DNA Pairing and Strand Exchange by the *Escherichia coli* RecA and Yeast Rad51 Proteins without ATP Hydrolysis

ON THE IMPORTANCE OF NOT GETTING STUCK*

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The bacterial RecA protein and the homologous Rad51 protein in eukaryotes both bind to single-stranded DNA (ssDNA), align it with a homologous duplex, and promote an extensive strand exchange between them. Both reactions have properties, including a tolerance of base analog substitutions that tend to eliminate major groove hydrogen bonding potential, that suggest a common molecular process underlies the DNA strand exchange promoted by RecA and Rad51. However, optimal conditions for the DNA pairing and DNA strand exchange reactions promoted by the RecA and Rad51 proteins *in vitro* are substantially different. When conditions are optimized independently for both proteins, RecA promotes DNA pairing reactions with short oligonucleotides at a faster rate than Rad51. For both proteins, conditions that improve DNA pairing can inhibit extensive DNA strand exchange reactions in the absence of ATP hydrolysis. Extensive strand exchange requires a spooling of duplex DNA into a recombinase-ssDNA complex, a process that can be halted by any interaction elsewhere on the same duplex that restricts free rotation of the duplex and/or complex, *I.e.* the reaction can get stuck. Optimization of an extensive DNA strand exchange without ATP hydrolysis requires conditions that decrease nonproductive interactions of recombinase-ssDNA complexes with the duplex DNA substrate.

Both the *Escherichia coli* RecA and eukaryotic Rad51 proteins bind readily to ssDNA[1] in the presence of ATP and promote DNA pairing and strand exchange with homologous duplex DNA targets *in vitro* (1–3). With long DNA substrates, complete strand exchange occurs in at least three distinct phases. The first phase is the formation of a RecA or Rad51 protein helical filament on the single-stranded DNA substrate. This is followed by alignment of homologous DNA sequences between the protein/ssDNA filaments and the duplex substrate. Alignment is followed closely by, or perhaps in concert with, a strand switch where the complementary strand of the duplex substrate is transferred to the DNA strand within the filament. None of these processes requires ATP hydrolysis (4–12). Once DNA alignment and strand transfer has begun, a third phase occurs in which the nascent hybrid duplex DNA can be extended over thousands of base pairs. For RecA protein, this third phase is generally coupled to the hydrolysis of ATP (13–15). DNA strand exchange is generally investigated in experiments employing long DNA substrates derived from bacteriophages (16–18). Short oligonucleotide fragments are used to investigate selectively the DNA pairing (second) phase of DNA strand exchange (19–22).

RecA and Rad51 are frequently cited as functional and structural homologs, based on amino acid sequence similarities (23), the apparent similarity in their filament structures (24), and the DNA pairing and strand exchange reactions they both promote. Both proteins require a single strand DNA-binding protein (bacterial SSB or eukaryotic RPA) for an optimal DNA strand exchange reaction. For both RecA and Rad51, the requirements for homologous pairing, other than the DNA substrates and recombinase, vary among reports but presumably include ATP (or certain ATP analogs), Mg^{2+} in sufficient concentration to coordinate the nucleotide cofactor, and a multivalent cation. This cationic species is usually additional magnesium but is often replaced with spermidine in experiments with yeast Rad51 protein (2). To date, there has been little attempt to compare directly the two proteins side by side. Our study initiates this potentially revealing comparison with an exploration of the effects of altered DNA substrates and solution conditions.

The altered DNA substrates were chosen to expand the range of useful substrates for these reactions and to provide additional insight into the DNA pairing process. In a previous report (21), we provided evidence that the molecular basis for homologous pairing is likely conserved among bacteria and yeast, based upon the capacity of both proteins to make use of DNA substrates containing base analogs with non-canonical functionality. The mechanism of DNA pairing remains uncertain, with the controversy focusing largely on two competing mechanisms. First is the R-form DNA hypothesis. In this model, the bound single strand within a RecA or Rad51 filament is aligned with a homologous duplex DNA (dsDNA) via non-Watson-Crick hydrogen bonding in its major groove, allowing a transient formation of a novel DNA triplex (Fig. 1) (25–29). The simultaneous binding of three DNA strands within a RecA filament (in some conformation) is well documented (30–37). However, efforts to demonstrate spontaneous formation of the R-form triplex structure *in vitro,* or to visualize it in the

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ATPγS, adenosine 5′-*O*-thiotriphosphate; SSB, single strand DNA-binding protein; RPA, replication protein A; DTT, dithiothreitol; MOPS, 4-morpholinepropanesulfonic acid; oligos, oligonucleotides; 2AP, 2-aminopurine.

electron microscope, have had no substantial success.

The alternative view is that homologous alignment is based simply on Watson-Crick pairing interactions (38–43). In principle, the dsDNA could approach via either the major or minor groove, although most studies have supported a minor groove first path (38–42). Protein binding interactions within the groove would extend the DNA and facilitate base flipping within one strand of the dsDNA, permitting a homology search based on Watson-Crick interactions between the flipped bases and the ssDNA bound within the RecA filament. A detailed study has verified key predictions of the base-flipping proposal (43).

For all of their similarities, the differences between RecA protein and Rad51 protein are at least as intriguing and potentially more informative. Rad51 protein can readily nucleate filament formation on either ssDNA or dsDNA (17, 44, 45), whereas RecA protein exhibits a very strong bias for nucleation on ssDNA (1). RecA protein hydrolyzes ATP at rates 30–50 times faster than Rad51 (2, 4). Both RecA and Rad51 proteins require nucleotide binding for activity. However, under most conditions RecA protein requires ATP *hydrolysis* for extensive strand exchange (9, 10, 15) whereas Rad51 protein does not (4, 5). The role of ATP hydrolysis in the reactions promoted by RecA is not completely understood, and a fundamental difference in the use of ATP by RecA and Rad51 proteins clearly exists that has not been explained (1, 15, 44, 46). Several reports have suggested that there are mechanistic differences in strand exchange activity, including the polarity of the reaction (3, 17, 47, 48). We also note that an extensive Rad51-promoted DNA strand exchange reaction is highly dependent on the addition of single strand DNA-binding proteins, with the cognate replication protein A (RPA) being quite effective (2, 18, 49). For RecA protein, the *E. coli* SSB protein is stimulatory, but significant reaction can occur in its absence.

The differences extend to the conditions under which optimal DNA pairing and strand exchange are observed. In general, RecA protein functions best in a buffer with added Mg^{2+}. The Mg^{2+} is required to form active complexes with ATP, to enhance DNA pairing interactions, and to optimize extensive strand exchange reactions (8, 9, 50–55). The Mg^{2+}/ATP ratio is important (9, 52), with ratios much in excess of 1.0 necessary for optimal DNA strand exchange. All reports of Rad51 protein-mediated DNA strand exchange also include Mg^{2+} in the reaction protocol, but high levels of Mg^{2+} permit only limited DNA strand exchange. Reported yields in Rad51-promoted DNA strand exchange reactions have been quite variable (3, 56–58). With the yeast Rad51 protein, high yields are generally observed only in the presence of the polycation spermidine (2, 17), whereas the best reactions with human Rad51 protein are seen when ammonium sulfate is included (18).

Our comparison of the DNA strand exchange activities of RecA and Rad51 proteins explores a wide range of conditions and several DNA substrates, but ultimately focuses on the DNA pairing process itself and the role of ATP. Based on the effects of Mg^{2+} on the DNA strand exchange reaction promoted by the mutant RecA K72R (which binds but does not hydrolyze ATP), we proposed a model previously (9) for extensive DNA strand exchange in the absence of ATP hydrolysis. With high levels of Mg^{2+}, DNA pairing was highly efficient but did not lead to extensive strand exchange because of topological barriers imposed by stable secondary DNA pairing events. Lower levels of Mg^{2+} supported less DNA pairing but allowed for the slow completion of extensive DNA strand exchange (9). In other words, extensive DNA strand exchange in the absence of ATP hydrolysis can be blocked if the fundamental DNA pairing process is sufficiently facile. A similar result has recently been obtained with the human Rad51, where efficient DNA strand exchange requires solution additions that weaken the interaction between the Rad51-ssDNA complex and the duplex DNA (18). In this report, we demonstrate that under conditions for DNA strand exchange that are optimized separately for the RecA and Rad51 proteins, the RecA protein pairs DNA more efficiently than does the Rad51 protein. However, the weaker DNA pairing function allows Rad51 to promote an extensive DNA strand exchange process efficiently with little or no ATP hydrolysis. We also continue our exploration of the range of DNA substrates tolerated in DNA strand exchange processes (21, 59).

EXPERIMENTAL PROCEDURES

Enzymes and Biochemicals—The *E. coli* RecA and SSB proteins and the yeast Rad51 and RPA proteins were purified and stored by published procedures (4, 49, 60–65). The concentrations of each were determined by UV absorption at 280 nm using the extinction coefficient of 2.23×10^4 M^{-1} cm^{-1} for RecA (61), 2.83×10^4 M^{-1} cm^{-1} for SSB (63), 0.30 mg^{-1} ml cm^{-1} for Rad51 (49), and 8.8×10^4 M^{-1} cm^{-1} for RPA (49). T4 polynucleotide kinase, *Pst*I endonuclease, and their accompanying buffers were purchased from Promega. ATPγS was from Roche Molecular Biochemicals. [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech. Tris base, boric acid, EDTA, magnesium acetate, potassium acetate, potassium chloride, sodium chloride, potassium glutamate, SDS, acrylamide, bisacrylamide, glycerol, 1 M hydrochloric acid, and 1 M acetic acid were purchased from Fisher. ATP, spermidine trihydrochloride, creatine phosphokinase, phosphocreatine, and bromphenol blue were purchased from Sigma. Dithiothreitol (DTT) was purchased from Research Organics Inc. Xylene cyanole FF was purchased from Eastman Kodak. Ficoll 400 was purchased from Amersham Pharmacia Biotech.

