDNA substrates appeared to be very slightly slower than the corresponding reactions involving circular ssDNA, but the reaction end points were very similar. All of the reactions shown in Fig. 4 were repeated at least three times with consistent results. Reaction efficiency decreased as the length of the heterologous insert increased, consistent with published results (5, 6, 11, 19, 47). In an experiment similar to that of Fig. 4, a 198-bp insert was not bypassed with either circular or linear ssDNA (data not shown).

Bypass is also observed when the 52-bp heterologous insertion is moved from the dsDNA to the linear ssDNA substrate (Fig. 3, *substrates B plus D*). The reactions were again monitored on an agarose gel (Fig. 5). The expected gapped duplex product was generated in each reaction. This bypass of heterology in the ssDNA substrate was somewhat less efficient than bypass of heterology in the duplex substrate for both circular and linear RecA nucleoprotein filaments, consistent with previous observations (6).

A RecA filament formed on linear ssDNA need only rotate about its long axis to relieve any torsional stress introduced in the DNA by dissociation of interior monomers. The molecular

mass of a contiguous RecA filament formed on the ssDNA substrates used in Figs. 4 and 5 is in excess of 90,000,000 daltons. To address any possibility that the mass itself is a barrier to free rotation, strand exchange reactions were conducted using ssDNA substrates that had been shortened by more than 60%, decreasing the mass of the filaments formed on them by a like amount (Fig. 3F). Where applicable, the 52-bp insertion was in the duplex substrate (Fig. 3H). With the shorter linear ssDNA substrate, strand exchange reactions generated the expected product whether the 52-bp insertion was present or not (Fig. 6). Both the rates and the end points of the reactions were comparable with the reactions involving the much longer linear ssDNA substrates in Fig. 4.

Testing Facilitated DNA Rotation: RecA Protein Can Unwind a Distal Segment of Duplex DNA as the Final Stage of a Four-strand Exchange Reaction—The question introduced in Fig. 2 was addressed in two experiments. The first was set up to determine if a complete DNA strand exchange could occur if the duplex region of the gapped duplex substrate extended beyond the end of DNA2, as in Fig. 2B. The experiment also addressed the effect of the length of the duplex extension in DNA1 on reaction efficiency. The second experiment was carried out to determine if severing the covalent link between the duplex extension and the remainder of the DNA strand (as in Fig. 2C) affected the reaction.

The substrates used in the first experiment, a gapped duplex substrate and a series of linear duplex substrates shortened progressively at the distal end, are illustrated in Fig. 7. In a standard RecA protein-mediated four-strand exchange reaction, the linear duplex DNA overlaps the single strand gap on the proximal end. The distal end of the linear duplex DNA
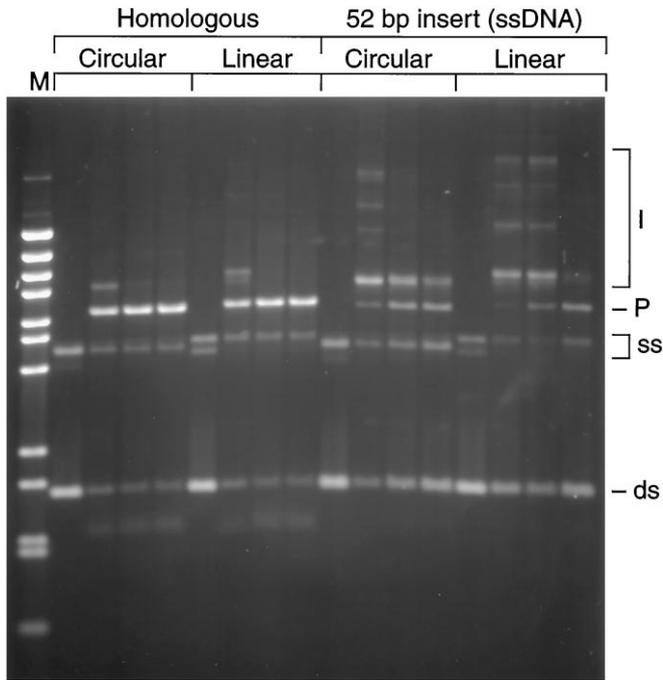
FIG. 5. **RecA protein-mediated DNA strand exchange past a 52-bp heterologous insert in a ssDNA substrate.** Reactions were carried out as described under "Materials and Methods." Reaction time points for each reaction were 0, 20, 40, and 90 min. Each reaction contained 10.2 μM M13mp8 (*Msc*I-*Bgl*II) duplex DNA fragment (substrate A in Fig. 2). The reactions labeled *Homologous* contained 20 μM M13mp8 ssDNA, circular or linearized as indicated above the individual reactions. Reactions labeled *52 bp insert (ssDNA)* contained 20.1 μM M13mp8.52 ssDNA, circular or linear as indicated. Abbreviations are the same as those in Fig. 4.