Oligonucleotide Substrates—The following oligonucleotides were purchased from Operon Technologies in polyacrylamide gel electrophoresis-purified form: A, AGTAGACTCAGCGAACTCACTGATCCAGTCTTAGCATCAGTCACGATACCTCGAGATACATACGGACGTA; B, TGATCCAGTCTTAGCATCAGTCACGATACCTCGAGATAC; C, GTATCTCGAGGTCTCGTGACTGATGCTAAGACTGGATCA; A*, AGTAGACTCAGCGAACTCACTG*X*TCC*X*GTCTT*X*GC*X*TC*X*GTC*X*CG*X*TX*CCTCG*X*G*X*TX*CATACGGACGTA; B*, TG*X*TCC*X*GTCTT*X*GC*X*TC*X*GTC*X*CG*X*TX*CCTCG*X*G*X*TX*C; C*, GT*X*TCTCG*X*GGT*X*TCGTG*X*CTG*X*TGCT*X**X*G*X*CTGG*X*TCA; A$_F$*, AGTAGACTCAGCGAACTCACT*X*ATCCA*X*TCTTA*X*CATCA*X*TCAC*X*ATACCTC*X*A*X*ATACATACGGACGTA; D, TGATCCAGTCTTAGCATCAGTCACGATACCTCGAGATACA; E, TGTATCTCGAGGTCTCGTGACTGATGCTAAGACTGGATCA; X, GTGCGAGATCTTGCGATGTCAGTCGTAGCCTCAGTTCGAC; and Y, GTCGAACTGAGGCTACGACTGACATCGCAAGATCTCGCAC. D and E differ from B and C only by the addition of a single nucleotide at their 3′ and 5′ ends, respectively. Lyophilized oligonucleotides were resuspended in TE (10 mM Tris-Cl (80% cation), 1 mM EDTA (pH 8.0)) and stored at −20 °C.

Oligonucleotides were 5′-^{32}P-labeled by incubating with T4 polynucleotide kinase (5–10 units) and [γ-^{32}P]ATP (0.5 μM) for 30 min at 37 °C in the buffer recommended by the enzyme supplier (final concentrations: 70 mM Tris-Cl (pH 7.6), 10 mM $MgCl_2$, and 5 mM DTT) with a final reaction volume of 50 μl. Unincorporated nucleotide was removed with the QIAquick Nucleotide Removal kit (Qiagen) according to the protocol provided by the manufacturer and eluted in no more than 50 μl of TE. The dsDNA 40-mer substrates were formed by annealing the two complementary ssDNA 40-mers and then heating to 85 °C for 5 min followed by slow cooling to 25 °C. Labeled double-stranded oligonucleotides were isolated from single strands by electrophoresis in 10% acrylamide, 1× TBE (45 mM Tris borate (50% cation), 1 mM EDTA (pH 8)) native gels. Radiolabeled bands were visualized by a 10-s exposure to x-ray film. Processed film was placed under the gel to facilitate band identification; the bands were then excised with a razor blade. DNA was extracted from excised intact gel slices by three successive incubations of at least 1 h in 1 ml of TE at 37 °C. After each incubation, the liquid was removed and the DNA concentrated in Microcon-10 concentrators (Amicon) for 30 min at 14,000 × *g* with the next extraction liquid being added to the previously concentrated material and then re-concentrated.

Unlabeled dsDNA 40-mers were generated by mixing equimolar amounts of the two complementary strands in TE at a final concentration of 1600 μM nucleotides. The duplex was annealed by incubating at 85 °C for 5 min followed by slow cooling to 25 °C. No further purification

of the duplex was performed. Oligonucleotide concentration was determined by UV absorption at 260 nm using the extinction coefficients provided by the manufacturer. All DNA concentrations are reported here in nucleotides, unless otherwise stated.

φX174 DNA Substrates—Circular single-stranded φX174 virion DNA (5386 bases in length) was purchased from New England Biolabs. Virion DNA concentrations were verified by UV absorption at 260 nm using the extinction coefficient, 27.8 A_{260} mg^{-1} ml cm^{-1}.

Linear double-stranded φX174 DNA was generated from a restriction digestion of φX174 replicative form I DNA (from New England Biolabs). Aliquots of this DNA (100 μg) were incubated with 200 units of *Pst*I in the supplied buffer (90 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, and 50 mM NaCl) in a final volume of 200 μl. This mixture was incubated for 2 h at 37 °C before a 20-min heat inactivation at 80 °C. The DNA was purified from the mixture using 2 QIAquick polymerase chain reaction purification spin columns (Qiagen) according to the protocol provided by the manufacturer. The DNA was eluted in a total volume of 150 μl of TE. Linear dsDNA concentrations were determined by UV absorption at 260 nm using the extinction coefficient, 20 A_{260} mg^{-1} ml cm^{-1}.

RecA-promoted Three Strand DNA Exchange of Oligonucleotide Substrates—DNA pairing reactions were carried out in 20 mM Tris-OAc buffer (80% cation) with added 1 mM DTT, 1% glycerol, and 1 mM Mg(OAc)$_2$ at 38 °C. DNA, protein, and NTP cofactor concentrations are listed in figure legends. The 70-mer ssDNA substrate was preincubated with RecA protein and ATPγS for 10 min. Additional reaction components were added to the preincubation as indicated. The reactions were initiated by the addition of the ^{32}P-labeled 40-mer dsDNA substrate and reagent additives as indicated with a final reaction volume of 150 μl. The final pH after addition of all reaction components was 7.55. Aliquots of 9 μl were removed at the indicated times and stopped with the addition of EDTA and SDS to final concentrations of 20 mM and 1%, respectively, in a final volume of 12 μl. A gel loading buffer (2.5% Ficoll (type 400), 0.08% bromphenol blue, 0.08% xylene cyanol FF, all final concentrations) was then added to each stopped aliquot, followed by electrophoresis on 10% acrylamide, 1× TBE gels. Bands were visualized by exposure to PhosphorImager screens (Molecular Dynamics) for 30 min and scanned on a Molecular Dynamics PhosphorImager (model 425E). Band intensities were calculated using the ImageQuant software (Molecular Dynamics). The percent products for each reaction was calculated as the combined signal from both product bands divided by the total labeled DNA signal for a given gel lane, and background was subtracted from all time points. Background was determined by performing the standard reaction as described but replacing the RecA protein with an equivalent volume of RecA protein storage buffer (20 mM Tris-HCl (80% cation), 0.1 mM EDTA, 10% (w/v) glycerol, and 1 mM DTT). The background, which generally reflected spontaneous pairing of unannealed substrate oligonucleotides (and exhibited no increase with time), was calculated as the average product percentage for all of the time points (less than 10% in all cases).

For the reaction series with 2AP-substituted oligonucleotide substrates, each time point was a separate reaction. The RecA protein and 70-mer concentrations were 18.7 and 56 μM, respectively. Reactions were initiated by the addition of 12.8 μM (final concentration) ^{32}P-labeled 39-mer dsDNA substrate and Mg(OAc)$_2$ (to 10 mM) yielding a final reaction volume of 12 μl. Reactions were stopped and treated as described above, except that stopped reactions were dialyzed into at least 100 ml of TE with 1-kDa molecular weight cut-off disposable dialyzers (The Nest Group) at 25 °C for 2 h prior to electrophoresis. For kinetic analysis, all reaction incubations were carried out at 30 °C. The ssDNA substrate was reduced to 44.8 μM and the RecA protein concentration to 14.9 μM.

RecA-facilitated Three Strand DNA Exchange of φX174 DNA Substrates—Three strand exchange reactions with large DNA substrates were carried out in the same solution conditions as the RecA protein-promoted DNA pairing reactions described above. DNA, protein, and NTP cofactor concentrations are listed in the figure legends. Preincubations were carried out at 38 °C for 10 min and included circular single-stranded φX174 DNA, RecA protein, 3 mM potassium glutamate, 12 mM phosphocreatine, and 10 units/ml creatine phosphokinase. Linear dsDNA was then added, followed by incubation at 38 °C for another 5 min. A 9.1-μl aliquot was removed to use as a zero time point. After another 5 min of incubation at 38 °C, the reaction was initiated by the addition of ATP and SSB. Aliquots of 10 μl were removed at the indicated times. All reactions were stopped by addition of 1/3 volume of a solution containing 15 mM EDTA, 1.25% SDS, 6.25% glycerol, and 0.05% bromphenol blue.

Time points were subjected to electrophoresis on 0.8% agarose, 1×

TAE (40 mM Tris-OAc (80% cation), 1 mM EDTA (pH 8.0)) gels. The substrate, product, and joint molecule bands were distinguishable after staining with ethidium bromide and exposure to ultraviolet light. Images were captured with a digital CCD camera using the GelExpert software (Nucleotech). Reaction progress was calculated as the percentage of nicked circular dsDNA product relative to total duplex DNA in a gel lane. Band intensities were quantitated with the ImageQuant software (Molecular Dynamics).

Rad51-promoted Three Strand DNA Exchange of Oligonucleotide Substrates—General DNA pairing reactions were carried out in 35 mM K-MOPS buffer (pH 7.2) with added 2.4 mM MgCl$_2$ and 1 mM DTT at 38 °C. DNA, protein, and NTP cofactor concentrations are listed in the figure legends. The preincubation mixture included 70-mer ssDNA substrate, Rad51 protein, ATP, and additives as indicated in the text. After 10 min, an initiation mixture consisting of the ^{32}P-labeled 40-mer dsDNA substrate and additives as indicated was introduced, with a final reaction volume of 150 μl. For all reactions in which the preincubation mixture was varied, the initiation mixture included 4 mM spermidine. Aliquots of 9 μl were removed at the indicated time points and treated as described above for the comparable RecA reactions.

For the reaction series with 2AP-substituted oligonucleotide substrates, each time point was a separate reaction. Reactions included 60 mM KCl and were initiated by the addition of ^{32}P-labeled 39-mer dsDNA substrate and spermidine HCl to 4 mM yielding a final reaction volume of 12.5 μl. Reactions were stopped, dialyzed, electrophoresed, and analyzed as described for the same reaction series using RecA protein. No changes in the reaction conditions were necessary to allow convenient kinetic analysis in the Rad51 experiments.