FIG. 6. **Heterology bypass with shorter ssDNA substrates.** Reactions were carried out as described under "Materials and Methods." Reaction time points for each reaction were 0, 20, 40, and 90 min. Reactions contained 20 μM M13mp8 (linearized with *Pac*I and *Bgl*II to generate a 2782-base fragment). The *Homologous* reaction also contained 14.4 μM of a *Ava*II-*Bgl*II dsDNA fragment derived from M13mp8. The *52 bp insert* reaction contained 14.7 μM of a *Ava*II-*Bgl*II dsDNA fragment derived from M13mp8.52. All DNA substrates are illustrated in Fig. 3, *F–H*, and abbreviations are the same as those in Fig. 4.

FIG. 7. **Substrates used in the first series of four-strand exchange reactions.** All reactions in this series used the same gapped duplex substrate, GD$_{2038}$. Linear duplex substrates either had distal ends identical to the distal end of the duplex region in GD$_{2038}$ (linear substrate cleaved at one end with *Ava*II) or were shortened on the distal end by 88, 199, 302, and 835 bp as indicated.

substrate also overlaps the single-stranded region of the gapped duplex or extends at least up to the duplex/single strand junction. After strand exchange enters the duplex region of the gapped duplex to convert a three-strand exchange to a four-strand exchange, the exchange reaction remains reciprocal through the end of the reaction. Gapped duplex and linear products accumulate as the reaction progresses (Fig. 8).

The effect of shortening the distal end of the linear duplex DNA substrate on a four-strand exchange reaction is shown in Fig. 8. In a reaction between a gapped duplex and a linear duplex lacking 88 bp from the distal end, separate linear and gapped duplex products formed (Fig. 8). The resolution of the two products indicated that the 88-bp on the distal end of the gapped duplex substrate were unwound in the last stage of the reaction. Products were evident within 40 min, although this reaction was less efficient than the reaction involving a full-length linear substrate (Fig. 8). As the length of the duplex region requiring unwinding increased, the efficiency of strand-exchange decreased somewhat further. However, linear duplex substrates lacking 199, 302, and 835 bp from the distal end all reacted to form detectable discrete products (Fig. 8). No unwinding was observed when the length of the distal segment to be unwound was increased to 3722 bp (data not shown).