Rad51-facilitated Three Strand DNA Exchange of φX174 DNA Substrates—Three strand DNA exchange reactions with φX174 DNA substrates were carried out in the same solution conditions as the Rad51-promoted DNA pairing reactions. DNA, protein, and NTP cofactor concentrations are listed in the figure legends. Preincubations (5 min at 38 °C) included circular single-stranded φX174 DNA, Rad51 protein, ATP, and additives as indicated. Yeast RPA was added to bring the reaction volume to 65.7 μl, followed by another 5 min at 38 °C. The reaction was initiated with the addition of linear double-stranded φX174 DNA in a mixture with additives as indicated. 10-μl aliquots were removed at the indicated times and subjected to electrophoresis and quantitation as described above.

Thermal Denaturation of Oligonucleotides—Thermal denaturation of the 39-mer dsDNA substrates (annealed oligonucleotides B and C) was carried out in 35 mM MOPS (pH 7.2) with 4 mM spermidine. These solution conditions are similar to those used for the Rad51-mediated DNA pairing assay. Conditions from the RecA assay were not used due to the temperature-dependent pH changes seen with Tris-based buffers. The concentration of each complementary oligonucleotide in every cuvette was 11.7 μM, giving a total DNA concentration of 23.4 μM. The volume in each experiment was 1.4 ml. Data were collected with a Cary 300 spectrophotometer (Varian) with a built-in thermal regulator in the cell block. The temperature was increased at 0.2 °C per min. Complementary renaturation experiments were also carried out in which the temperature was decreased at the same rate. Data points were collected every 0.5 min and averaged for 3 s. Melting temperatures were calculated in triplicate as the temperatures at which the transition was 50% complete.

RESULTS

Experimental Design—The *E. coli* RecA and *Saccharomyces cerevisiae* Rad51 proteins were compared in two different reaction systems. The first involves a DNA strand exchange using relatively short synthetic oligonucleotide substrates. This reaction does not require ATP hydrolysis for either protein. The second reaction is DNA strand exchange with long (5386 base pairs) DNA substrates derived from bacteriophage φX174. With these substrates, ATP hydrolysis is usually required for extensive DNA strand exchange promoted by RecA protein (10) but not for the reaction promoted by the yeast Rad51 protein (4). Most of this work focuses on solution requirements for the reactions, although some comparative work was also done with DNA substrates containing base analogs. These proteins clearly have different solution requirements for optimal reactions. To keep the reactions as comparable as possible, the DNA substrates employed and the reaction temperature (38 °C) were kept constant except where noted.
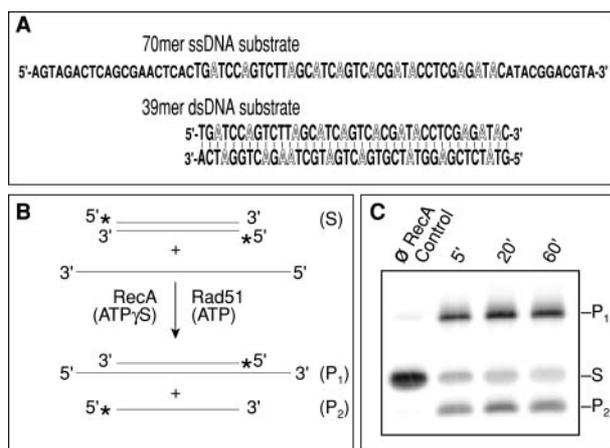
FIG. 1. **The DNA pairing and strand exchange reaction with oligonucleotide substrates.** *A,* sequences of the DNA oligonucleotides used in these experiments. In the 2AP-substituted oligonucleotides described later, all of the *highlighted* A residues were replaced with 2AP. *B,* the DNA strand exchange reaction promoted by RecA or Rad51 proteins with the DNA oligonucleotide substrates in *A*. *C,* a typical reaction, this one promoted by the RecA protein. This reaction is carried out under standard RecA reaction conditions (see "Experimental Procedures"), with 18.7 μM RecA protein, 56 μM 70-mer DNA, 12.8 μM 39-mer duplex, 10 mM Mg(OAc)$_2$, and 3 mM ATPγS. A single-stranded DNA-binding protein was not added to reactions of this type.

The oligonucleotide reaction is illustrated in Fig. 1. Duplex substrates identical except for the addition of 1 base pair on one end (40-mers; see under "Experimental Procedures") were used in many experiments. The 70-mer ssDNA substrates were short enough to be chemically synthesized but long enough to allow efficient binding by RecA protein (66, 67). The length difference between substrates allows for differentiation of substrate and product duplexes after electrophoresis. In the RecA reactions, the non-hydrolyzable ATP analog ATPγS was used to help stabilize the RecA filaments. For the reactions with the 39-mer duplexes, there were two DNA substrates, one with normal DNA bases and one with 2-aminopurine (2AP) replacing all but one adenosine in the pairing region in all three oligonucleotides. Thus, 2AP appears in at least one strand at 20 of 39 positions.

With such a short length of sequence exchanged, the processes of homologous pairing and strand exchange are indistinguishable in an electrophoretic assay (19). The use of oligonucleotides as substrates for DNA pairing provides a way to examine the pairing efficiency of different types of sequences without the topological and structural constraints that can affect DNA strand exchange reactions with longer DNAs.

The DNA strand exchange with longer DNA substrates derived from bacteriophage ϕX174 is illustrated in Fig. 2. These reactions have been independently optimized for RecA protein and Rad51 protein, by using conditions that are established in subsequent figures. In the RecA reaction, products (nicked duplex circles) appear only after a lag of somewhat more than 10 min and then increase steeply until reaction completion by 30 min (Fig. 2, *B* and *D*). Intermediates (joint molecules) appear earlier, hit a maximum at early times, and then decline as they are converted to products (Fig. 2, *B* and *E*). This reaction profile, seen only when ATP is hydrolyzed, features a relatively synchronized (for this type of reaction) conversion of substrates to products for all of the DNAs in the solution. Initiations occur over a span of about 15 min and products appear over a similar time span, suggesting that each of the individual strand exchange reactions in the test tube is directed around the ϕX174 DNA circle at approximately the same rate.

The kinetics of the Rad51 protein-promoted reaction are much different, and a typical example is illustrated in Fig. 2.
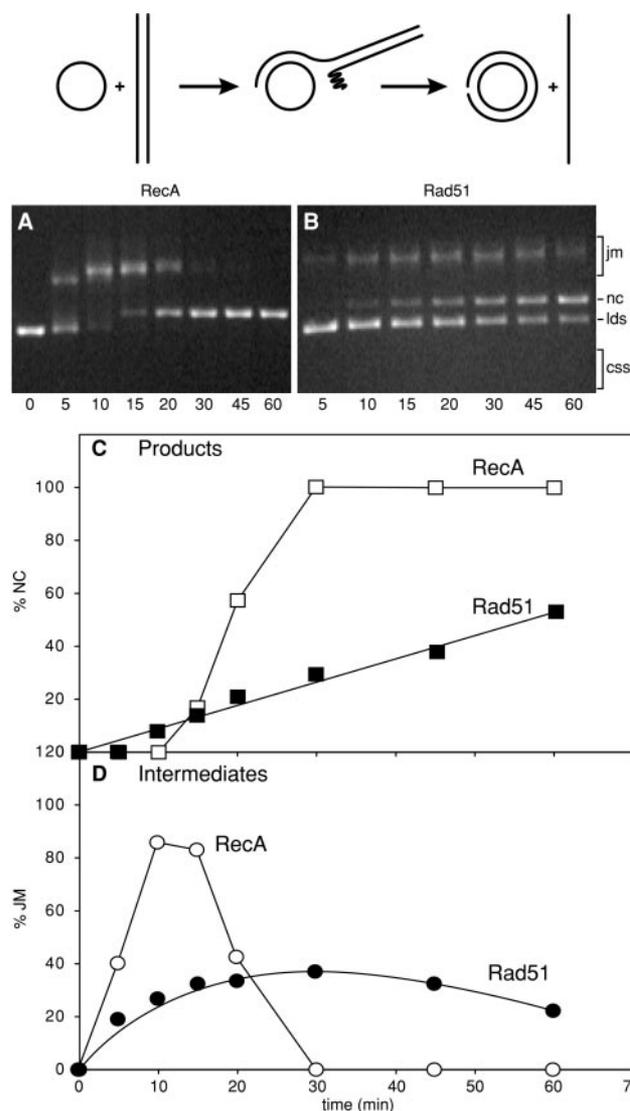

FIG. 2. **The extensive DNA strand exchange reaction with bacteriophage ϕX174-derived DNA substrates.** The reaction is illustrated at the *top*. *A* and *B* show gels with reactions promoted by RecA and Rad51 proteins, respectively, carried out as described under "Experimental Procedures." The RecA reaction contained 7.1 μM RecA protein, 21.3 μM circular ssDNA, 21.3 μM linear duplex DNA, 3 mM ATP, 11 mM Mg(OAc)$_2$, and 2.13 μM SSB. The Rad51 reactions featured the same DNA concentrations, 6.45 μM Rad51 protein, 0.75 μM yeast RPA, 2 mM ATP, 2.4 mM Mg(OAc)$_2$, 4 mM spermidine HCl, and 30 mM KCl. The generation of products (nicked circular DNA duplexes) and intermediates (branched joint molecules) for these reactions are plotted in *C* and *D*, respectively. *nc*, nicked circular DNA (products); *jm*, joint molecules (intermediates); *lds,* linear double-stranded DNA (substrate); *css*, circular single-stranded DNA (substrate).

Product formation again exhibits a lag (relatively short in this example, Fig. 2, *C* and *D*). Products are then formed at a slow but fairly steady rate that leads to gradual accumulation. The accumulation continues beyond the 60-min span of this experiment (data not shown) and can eventually approach the product generation of the RecA reaction. Reaction intermediates are also seen, generated more slowly than in the RecA-mediated process and remaining at a low level for the entire reaction course shown (Fig. 2, *C* and *E*). The intermediate-product relationship of the joint molecules and nicked circular DNA bands is not as obvious with the kinetics seen in the Rad51 gel. The early appearance of products in at least some of the Rad51 protein-promoted reactions suggests that the progression of strand exchange around the ϕX174 circle can be comparable to or sometimes even faster here than in the RecA protein-pro-

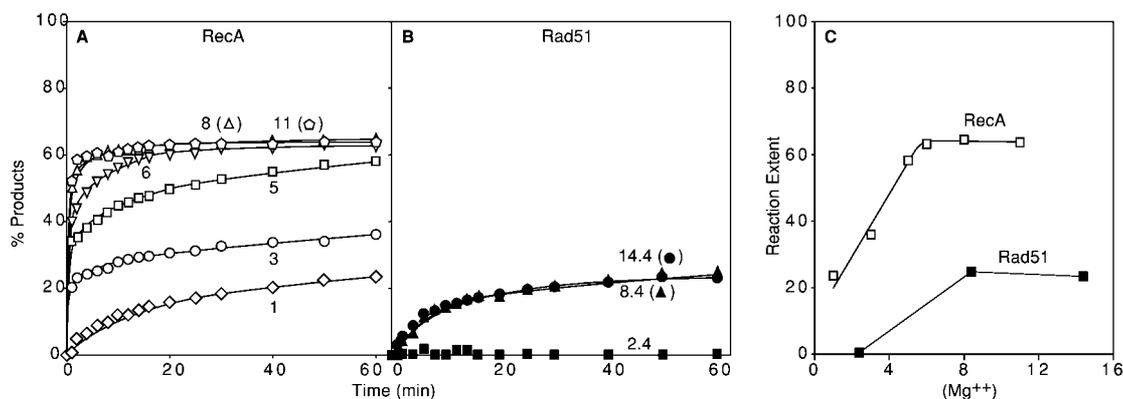*RecA and Rad51-promoted DNA Pairing*

FIG. 3. **Effects of Mg^{2+} on the DNA strand exchange with oligonucleotide substrates.** Reactions were carried out as described under "Experimental Procedures." The 40-mer duplex is the duplex formed by annealing oligonucleotides D and E under "Experimental Procedures." RecA reactions (*A*) contained 31.5 μM 70-mer ssDNA substrate (0.45 μM molecules), 12 μM ^{32}P-labeled 40-mer dsDNA substrate (0.15 μM molecules), 10.5 μM RecA protein, and 3 mM ATPγS. Rad51 reactions (*B*) contained the same concentrations of DNA substrates, 9.55 μM Rad51 protein, 2 mM ATP, and 60 mM KCl (no spermidine). Reactions also contained the concentration of Mg(OAc)$_2$ (in mM) indicated by the *numbers* next to each progress curve. The extents of strand exchange observed after 60 min of reaction are summarized in *C*.

moted reaction. However, the DNA pairing leading to intermediate formation is much slower than with RecA protein. Part of the thesis of this study is that this reduced DNA pairing is necessary to enable efficient DNA strand exchange over long DNA distances when ATP hydrolysis is not coupled to the process. This conclusion is derived in part from the comparative examination of solution condition effects to follow.

Mg^{2+} Ion Is Sufficient to Optimize RecA but Not Rad51-mediated Reactions—The effects of added Mg^{2+} ion on DNA pairing as measured by the reactions with short oligonucleotides is shown in Fig. 3. For RecA protein, an optimal DNA pairing process is seen at Mg^{2+} concentrations over 6 mM, with over 60% of the duplex DNA substrates rapidly converted to products. The single-stranded 70-mers are in 3-fold excess relative to the duplex 40-mers in these experiments. The Rad51-mediated reaction is considerably slower and proceeds to a much lower extent even when over 8 mM Mg^{2+} is added. Reaction extents after 60 min are summarized in Fig. 3*C*.

The differences between RecA and Rad51 are even more dramatic when the DNA strand exchange with ϕX174 substrates is examined (Fig. 4). For RecA protein, 6 mM Mg^{2+} is enough to effect 100% product generation, with just a little more needed for optimal rates. Rad51 protein, in contrast, exhibits little reaction with any level of Mg^{2+}. Some products are generated after 60 min in the reactions with 8.4 or 14.4 Mg^{2+} (difficult to see in these reproduced gels), but the levels remain under 10%. Slow generation of somewhat higher levels of products is seen with longer incubations (not shown).

Spermidine Stimulates Rad51 Protein Reactions but Not Those of RecA Protein—The effects of added spermidine on the Rad51 protein-promoted DNA pairing with short oligonucleotide substrates is shown in Fig. 5. With 2.4 mM Mg^{2+} (just over the amount needed to chelate the ATP), there is no reaction in the absence of spermidine. Additions of 2–6 mM spermidine in the initiation mixture produce reaction extents that are comparable to those seen with RecA protein in Fig. 3. The effects of Mg^{2+} alone and spermidine (in the presence of 2.4 mM Mg^{2+}) are compared in Fig. 5*B*. Higher levels of Mg^{2+} slightly inhibit the reaction when spermidine is present (Fig. 5*C*). At all levels of Mg^{2+}, added spermidine enhances the reaction, although the enhancement is mitigated as the Mg^{2+} concentration increases. The spermidine and Mg^{2+} appear to be partially competitive in their effects. The spermidine effects are greatest at relatively low concentrations of Mg^{2+}. Mg^{2+} cannot be eliminated entirely (no reaction occurs, even when the other conditions are optimized, data not shown), as it is probably required to form active complexes with ATP.

A similar stimulatory effect of spermidine is seen in Rad51 protein-promoted DNA strand exchange with ϕX174-derived substrates (Fig. 6). Low levels of Mg^{2+} alone do not allow for product formation, as already seen. However, the addition of 2–6 mM spermidine HCl allows an efficient reaction to occur, with maximum reaction seen at 4 mM. The spermidine appears to be required for an optimal DNA pairing and strand exchange reaction with Rad51 protein, and it cannot be replaced by Mg^{2+} in this function.

The results seen with RecA protein are quite different. In the reaction with the short oligonucleotides, spermidine has a slightly inhibitory effect (about 10% decrease in extent in a 120-min reaction), even when the concentration of Mg^{2+} remains limiting at only 1 mM (data not shown). At normal (11 mM) concentrations of Mg^{2+}, the DNA strand exchange with ϕX174 substrates is also inhibited by spermidine (Fig. 7). The inhibition increases as the spermidine concentration is increased from 2 to 6 mM (not shown).

When Mg^{2+} levels are kept low (comparable to the ATP concentration), RecA protein will promote a very slow generation of complete products in the ϕX174 DNA reaction without ATP hydrolysis (9). We therefore tried to see if spermidine would enhance this reaction. With ATPγS and 1 mM Mg^{2+}, spermidine still inhibited the DNA pairing process as seen in the formation of pairing intermediates (Fig. 8, *A* and *B*). Therefore, the spermidine does not enhance the ATP hydrolysis-independent reaction of RecA protein. The one case where a positive effect of spermidine is seen with RecA is when ATP is hydrolyzed, but the Mg^{2+} level is limiting (Fig. 8, *C* and *D*). The generation of products increases markedly in this case.

KCl Inhibits Rad51-mediated DNA Pairing, Yet Is Required for Extensive DNA Strand Exchange—The Rad51 protein also exhibits a requirement for KCl in many of its reactions, and most of the Rad51 reactions to this point include added KCl. This effect can be seen in Fig. 9. In the absence of KCl, but with conditions otherwise optimized, there is no DNA strand exchange observed with the ϕX174-derived DNA substrates. The optimum reaction is reached between 30 and 60 mM KCl. The same reaction promoted by the RecA protein is not detectably different in the presence or absence of 60 mM KCl (Fig. 9*B*).

In the simpler DNA pairing reactions with oligonucleotides, the added KCl inhibits the Rad51 protein-promoted reaction by factors of 1.5–2 (Fig. 10, *A* and *B*), in contrast to its positive effects on the strand exchange using much larger substrates as described above. The RecA protein-promoted reaction with the oligonucleotides is enhanced somewhat by KCl, but only in
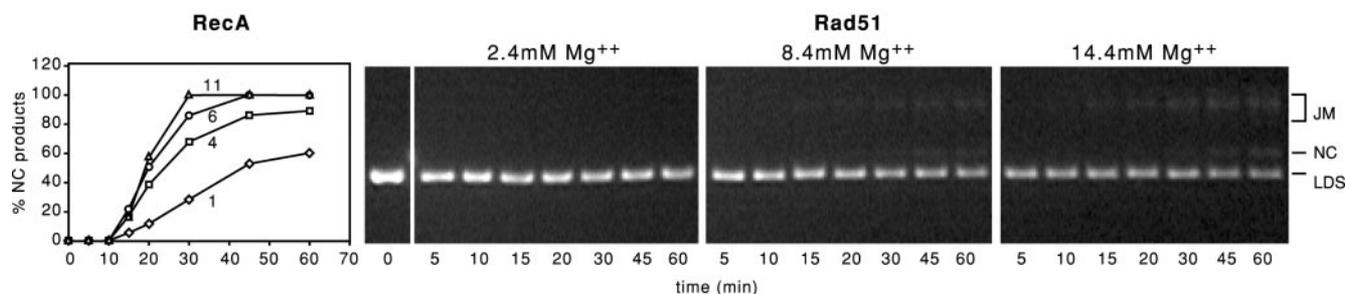
FIG. 4. **Effects of Mg²⁺ on extensive DNA strand exchange with bacteriophage φX174-derived DNA substrates.** Reactions were carried out as described under "Experimental Procedures." The RecA reactions contained 7.1 μM RecA protein, 21.3 μM circular ssDNA, 21.3 μM linear duplex DNA, 3 mM ATP, 2.13 μM SSB, and the concentration of Mg(OAc)₂ (in mM) indicated by the *number* near each progress curve in the *1st panel*. The Rad51 reactions featured the same DNA concentrations, 6.45 μM Rad51 protein, 0.75 μM yeast RPA, 2 mM ATP, 60 mM KCl, and the indicated concentration of Mg(OAc)₂. No spermidine was included. *NC,* nicked circular DNA; *JM,* joint molecules; *LDS,* linear double-stranded DNA.
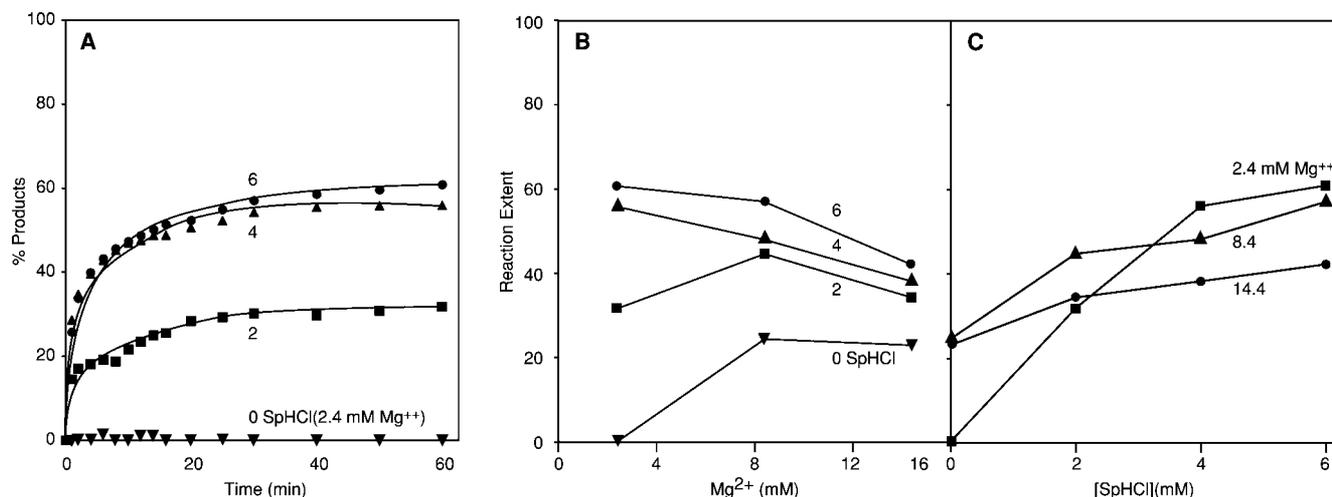