We investigated the possibility that the relatively low level of completed products in the nonreciprocal exchange reactions in Fig. 8 was due to nuclease contamination. An endonuclease could convert molecules in which strand exchange had stalled at the distal extension in the duplex of DNA1 to molecules migrating like products if it cleaved off the DNA branch. This would generate products with a ssDNA gap shorter than those expected if the products were generated by unwinding the distal extension, since the distal extension would still be present. Alternatively, an exonuclease could act on the interrupted
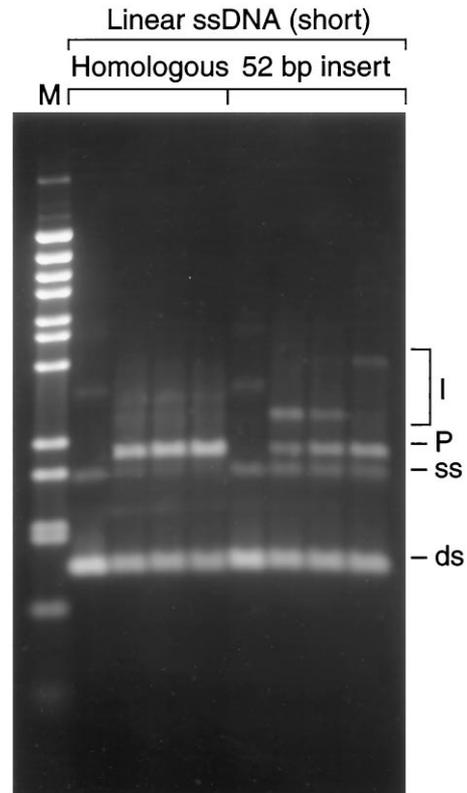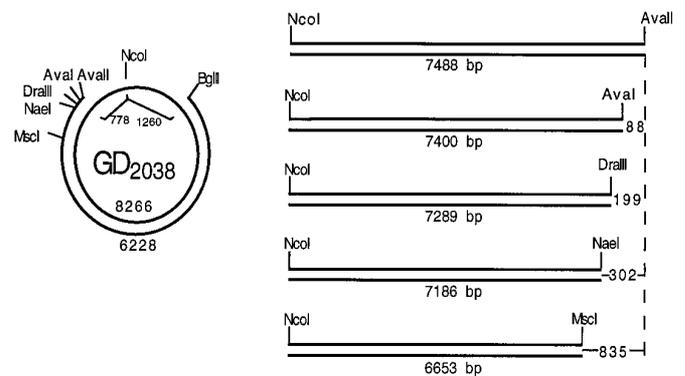
strand in the gapped circle, creating gaps of variable size and sometimes removing the DNA branch. To determine the length of the ssDNA gap in the products, the product bands from some reactions were excised from the gel, and the DNA was examined by electron microscopy. Representative gapped duplex products from strand exchanges with distal unwinding of 835 and 88 bp are shown in Fig. 9, *panels A* and *B* and *panels C* and *D*, respectively. The length of the ssDNA gap in products such as those in Fig. 9, *A* and *B*, was measured and found to be consistent with complete unwinding of the 835-bp distal region.
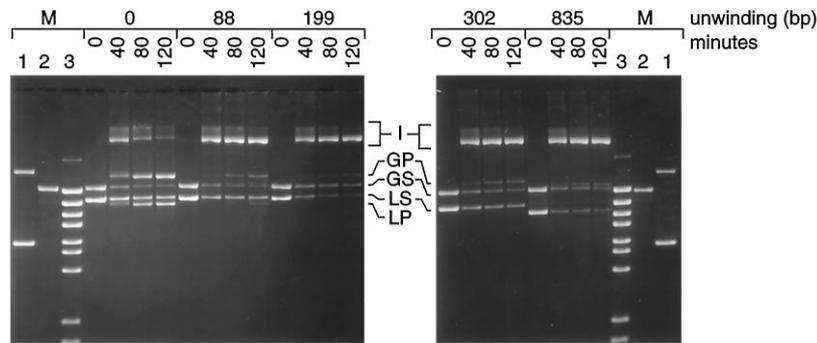
FIG. 8. **Unwinding of a distal segment of duplex DNA during a four-strand exchange reaction.** Reactions were carried out as described under "Materials and Methods." Markers (*M*, *lanes 1–3*) were as follows: nicked plus supercoiled M13mp8.1037 (*lane 1*); GD$_{2038}$ (*lane 2*); *Bst*EII digestion of bacteriophage λ DNA (*lane 3*). Individual reactions are identified by the length of the distal extension in DNA1. The first reaction on the *left* (designated *0*) was a fully reciprocal four-strand exchange reaction, while the linear duplexes (DNA2) in the remaining reactions lacked 88, 199, 302, and 835 bp, respectively, at the distal end relative to the duplex region in DNA1. Reactions contained 4.2 μM RecA protein, 1.0 μM SSB, and 20 μM GD$_{2038}$. Reaction 1 contained 15 μM *Ava*II-*Nco*I-cut M13mp8.1037; reaction 2 contained 15 μM *Ava*I-*Nco*I-cut M13mp8.1037; reaction 3 contained 15 μM *Dra*III-*Nco*I-cut M13mp8.1037; reaction 4 contained 15 μM *Nae*I-*Nco*I-cut M13mp8.1037; and reaction 5 contained 13 μM *Msc*I-*Nco*I-cut M13mp8.1037. Reaction volumes were 55 μl. Time points in each reaction (12-μl aliquots) were 0, 40, 80, and 120 min (from *left* to *right* as indicated). *GS* and *LS*, gapped circular and linear duplex DNA substrates, respectively; *GP* and *LP*, gapped circular and linear duplex DNA products, respectively; *I*, reaction intermediates.

The measured length of the gap was 1770 ± 90 nucleotides (nine molecules measured), compared with a gap of 1613 nucleotides that would be generated by unwinding of the distal extension. No molecules were observed with a gap size approximating the 778-nucleotide gap expected if a nuclease simply removed the DNA branch. When the entire 835-bp unwinding reaction mixture was spread for electron microscopy, the predominant species was found to be a branched intermediate, apparently stalled at the junction with the distal duplex extension in DNA1 (Fig. 9, *E* and *F*). This is consistent with the results in Fig. 8. There was no evidence of unusual variability in gap size of the sort that might result from a contaminating exonuclease, either in the complete reaction mixture or in the gapped DNA in the product band. We conclude that the products generated in the reactions requiring nonreciprocal exchange resulted from unwinding of the distal segment rather than from nuclease action.

Similar EM results were obtained in an examination of products such as those in Fig. 9, *C* and *D*. The measured gap size (970 ± 70 nucleotides; 14 molecules measured) is more consistent with the gap expected if the distal 88 bp is unwound (866 nucleotides) than with the gap expected if the branch is removed by nuclease action (778 nucleotides). However, the differences in gap sizes are smaller and less meaningful in this instance than they are when unwinding of 835 bp is required.