FIG. 5. **Effects of spermidine on Rad51 protein-promoted DNA strand exchange with oligonucleotide substrates.** Reactions were carried out as described under "Experimental Procedures." Reactions contained 31.5 μM 70-mer ssDNA substrate (0.45 μM molecules), 12 μM ³²P-labeled 40-mer dsDNA substrate (0.15 μM molecules), 9.55 μM Rad51 protein, 2 mM ATP, and 60 mM KCl. Reactions in *A* contained 2.4 mM Mg(OAc)₂, along with the concentration of spermidine HCl (*SpHCl*) (in mM) indicated by the *numbers* next to each progress curve. A series of such reactions with concentrations of spermidine HCl or Mg(OAc)₂ indicated are summarized in *B* and *C*, with reaction extents at 60 min plotted.
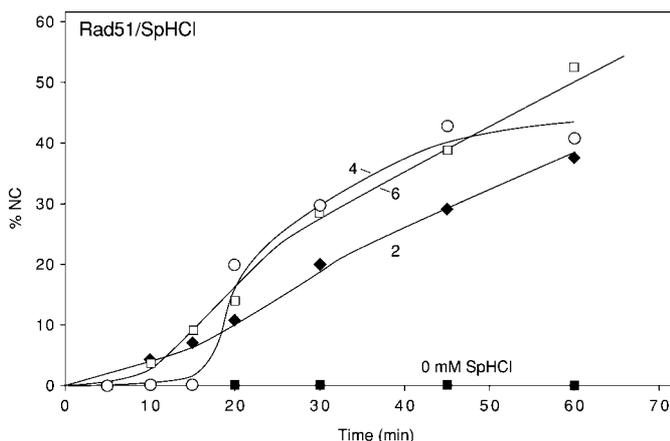

FIG. 6. **Effects of spermidine on Rad51 protein-promoted DNA strand exchange with bacteriophage φX174-derived DNA substrates.** Reactions were carried out as described under "Experimental Procedures." Reactions contained 6.45 μM Rad51 protein, 0.75 μM yeast RPA, 21.3 μM circular ssDNA, 21.3 μM linear duplex DNA, 2 mM ATP, 2.4 mM Mg(OAc)₂, and 60 mM KCl. Concentrations of spermidine HCl were 0 (■), *2* (♦), *4* (○), or *6* (□) mM.
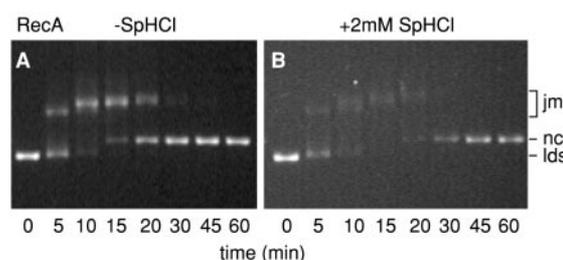

FIG. 7. **Effects of spermidine on RecA protein-promoted DNA strand exchange with bacteriophage φX174-derived DNA substrates.** Reactions were carried out as described under "Experimental Procedures" and contained 7.1 μM RecA protein, 21.3 μM circular ssDNA, 21.3 μM linear duplex DNA, 3 mM ATP, 11 mM Mg(OAc)₂, and 2.13 μM SSB. The reaction in *B* also contained 2 mM spermidine HCl (*SpHCl*). Higher (4 or 6 mM) concentrations of spermidine HCl had greater inhibitory effects, further delaying the generation of products (not shown). *nc,* nicked circular DNA; *jm,* joint molecules; *lds,* linear double-stranded DNA.

reactions with relatively low concentrations of Mg²⁺ (Fig. 10, *C* and *D*).

Rad51 Protein-promoted DNA Pairing Is More Susceptible to Inhibition by Heterologous Duplex DNA than Comparable Re-actions Promoted by RecA Protein—A heterologous duplex DNA was constructed by annealing oligos X and Y (see under "Experimental Procedures"). This was then added in 1-, 2-, or 5-fold excess relative to the homologous DNAs in the oligonucleotide reaction of Fig. 1. The reaction promoted by RecA protein was unaffected by this addition, in the presence of either 1 or 11 mM magnesium acetate (Fig. 11*A*). The reaction promoted by Rad51 protein, was reduced by 1/4 to 1/3 by an

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FIG. 8. **Effects of spermidine on RecA protein-promoted DNA strand exchange with bacteriophage φX174-derived DNA substrates, under otherwise suboptimal reaction conditions.** Reactions were carried out as described under "Experimental Procedures" and contained 7.1 μM RecA protein, 21.3 μM circular ssDNA, 21.3 μM linear duplex DNA, 3 mM ATP or ATPγS, 1 mM Mg(OAc)₂, 2.13 μM SSB, and the indicated concentrations of spermidine HCl. Each gel lane is an independent reaction incubated at 38 °C for 120 min. Quantification of the production of joint molecule intermediates in *A* is provided in *B*, and the production of nicked circular products in *C* is quantified in *D*. *jm*, joint molecules; *nc*, nicked circular DNA; *lds*, linear double-stranded DNA; *css*, circular single-stranded DNA; *lss*, linear single-stranded DNA.
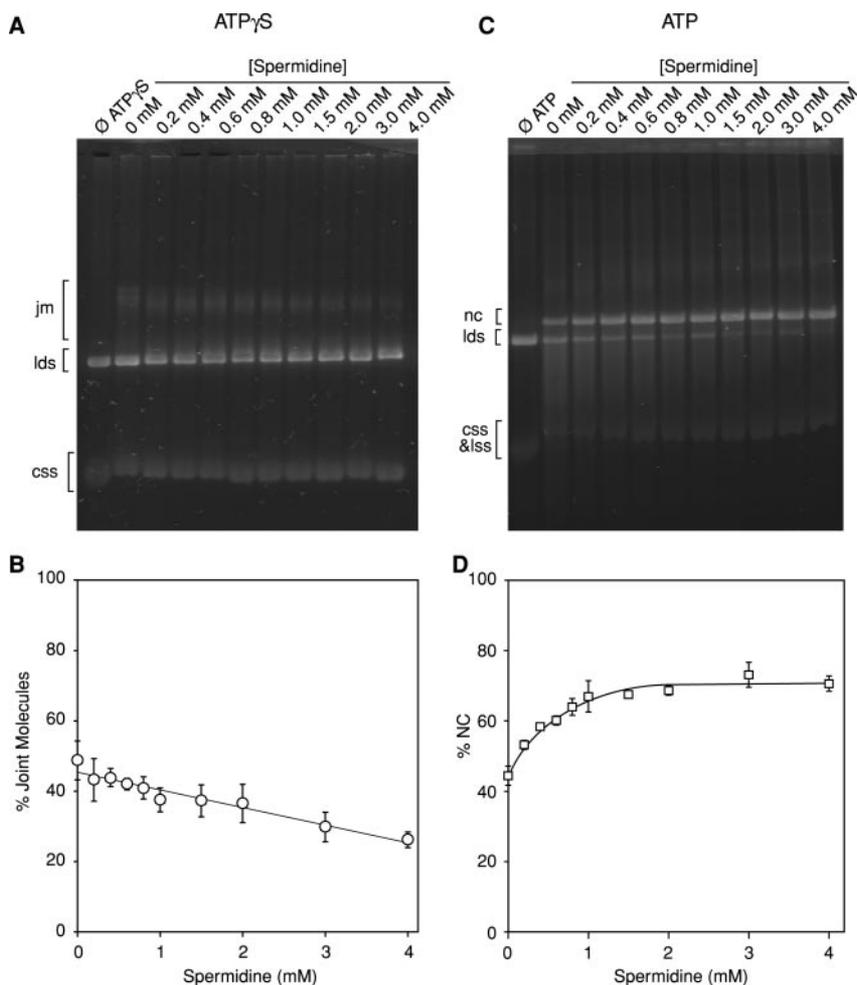
amount of heterologous DNA equivalent to the homologous substrate, although additional heterologous DNA had no evident additional effect. The effect was seen in reaction extent primarily.

The RecA Protein and Rad51 Protein Both Promote Efficient DNA Strand Exchange with Oligonucleotide Substrates Containing Extensive Substitution with 2-Aminopurine in Place of Adenine—For this set of reactions, the 40-mer duplex was shortened by 1 base pair, and two sets of DNA substrates were synthesized and purified. One set, consisting of the 70-mer oligo A, and the 39-mer duplex made by annealing oligos B and C, contained only normal DNA bases. The other, derived from oligos A*, B*, and C* was identical, except all of the adenosine residues in the pairing region on all strands were substituted by 2-aminopurine. This left at least one 2-aminopurine residue at 20 of the 39 positions involved in DNA pairing (Fig. 1). The oligonucleotide reactions used to explore the effects of 2AP substitutions were carried out under conditions that were separately optimized for each protein.

The 2-aminopurine residues pair with thymine, but eliminate much of the non-Watson-Crick hydrogen bonding in the major groove upon which the R-form triplex hypothesis for DNA pairing depends (Fig. 12). These substitutions reduced the melting temperature (T_m) for the 39-mer duplex from 78.8 ± 0.3 to 72.0 ± 0.1 °C (data not shown). Denaturation experiments in which the temperature was systematically increased were followed by renaturation experiments in which the temperature was systematically decreased, and the transitions observed were identical in both cases.

The oligonucleotide strand exchange experiments were little affected by the substitutions (Fig. 13). RecA protein converted

89.3 ± 3.9% (*n* = 3) of the input DNA substrates to products when all DNAs contained only normal DNA bases. When 2AP DNA substrates were used in an otherwise identical assay, the yield of products was comparable, 84.6 ± 7.2% (*n* = 4). As already noted, RecA-promoted DNA pairing reactions were quite rapid, which complicates adequate monitoring of their kinetics. To investigate the kinetics of the DNA pairing reactions with RecA protein, additional experiments were conducted at a lower temperature (30 °C) and reduced ssDNA and RecA concentrations (see "Experimental Procedures") to permit more detailed observation of the initial stages of the reaction. Both reactions again yielded products efficiently under the more stringent set of conditions (85.8 and 65.2% for the normal and 2AP reactions, respectively). The observed half-times of the reactions were equivalent within error (data not shown). This could mean either the normal reaction with its higher yield was faster or that the 2AP substrates generated more nonproductive complexes with the basic rate of the reaction being unaltered. In any case, the differences are small.

The slower Rad51-mediated DNA strand exchange is also similar with respect to both rate and extent when the normal and 2AP DNA substrates are compared (Fig. 13*B*). After 60 min, Rad51 converted 73.5 ± 3.5% (*n* = 3) of the substrates to products when normal substrates were used and 61.0 ± 4.1% (*n* = 3) when 2AP substrates were used. For Rad51, there was no need to alter the reaction conditions to study the kinetics of Rad51-promoted DNA strand exchange. In the detailed time course (not shown), there was no evident difference in the half-time for maximum product formation. Given the difference in reaction extents, this could again reflect either a somewhat faster reaction with the normal DNA substrates or an increase
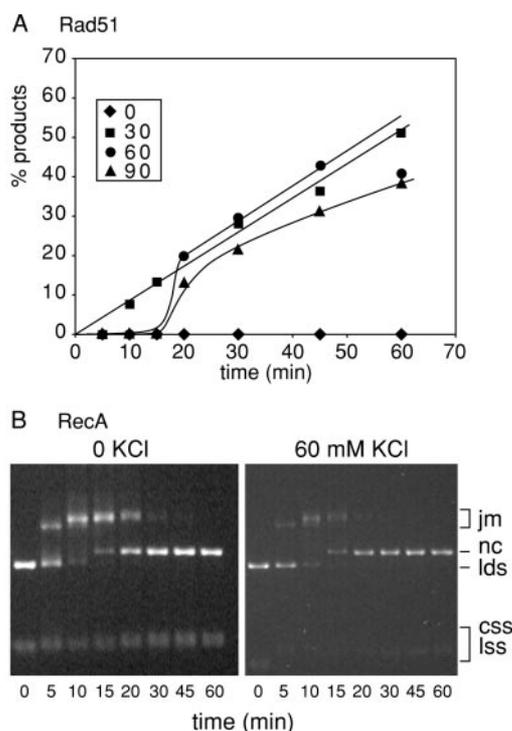
FIG. 9. **Effects of KCl on Rad51 protein-promoted DNA strand exchange with bacteriophage φX174-derived DNA substrates.** Reactions were carried out as described under "Experimental Procedures." The Rad51 reactions in *A* contained 21.3 μM circular ssDNA, 21.3 μM linear duplex DNA, 6.45 μM Rad51 protein, 0.75 μM yeast RPA, 2 mM ATP, 2.4 mM Mg(OAc)$_2$, 4 mM spermidine HCl, and the indicated concentration of KCl (in mM). The RecA reactions shown in *B* contained 7.1 μM RecA protein, the same concentrations of DNA substrates, 3 mM ATP, 11 mM Mg(OAc)$_2$, 2.13 μM SSB and the indicated concentration of KCl. *nc*, nicked circular DNA; *jm*, joint molecules; *lds*, linear double-stranded DNA; *css*, circular single-stranded DNA; *lss*, linear single-stranded DNA.

in the formation of unproductive complexes with the 2AP-containing substrates. For both proteins, pairing was dependent upon both active recombinase and nucleotide cofactor. Overall, the 2AP substitutions produced a small reduction in reaction extent with at most a comparable effect on reaction rates.

The 2AP substitutions have an enhanced capacity to mispair during RecA or Rad51 protein-mediated DNA strand exchange and thereby reduce the fidelity of the process. To demonstrate this directly, additional substrates were designed with specific heterologous base substitutions and then incorporated into the strand exchange assay (Fig. 13*C*). All seven Gs in the pairing region of the 70-mer ssDNA substrate (Oligo A$_F$* under "Experimental Procedures") were replaced with 2APs (creating 2AP:C pairs in a DNA strand exchange product when paired with duplex BC). RecA protein-promoted DNA pairing with the unmodified duplex substrate occurs, although the final product yield is reduced 2-fold. 2AP can pair with C in a duplex (68), although it must adopt a wobble geometry (Fig. 13*C*).

DISCUSSION

This comparison of the DNA pairing and strand exchange activities of the *E. coli* RecA protein and the yeast Rad51 protein leads to three related conclusions. First, the capacity of a RecA class recombinase to promote extensive DNA strand exchange optimally without ATP hydrolysis often involves conditions that are suboptimal for DNA pairing. Optimized pairing can thus have a negative effect on the more extensive DNA strand exchange reaction, leading to nonproductive DNA-DNA interactions. Second, the capacity of both RecA and Rad51

proteins to utilize DNA substrates that are extensively substituted with base analogs during DNA pairing reactions adds to the evidence that the fundamental DNA transactions are the same for both systems. Third, the solution conditions required for optimal reactions are substantially different for each recombinase. The conditions employed for RecA protein are of limited value in working with a new recombinase, and a thorough exploration of solution conditions is warranted whenever a new protein in this class is isolated.

The Importance of Not Getting Stuck (Avoiding Nonproductive Interactions), Extensive DNA Strand Exchange Reactions in the Absence of ATP Hydrolysis—We propose that a variety of nonproductive interactions between the duplex DNA and the recombinase-ssDNA complex can block or inhibit extensive DNA strand exchange reactions in the absence of ATP hydrolysis. This can lead to a situation that is at first counterintuitive, *i.e.* conditions that inhibit DNA pairing can sometimes facilitate a more extensive DNA strand exchange. The problem is illustrated in the model of Fig. 14. For a long DNA strand exchange reaction, the initial pairing of the recombinase-ssDNA complex (the nucleoprotein filament) at one end of a linear duplex must be followed by free rotation of the DNA and the filament so that additional DNA can be spooled into the filament (Fig. 14, *panel I*). Any stable interaction of the duplex DNA with the filament, at a location other than the branch point where the DNA is being spooled in, will interfere with this spooling process and effectively halt strand exchange. We suggest that there are two general types of nonproductive interactions. First, a too-rapid DNA pairing can lead to secondary DNA pairing interactions (Fig. 14, *panel II*), preventing the extension of the first hybrid duplex formed in a strand exchange reaction. Alternatively, regardless of the rate of pairing, some other type of interaction may occur in which the DNA simply gets "stuck" (Fig. 14, *panel III*). This could be a nonspecific DNA pairing within the filament groove or some other type of interaction on the exterior of the filament. Nonproductive interactions of a duplex DNA with a recombinase-ssDNA complex could also occur before the first DNA pairing event and limit the efficiency of simpler DNA pairing reaction with oligonucleotides (as is seen in the inhibition of Rad51-mediated DNA pairing by heterologous duplexes). Solution conditions can enhance ATP hydrolysis-independent strand exchange by eliminating or at least minimizing such interactions.

Two observations in the present study support the scenario of Fig. 14. First, the inherent DNA pairing activity of the Rad51 protein is generally slower than that of the RecA protein, when conditions are independently optimized for both proteins. This can be seen in the best DNA pairing reactions presented in Figs. 10 and 11. Under the optimized conditions, Rad51 protein promotes extensive DNA strand exchange with the φX174-derived DNA substrates, with or without ATP hydrolysis. In contrast, if RecA protein conditions are optimized by adding magnesium to concentrations of 10 or 11 mM, RecA protein does not generate products in the reaction of Fig. 2 without ATP hydrolysis. Second, the extensive strand exchange activity of Rad51 protein exhibits a strong requirement for the inclusion of 30–60 mM potassium, whereas the DNA pairing activity of Rad51 using short oligonucleotides is reduced 1.5–2-fold when the same KCl concentrations are used.