The yield of discrete products generated in these reactions depended upon the concentrations of magnesium ion, linear dsDNA substrate, and SSB as well as the length of time the reactions were followed. The reactions were optimal at magnesium acetate concentrations of between 6 and 12 mM (data not shown). A stronger reaction than that observed in Fig. 8 could be observed, at least for the shorter distal segments, if the ratios of SSB and linear dsDNA to other reaction components was increased, and the reaction time course was extended (Fig. 10). The effect of SSB was especially important (data not shown). Doubling the SSB:DNA substrate ratio (as was done in Fig. 10 relative to Fig. 8) greatly increased the yield of products at all time points, despite the fact that the SSB concentration under either set of conditions is greatly in excess relative to the amount of single-stranded DNA available for binding. We hypothesize that the excess SSB is required to ensure that the separated complementary strands do not reanneal. Adding the excess SSB prior to RecA protein, which should inhibit the nucleation of RecA filaments, had a substantial inhibitory effect on the reaction (Fig. 10).

As a final control, we tested the possibility that simple instability of the branched reaction intermediates could contribute to the yield of products. A set of reactions was carried out with the DNA and RecA protein concentrations of Fig. 10 but with a wide range of magnesium acetate and SSB concentrations, encompassing the optimum for both variables. Incubation at 37 °C for 18 h, enough time for the ATP regeneration system to be long depleted, did not increase the yield of products over that observed at 4 h but instead decreased it somewhat (data not shown). The yield of branched DNA intermediates did not decrease. We conclude that the intermediates do not undergo significant breakdown under these conditions and that spontaneous reannealing of the products may occur and lead to an underestimation of overall reaction efficiency.

Unwinding of the Distal Duplex DNA Segment Requires a Covalent Link to DNA Undergoing a Reciprocal Strand Exchange—The nonreciprocal DNA unwinding could represent a cryptic helicase activity of the RecA protein monomers bound to the DNA being unwound or a contaminating helicase, perhaps activated in some way by the strand exchange process. Alternatively, if the four-strand DNA exchange is brought about by facilitated DNA rotation, the distal segment might be unwound indirectly as a result of the torsional stress generated by the rotation of the DNA strands upstream of it. To distinguish between these possibilities, a gapped duplex substrate was constructed with a nick on the exchanged strand, separating the regions of reciprocal and nonreciprocal exchange. The nick would preclude the transmission of any torsional stress generated in the reciprocal strand exchange reaction into the distal duplex DNA segment. It should not affect unwinding of the duplex segment if unwinding is mediated directly by the RecA protein that is bound to it. The substrates for this series of experiments are illustrated in Fig. 11. The different gapped duplexes are designated GS$_1$–GS$_4$.