Two published observations (9, 18) complement these results. First, RecA protein will promote complete strand exchange with long DNA substrates in the absence of ATP hydrolysis but only when the magnesium concentration is lowered so much that the basic DNA pairing activity of RecA is weakened (9). RecA protein then generates complete products, albeit at quite low yields and over a much longer time frame

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FIG. 10. **Effects of KCl on DNA strand exchange reactions with oligonucleotide substrates.** Reactions were carried out as described under "Experimental Procedures." Rad51 reactions (*A* and *B*) contained 31.5 μM 70-mer ssDNA substrate (0.45 μM molecules), 12 μM ^{32}P-labeled 40-mer dsDNA substrate (0.15 μM molecules), 9.55 μM Rad51 protein, 2 mM ATP, 2.4 mM Mg(OAc)$_2$, 4 mM spermidine HCl. Reactions also contained 0 (▲), 20 (□), 40 (▲), 60 (×), 80 (●), 100 (○), or 120 (■) mM KCl. Reaction extents at 60 min are summarized in *B*. RecA reactions (*C* and *D*) contained 10.5 μM RecA protein, the same concentrations of DNA substrates, and 3 mM ATPγS. *Open* and *closed symbols* reflect reactions carried out in the presence and absence of 60 mM KCl, respectively. Progress curves in *C*, from *bottom* to *top* in each series (*open* or *closed symbol* reactions), reflect reactions carried out at 1, 3, or 11 mM Mg(OAc)$_2$, respectively.
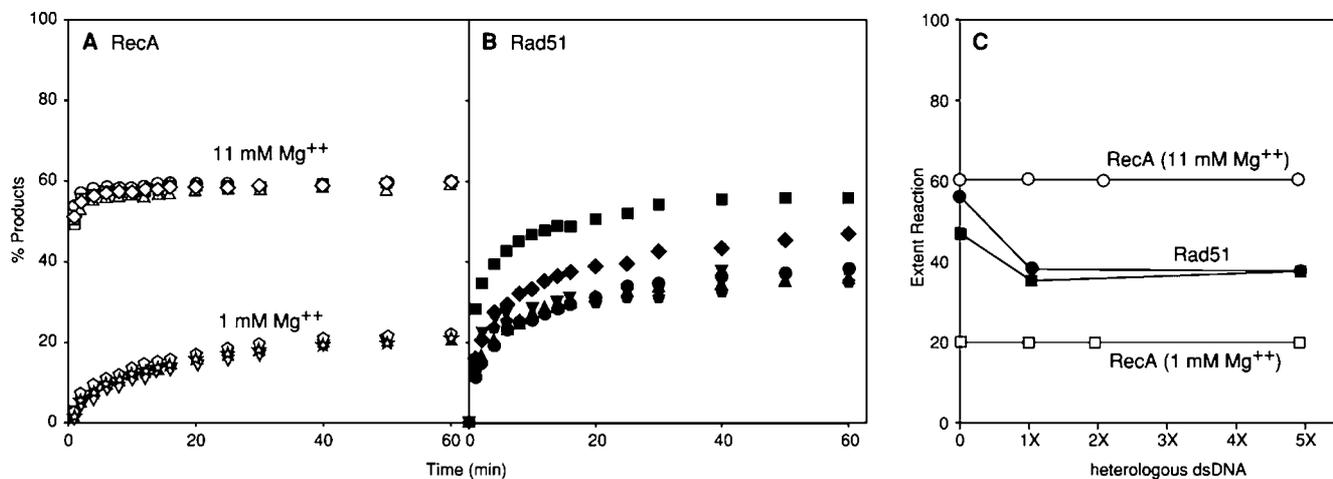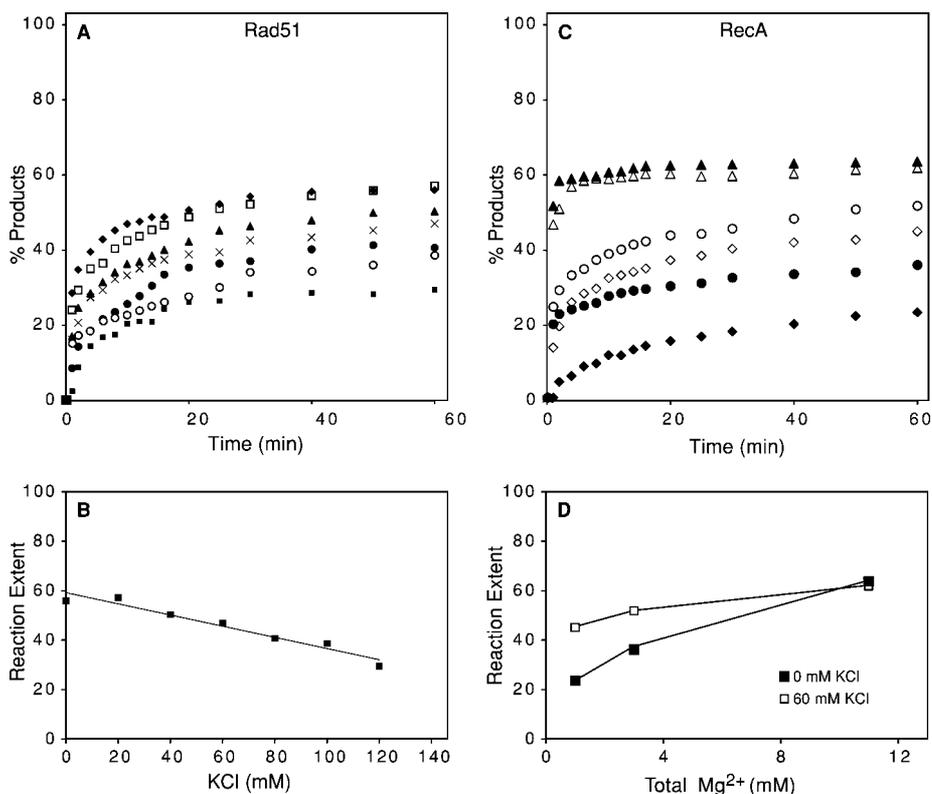

FIG. 11. **Inhibition of DNA strand exchange reactions with oligonucleotide substrates by addition of heterologous duplexes.** Reactions were carried out as described under "Experimental Procedures." *A,* RecA reactions contained 31.5 μM 70-mer ssDNA substrate (0.45 μM molecules), 12 μM ^{32}P-labeled 40-mer dsDNA substrate (0.15 μM molecules), 10.5 μM RecA protein, and 3 mM ATPγS. Reactions were carried out in the presence of 1 or 11 mM Mg(OAc)$_2$ as indicated. Reactions also contained either 0, 12, 24, or 60 μM of a heterologous duplex DNA constructed by annealing oligos X and Y under "Experimental Procedures." The various symbols are not assigned, since there was no significant difference in the progress curves due to addition of the heterologous DNA. *B,* Rad51 reactions contained the same concentrations of DNA substrates, 9.55 μM Rad51 protein, 2 mM ATP, 2.4 mM Mg(OAc)$_2$, either 0 or 60 mM KCl, and 4 mM spermidine HCl. Reactions also contained either 0, 12, or 60 μM of the same heterologous duplex DNA. Reactions are denoted with ■ or ♦ for those with 0 or 60 mM KCl, respectively, and with no competing heterologous DNA. The remaining reaction symbols are not specified since there was no significant difference in the progress curves. Reaction extents at 60 min for all reactions are summarized in *C*.

than is seen when ATP is hydrolyzed. Second, efficient DNA strand exchange with the human Rad51 protein appears to depend on the addition of salts that lessen unproductive interactions between the hRad51-ssDNA complexes and duplex DNA (18).

For RecA protein, the observation of at least some products in long DNA strand exchanges exhibits a correlation with reduced DNA pairing, suggesting that secondary DNA pairing (Fig. 14, *panel II*) is a major source of obstruction for DNA strand exchange without ATP hydrolysis in this system (9). For Rad51 protein, some results (such as the inhibition seen with

heterologous duplex DNA) suggest that other types of nonproductive interactions, perhaps including nonspecific DNA-DNA interactions, may play a significant role in impeding the progress of a DNA strand exchange reaction.

RecA and Rad51 Proteins Share a Common DNA Pairing Mechanism—Several mechanisms have been proposed for the DNA pairing process promoted by RecA, Rad51, and related proteins. The strongest evidence now favors a mechanism involving standard Watson-Crick interactions via base flipping (43). Major groove hydrogen bonding potential is the source of DNA pairing fidelity in the proposed R-form DNA triplex al-
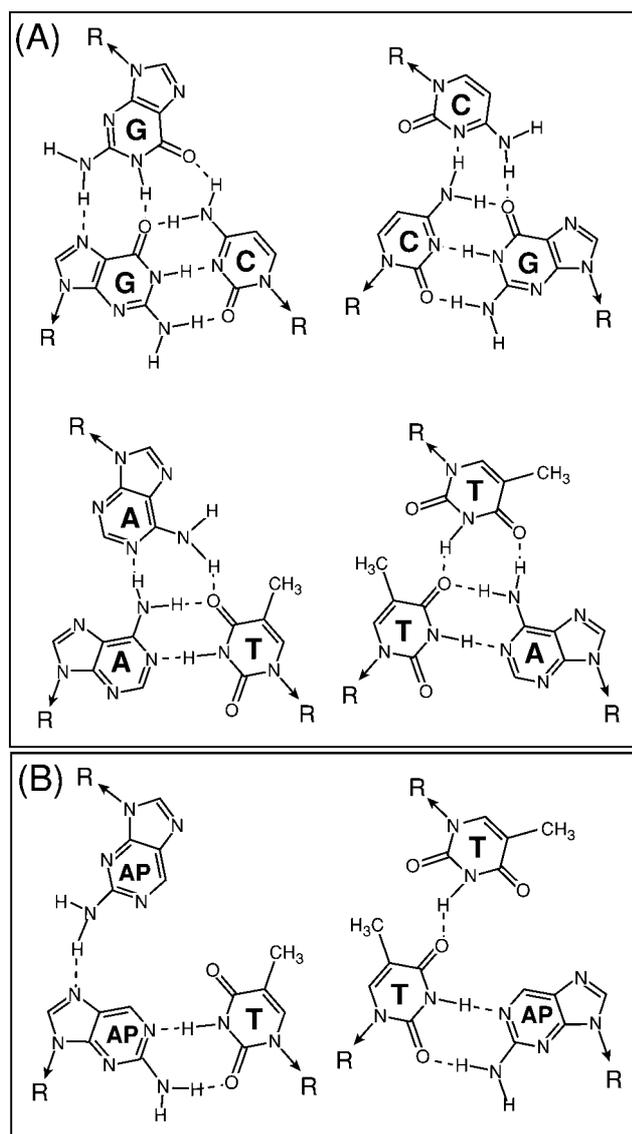
FIG. 12. **R-form DNA.** *A,* proposed R-form DNA triplets. The proposed triplex DNA pairing intermediate has like strands arranged in parallel. The non-Watson-Crick hydrogen bonds potentially provide a unique readout for each base pair to ensure a high degree of fidelity in homologous alignment. This triplex structure is distinct from any stable triplex DNA structure yet characterized (70). *B,* triplets in which 2AP is substituted for *A.*