In a fully reciprocal four-strand exchange reaction, with GS$_1$, the expected products were generated (Fig. 12*A*, *reaction 1*). Next, a strand exchange reaction was conducted with a gapped duplex substrate (GS$_2$) containing a segment of duplex DNA extending 183 bp beyond the distal end of the linear duplex. As expected from the results in Fig. 8, the efficiency of the overall strand exchange is reduced by the requirement that the 183-bp segment be unwound. However, the generation of discrete products indicated that RecA protein can unwind this distal duplex segment without reciprocal exchange (Fig. 12*A*, *reaction 2*). When the distal duplex segment was separated from the
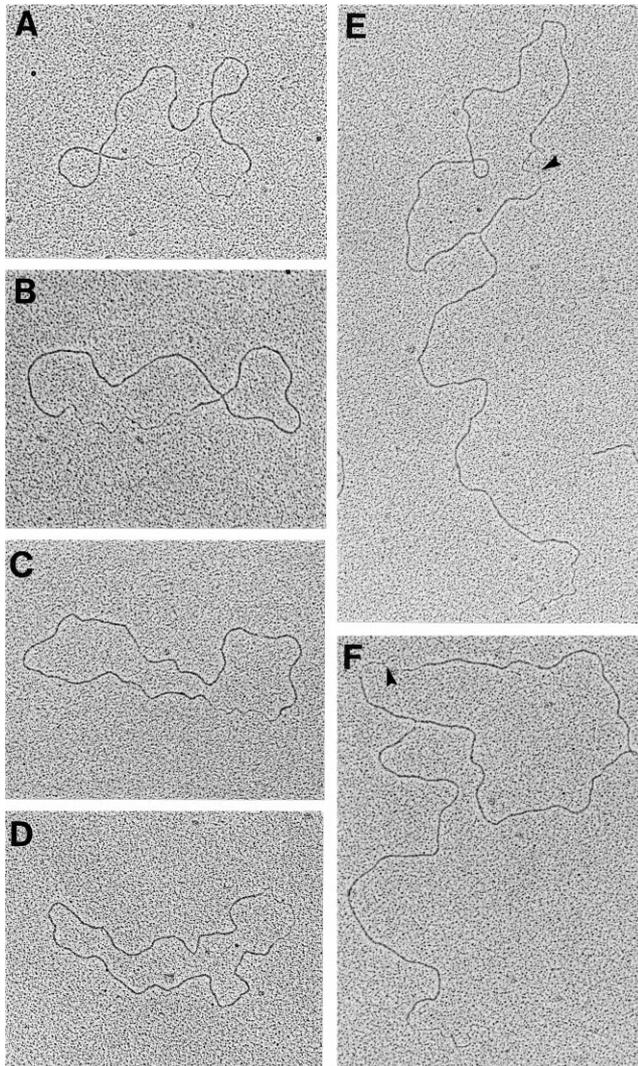
FIG. 10. **Improved yields of reaction products.** Reactions were carried out as described under "Materials and Methods" and contained 2.5 μM RecA protein, 1.0 μM SSB, 10 μM GD_{2038}, and a 20 mM concentration of the appropriate linear dsDNA illustrated in Figs. 7 and 8. The lengths of the distal segments in the gapped DNA are indicated for each reaction. In the first reaction on the *left*, the standard protocol was altered only in that SSB was added to the reaction prior to RecA protein. Abbreviations are the same as those in Fig. 8.
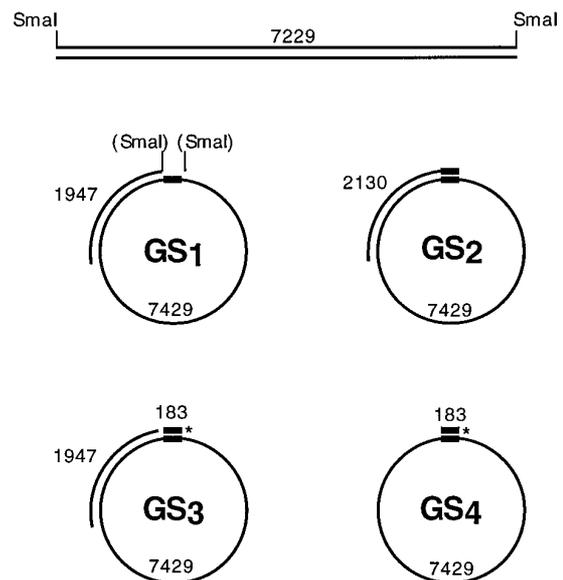
FIG. 11. **Substrates used in the second series of four-strand exchange reactions.** All reactions in this series used the same linear duplex substrate, *Sma*I-linearized M13mp8, while the gapped duplex substrate varied. GS_1 included 1947 bp of duplex DNA with a distal end identical to the distal end of the linear substrate. GS_2 had 2130 bp of duplex DNA, 1947 bp matching the linear substrate and another 183 contiguous bp extending beyond the distal end of the linear substrate. GS_3 was identical to GS_2 except that a nick divided the matching and distal extension segments of the exchanged strand, and the 183-nucleotide fragment was radiolabeled. GS_4 had only the 183 bp of dsDNA corresponding to the distal extension, including the radiolabel.

FIG. 9. **Gapped duplex products of the DNA strand exchange reactions; nonreciprocal unwinding of distal extensions of 88 and 835 bp.** Reactions were carried out as described under "Materials and Methods" and in the legend to Fig. 3, except that reaction volumes were 30 μl. The reaction products comigrating with gapped duplexes at the 120-min time point were excised and eluted from a gel and spread for electron microscopy. *A* and *B*, gapped duplex products from a strand exchange reaction requiring the unwinding of an 88-bp distal extension. *C* and *D*, gapped duplex products from a strand exchange reaction requiring the unwinding of an 835-bp distal extension. *E* and *F*, intermediates from a reaction such as that in *panels A* and *B*. An aliquot was taken from the reaction at 120 min, and the entire reaction was cross-linked, deproteinized, and spread as described under "Materials and Methods." The molecules shown are typical of the reaction intermediates observed and were the predominant species present. The intermediates in *panels E* and *F* exhibit the expected fine structure for the experiment (see Fig. 2); each intermediate has a single strand gap (see *arrows*) and a single strand region at the end of the DNA tail.

remainder of its strand in the gapped duplex substrate (GS_3), the efficiency of the subsequent four-strand exchange reaction was improved (Fig. 12*A*, *reaction 3*). A three-strand exchange reaction also proceeds efficiently when the distal end of the linear duplex substrate does not overlap the same 183 bp representing the only duplex region in the gapped duplex GS_4 (Fig. 12*A*, *reaction 4*).

The fate of the 183-nucleotide DNA strand cannot be ascertained in reactions 3 and 4 of Fig. 12*A*. These reactions were therefore repeated, except that the 183-bp fragment of the gapped duplex substrate included a radiolabel to facilitate product identification (Fig. 12*B*). In the reaction corresponding
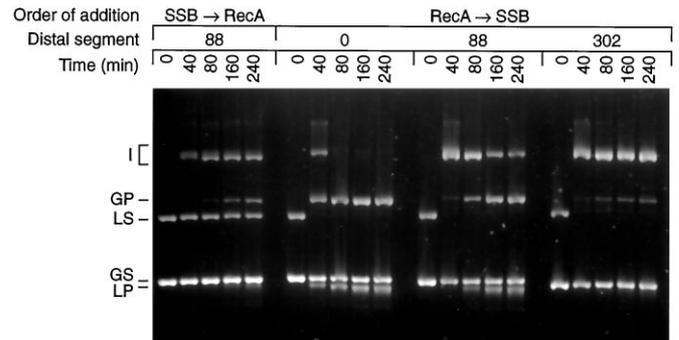
to Fig. 12*A*, *reaction 3*, only the substrate and the gapped duplex product was radiolabeled. No free 183-bp fragment was observed (Fig. 12*B*, *reaction 3*). This indicates that the 183-bp distal duplex segment was not unwound when it was separated by a nick from the region of reciprocal exchange. The result was the same when the strand exchange reaction occurring proximal to the 183-bp duplex segment was limited to three strands Fig. 12*A*, *reaction 4*, and 12*B*, *reaction 4*). The 183-bp fragment was not unwound under any circumstances unless it was covalently linked to a strand involved in a reciprocal four-strand exchange reaction proximal to it.

Additional control reactions were conducted to further clarify the conditions required to separate the 183-bp fragment from the gapped duplex substrate. Under the same reaction conditions, but without a linear duplex substrate, the radiolabeled
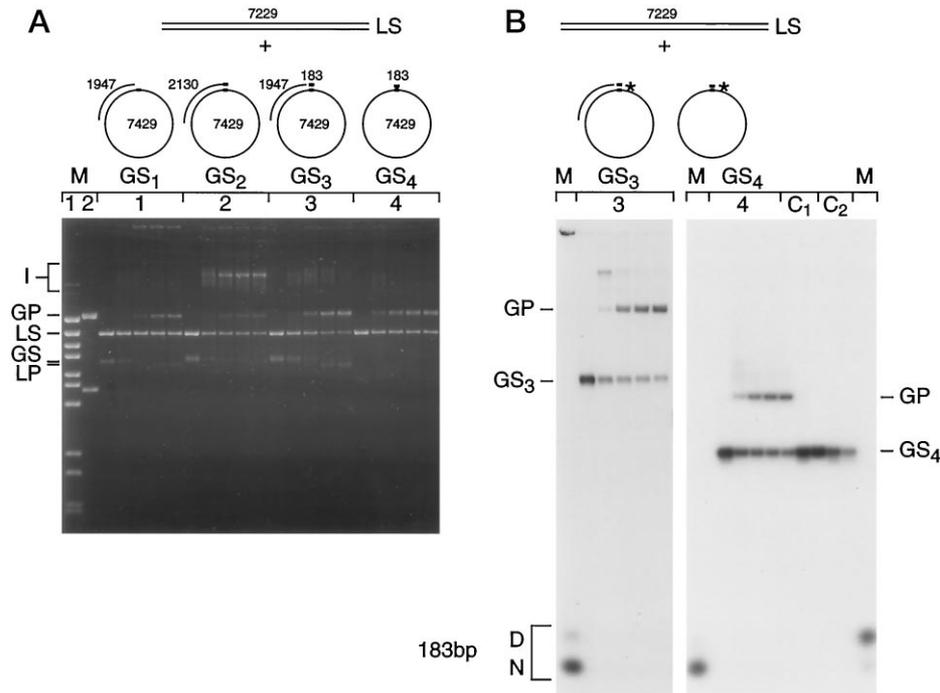
FIG. 12. **Effect of a nick separating the reciprocal and nonreciprocal regions on distal nonreciprocal unwinding.** Reactions were carried out as described under "Materials and Methods" and contained 2.6 μM RecA protein, 0.8 μM SSB, and 15.4 μM *Sma*I-linearized M13mp8, except as noted below. Reaction 1 contained 10 μM GS$_1$; reaction 2 contained 10 μM GS$_2$; reaction 3 contained 10 μM GS$_3$; and reactions 4, C$_1$, and C$_2$ contained 8.1 μM GS$_4$. Total reaction volumes were 25 μl for control reactions (C$_1$ and C$_2$) in *panel B* and 60 μl for all other reactions. Aliquots (10 μl) were removed at the indicated times. *A*, reactions 1–4 were strand exchange reactions between *Sma*I-linearized M13mp8 and the different gapped duplexes, GS$_1$, GS$_2$, GS$_3$, and GS$_4$, respectively (Fig. 11). Time points in each reaction were 0, 30, 60, 90, and 120 min. Markers in lane M$_1$ came from a *Bst*EII digestion of bacteriophage λ DNA. Markers in lane M$_2$ were nicked plus supercoiled M13mp8.198. *B*, reactions 3 and 4 were the same as in *panel A*, except that the short 183 nucleotide fragment was labeled (*). Reactions C$_1$ and C$_2$ were control reactions with GS$_4$. Reactions C$_1$ and C$_2$ contained no *Sma*I-linearized M13mp8, and reaction C$_2$ contained no RecA protein or SSB. Time points in reactions C$_1$ and C$_2$ were 0 and 120 min. Markers *N* and *D* are radiolabeled native and heat-denatured 183-nucleotide single strand fragments, respectively, identical in sequence to the 183-nucleotide distal fragment in the exchanged strand of substrate GS$_3$.
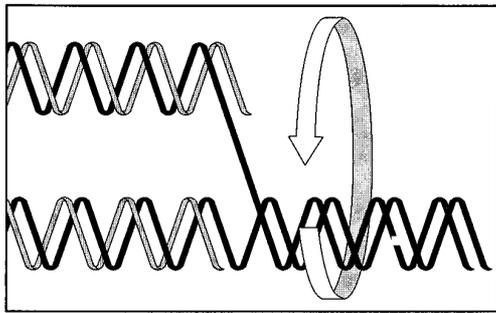