FIG. 13. **DNA strand exchange with 2AP-substitued oligonucleotide DNA substrates.** *A,* typical RecA protein-promoted DNA strand exchange reactions with the normal or 2AP-substituted DNA substrates. Standard reactions were carried out at 37 °C as described under "Experimental Procedures" and contained the 70-mer ssDNA substrate (56 μM), RecA protein (18.7 μM), ATPγS (3 mM), 10 mM Mg(OAc)$_2$, and 12.8 μM ^{32}P-labeled 39-mer dsDNA substrate in a final reaction volume of 12 μl. *B,* Rad51 protein-promoted DNA strand exchange reactions with the normal or 2AP-substituted DNA substrates. Standard reactions were carried out at 37 °C as described under "Experimental Procedures" and contained the 70-mer ssDNA substrate (56 μM), Rad51 protein (17 μM), ATP (2 mM), the ^{32}P-labeled 39-mer dsDNA substrate (12.8 μM), and 4 mM spermidine in a final reaction volume of 12.5 μl. *C,* reaction of oligonucleotide A$_F$* with the duplex formed by annealing oligos *B* and *C*. This results in the formation of seven 2AP:C base pairs in the projected heteroduplex product. *D,* the 2AP:C base pair forms two hydrogen bonds but adopts a wobble geometry. For all reactions shown, each reaction time point represents a separate reaction incubated for the indicated time. *C,* cytosine.

ternative. However, the rates of DNA strand exchange promoted by either the RecA or Rad51 recombinases are little affected by substituting adenosine nucleotide bases with 2-aminopurine bases that eliminate much of the non-Watson-Crick hydrogen bonding potential of the DNA major groove. The effects of the substitutions on the final yield of products are measurable but small. These and previous studies (21, 59) indicate that a substantial reduction of the potential major groove hydrogen-bonding utilized in the proposed R-form triplex pairing is not enough to affect the rate of DNA strand exchange. Because of the effects on DNA stability, we are unable to create a duplex DNA where the 2AP is present at every position in the paired region. Thus, we cannot eliminate the possibility that the GC base pairs somehow compensate for the missing major groove interactions. However, the minimal effects of base analogs that affect the major groove hydrogen bonding potential, both here and in previous studies (21, 59), are most easily accommodated by a mechanism that does not involve an R-form triplex.
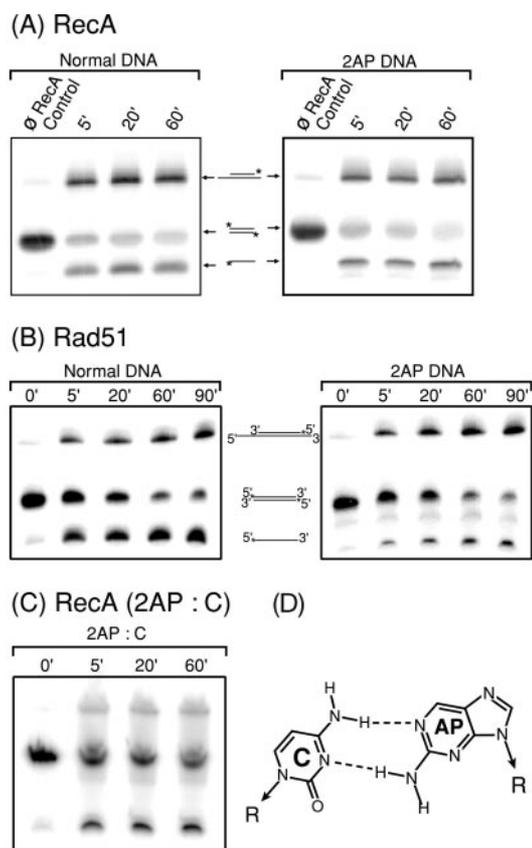
The limited major groove hydrogen bonding potential in DNA substrates containing 2AP also has the potential to compromise the fidelity of homologous recognition in several ways. A 2AP in the single strand can be aligned with almost any base pair to form a plausible base triple (with one hydrogen bond) that might work within the R-form triplex hypothesis. This is especially evident in the case of G:C base pairs. The functional groups in the major groove of G:C base pairs present essentially the same hydrogen bonding potential for 2AP in a single-stranded DNA as do 2AP:T pairs. Thus, during a sampling process of homologous alignment via an R-form triplex, a 2AP base should in principle align with a G:C pair as well as with a 2AP:T pair. A 2AP in a single strand should also permit a reaction with either a 2AP:T base pair or a G:C pair. A 2AP:C base pair can be formed with two hydrogen bonds, although its geometry would differ from that of a standard Watson-Crick base pair. In fact, 2AP is utilized as a substrate for DNA polymerase and can be paired across from C, although not as frequently as from T (68, 69). We found that the presence of 2AP at seven positions where it would pair with C in the strand
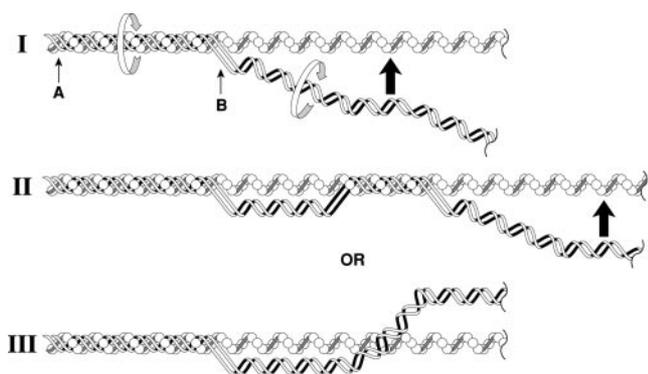
FIG. 14. **Barriers to extensive DNA strand exchange in the absence of ATP hydrolysis.** A recombinase (Rad51 protein or RecA protein) forms helical filaments on ssDNA. When the end of a homologous duplex DNA is aligned with the bound single strand, additional duplex DNA must be spooled into the filament to extend the paired region. This spooling requires rotation of the filament and/or the duplex DNA (*panel I*). If the duplex DNA interacts with the filament at some point downstream, either by a secondary pairing interaction (*panel II*) or some unproductive interaction with the filament (*panel III*), the continued spooling process to extend the paired region will be impeded.

exchange product still allowed for significant DNA strand exchange, furthering the argument that fidelity is reduced with 2AP. A similar strand exchange reaction with normal DNA, designed to generate seven mismatches at random locations, failed to yield detectable levels of products (data not shown). This result indicates an enhanced capacity of 2AP for misalignment in RecA-mediated DNA strand exchange reactions. Nevertheless, the effect on the rates or extents of DNA strand exchange with oligonucleotides was small.

The present result may provide some new avenues for future experimentation. The base 2-aminopurine has fluorescence properties that have been used previously to probe the kinetics of DNA pairing reactions promoted by this class of proteins (43), using oligonucleotides with a much smaller number of substitutions. The capacity to generate completely functional DNAs with broad substitutions of 2-aminopurine, as demonstrated here, should simplify the design of a variety of novel DNAs for real time monitoring of DNA strand exchange. RecA protein can promote DNA strand exchange with DNA substrates containing a wide array of base analogs (21, 59), as long as the analogs form a sufficiently stable base pair compatible with a Watson-Crick duplex DNA. This capacity extends to the Rad51 protein (Ref. 21 and the present work), adding to the list of observations suggesting that the fundamental DNA pairing processes promoted by RecA and Rad51 are similar.

Optimal Solution Conditions for DNA Strand Exchange Are Different for RecA and Rad51—The results of the present work should help to rationalize the great variability in published reports (3, 19, 45, 48, 49, 56–58) of the reactions of yeast and human Rad51 proteins. The yeast Rad51 protein promotes an optimal DNA strand exchange reaction only when small amounts of KCl and spermidine are present. For the yeast Rad51 protein, an apparently efficient DNA strand exchange in the presence of only potassium and magnesium ions has been reported (48), but these reactions involved much longer time courses and DNA substrates (pBluescriptSK, 2958 base pairs) a little more than half the size of the ϕX174 DNAs used here. Neither KCl nor spermidine is required for RecA protein, and they have little effect on RecA activities under most conditions. If the basic DNA transactions during DNA strand exchange are conserved among the reactions promoted by this class of proteins, the observed changes in optimal conditions suggest significant differences in the manner in which these proteins interact with DNA.

One of the major differences between RecA and Rad51 is seen in their utilization of ATP. For RecA protein, conditions have not yet been found that allow a long DNA strand exchange at high efficiency without ATP hydrolysis. When RecA protein hydrolyzes ATP, it promotes a robust DNA strand exchange that is unidirectional (10), bypasses DNA heterologous insertions in the DNA substrate that can extend over a few hundred base pairs (9, 11), and promotes strand exchange reactions involving four DNA strands (9, 12, 60). The Rad51 protein hydrolyzes ATP (albeit much more slowly than RecA), but the hydrolysis appears to have little effect on the strand exchange reactions. Rad51 has exhibited little capacity to bypass heterologous insertions in its substrate DNA (17, 48) or to promote four strand exchange reactions.[2] The Rad51 protein does not require ATP hydrolysis to promote a robust and extensive three strand exchange reaction such as that in Fig. 2.

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