FIG. 13. **Possible mechanism for nonreciprocal strand exchange.** The two duplex regions on the *left* may be rotated about each other as shown, unwinding the distal extension region (*right*) by means of the torsional stress generated.

183-bp fragment remained part of the gapped duplex when RecA protein was bound to it under otherwise identical reaction conditions (Fig. 12*B*, *reaction C$_1$*). In addition, under the same conditions, but without linear duplex substrate, RecA protein, and SSB, the 183-bp fragment remained part of the gapped duplex (Fig. 12*B*, *reaction C$_2$*), demonstrating that the substrate itself has no inherent instability. These results indicate that the unwinding of the nonreciprocal region was not caused by a direct, helicase-like, unwinding of the 183 bp.

DISCUSSION

Two sets of experiments with quite different experimental rationales are described in this report. They are unified only in that they both address the role of ATP hydrolysis in RecA protein-mediated DNA strand exchange. The first set of experiments demonstrates that the RecA redistribution model can-not explain the bypass of heterologous DNA insertions, one of the key effects of ATP hydrolysis. The second set of experiments presents a reaction that is readily explained only by a facilitated DNA rotation process. A coupling of ATP hydrolysis to rotation of segments of one DNA substrate about the other (as illustrated in detail elsewhere (3, 11, 26)) would expand the constellation of established RecA protein functions to include the role of the molecular motor.

The bypass of heterologous insertions during RecA protein-mediated DNA strand exchange is affected little when the ssDNA substrate is converted from a circular to a linear form. Insertions of at least 121 bp are bypassed with good efficiency. Good heterology bypass is also mediated by relatively short RecA filaments formed on linear ssDNA that is 2800 bases in length. These results provide a simple argument that heterology bypass does not occur by means of RecA monomer redistribution (as in Fig. 1). In a linear RecA nucleoprotein filament, any DNA underwinding uncovered by RecA dissociation should be readily relaxed by simple rotation of the nucleoprotein filament about its long axis. Since the DNA within a RecA filament is underwound by nearly 40% when ATP is being hydrolyzed (30), the release of 18 bp by dissociation of six RecA monomers could in principle be translated into strand separation over a stretch of 8 bp if filament rotation were constrained. The unwinding and bypass of a 52-bp insertion would therefore require the dissociation of a stretch of at least 40 RecA monomers, vacating 120 bp total, assuming that *all* of the released underwinding was translated into strand separation precisely at the heterologous insertion. The bypass of a 121-bp insertion would require the dissociation of over 90 RecA monomers, vacating over 270 bp of DNA. Rotation of a linear filament at its

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free ends would relax any DNA released by RecA dissociation, preventing the translation of underwinding into strand separation. The linear filaments used in this study should not promote the observed bypass of heterologous insertions if ATP hydrolysis serves only to redistribute RecA monomers.

In addition, there is no evidence that filament gaps of the required size are ever created in the interior of a RecA filament. Filament assembly processes (filament extension on the end corresponding to that nearest the 3′-end of a ssDNA lattice) are very rapid on either ssDNA or dsDNA when a primer in the form of an appropriate filament end is present (3, 48). Disassembly at the opposite end of the filament is generally much slower (3, 20–22, 48). Any significant gap in the filament interior should be quickly filled. To augment this point, we note that doubling the concentration of RecA protein (to twice the amount needed to saturate the ssDNA) does not inhibit the bypass of a 52-bp heterologous DNA insertion,[2] as it might if partial disassembly of the filament were a requirement for bypass.

The heterology bypass with linear DNA substrates is readily explained by the facilitated DNA rotation model (3, 11, 12, 16). A second set of experiments addresses the facilitated DNA rotation model more directly. RecA protein promotes the complete unwinding of hundreds of base pairs of duplex DNA as the final stage of an otherwise reciprocal four-strand exchange reaction. The unwinding occurs only if both strands are contiguous with DNA strands directly involved in the four-strand exchange portion of the reaction. The unwinding mechanism therefore involves an indirect application of torsional stress, as opposed to a direct helicase-like melting of the DNA by bound RecA. The experiments in Figs. 7–12 were inspired in part by the work of Jwang and Radding (27), and our results are consistent with their work. These results are also readily explained by facilitated DNA rotation of the DNA substrates coupled to ATP hydrolysis (16). Unwinding of the duplex DNA segments would occur as a byproduct of rotating the hybrid DNA segments, as illustrated in Fig. 13. This mechanism requires no disassembly of RecA filaments to bring about the unwinding.

The bypass of heterologous insertions during three-strand exchanges displays a complete requirement for ATP hydrolysis (6). We infer that the unwinding of the duplex segments documented in the present study also requires ATP hydrolysis. We cannot directly test this, however, because the four-strand exchange that must precede the duplex unwinding also requires ATP hydrolysis (7, 11).

This work must be considered in the context of a range of recent findings. When ATP is not hydrolyzed by RecA protein (*e.g.* when the RecA K72R mutant protein is used in place of the wild type), some DNA strand exchange occurs, but it is limited in extent (4, 8–11) and exhibits other limitations enumerated in the Introduction. The RecA redistribution model posits that DNA strand exchange without ATP hydrolysis is limited only by discontinuities in the RecA filament and that ATP hydrolysis is needed *only* for RecA monomer redistribution to repair the discontinuities. The exchange of RecA monomers between free and bound forms that is required by this model has been observed during DNA strand exchange (26). However, DNA strand exchange is blocked in mixed filaments of wild type and K72R mutant protein under conditions where a facile exchange of RecA monomers between free and bound forms is observed (26). Also, the addition of excess RecA K72R protein to reactions (sufficient to fill in any filament discontinuities) does not overcome the barrier to more extensive strand exchange. RecA

redistribution is therefore not sufficient in itself to overcome the limited extent of strand exchange seen when ATP is not hydrolyzed. We demonstrate here that RecA redistribution also cannot account for the requirement for ATP hydrolysis in the bypass of heterologous DNA insertions. No effort has been made to explain how RecA redistribution might explain the requirement for ATP hydrolysis in four-strand exchange reactions. The role of RecA monomer exchange in filament interiors (25) in the mechanism of DNA strand exchange, if any, remains to be elucidated.

The facilitated DNA rotation model replaces the discontinuous filament with a discontinuous DNA pairing intermediate. Segments in which the duplex DNA substrate is interwound with the ssDNA within the filament alternate with segments in which the duplex DNA is looped outside the filament (3, 11, 26). Since homologous DNA pairing occurs in the segments contained within the RecA filament, this idea does not conflict with any current proposal for the alignment of three DNA strands by RecA protein. In the absence of ATP hydrolysis, the DNA substrates are paired, and strand exchange is promoted by binding energy within the filament groove, but it can proceed only up to the first external loop. We point out that discontinuous DNA pairing intermediates of the required type are formed under conditions where ATP hydrolysis (and therefore extensive DNA strand exchange) is blocked and can be observed directly by electron microscopy (11, 49). Beginning with these intermediates, facilitated DNA rotation provides a comprehensive explanation for the observed effects of ATP hydrolysis on DNA strand exchange (3, 16) as well as for the quantitative relationship between the rates of branch movement and ATP hydrolysis (12).

Traditionally, bacterial recombination has been explored within a paradigm defined by conjugational recombination (17, 50). However, a molecular motor function for the RecA ATPase is best rationalized within a paradigm focused on recombinational DNA repair, where unidirectionality and the capacity to bypass structural barriers in the DNA would be critical features of DNA strand exchange (15, 16). The ATP binding site represents the most conserved portion of the RecA protein structure (3), yet ATP hydrolysis appears to have no role in the fundamental process of DNA pairing. DNA pairing and significant DNA strand exchange is promoted by a RecA mutant protein (K72R) that binds but does not hydrolyze ATP (8, 11). However, *in vivo*, this same mutation results in a RecA null phenotype (51). At least one other RecA mutant protein, with partially compromised DNA binding and ATP hydrolytic functions, will function adequately in conjugational recombination *in vivo* but will not function in recombinational DNA repair (52). These and other results (3, 15) indicate that recombination leading to repair is an inherently more demanding process than recombination during conjugation and that at least some of the molecular features of RecA protein evolved specifically to address the exigencies of repair. If the biochemistry of RecA protein and other bacterial Rec proteins is to be understood in all its complexity, we believe that the paradigm within which that understanding is sought should be shifted from conjugational recombination to recombinational DNA repair.

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