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 spoiling 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 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), Mg²⁺ 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
RecA and Rad51-promoted DNA Pairing

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·mL^−1 cm^−1 for Rad51 (49), and 8.8 × 10^4 M^−1 cm^−1 for RPA (49). T4 polynucleotide kinase, PhoI endonuclease, and their accompanying buffers were purchased from Promega. ATP·S was from Roche Molecular Biochemicals. 32P-ATP was purchased from Amersham Pharmacia Biotech. Tris base, boric acid, EDTA, magnesium 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, spermine trihydrochloride, creatine phosphokinase, phosphocreatine, and bromophenol blue were purchased from Sigma. Dithiothreitol (DTT) was purchased from Research Organizations 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, AGTAGACTCAGGAGACTCAGGACTCTCTGACGAGACGCTGAGAATAC; B, TGATCCACGTCTACGATCCGTTCGATCCGATAC; C, GTACTGCCTGTCTGACGACTCAGAAGACGCTGAGAATAC; D, TGATCCAGTCTGACGACTCAGAAGACGCTGAGAATAC; E, TGACTGACGACTCAGAAGACGCTGAGAATAC; F, TGACTGACGACTCAGAAGACGCTGAGAATAC; G, TGACTGACGACTCAGAAGACGCTGAGAATAC; H, TGACTGACGACTCAGAAGACGCTGAGAATAC; I, TGACTGACGACTCAGAAGACGCTGAGAATAC; J, TGACTGACGACTCAGAAGACGCTGAGAATAC; K, TGACTGACGACTCAGAAGACGCTGAGAATAC; L, TGACTGACGACTCAGAAGACGCTGAGAATAC; M, TGACTGACGACTCAGAAGACGCTGAGAATAC; N, TGACTGACGACTCAGAAGACGCTGAGAATAC; O, TGACTGACGACTCAGAAGACGCTGAGAATAC; P, TGACTGACGACTCAGAAGACGCTGAGAATAC; Q, TGACTGACGACTCAGAAGACGCTGAGAATAC; R, TGACTGACGACTCAGAAGACGCTGAGAATAC; S, TGACTGACGACTCAGAAGACGCTGAGAATAC; T, TGACTGACGACTCAGAAGACGCTGAGAATAC; U, TGACTGACGACTCAGAAGACGCTGAGAATAC; V, TGACTGACGACTCAGAAGACGCTGAGAATAC; W, TGACTGACGACTCAGAAGACGCTGAGAATAC; X, TGACTGACGACTCAGAAGACGCTGAGAATAC; Y, TGACTGACGACTCAGAAGACGCTGAGAATAC; Z, TGACTGACGACTCAGAAGACGCTGAGAATAC.

Experimental Procedures

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 Mg2+ 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 Mg2+, 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 Mg2+ 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).

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 RecA-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, 47). We also continue our exploration of the range of DNA substrates tolerated in DNA strand exchange processes (21, 59).
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 n mole/μl, unless otherwise stated.

**RecA and Rad51-promoted DNA Pairing**

RecA-facilitated Three Strand DNA Exchange of Oligonucleotide Substrates—DNA pairing reactions were carried out in 20 mM Tris-OAc buffer (pH 7.4) with added 1 mM DTT, 1% glycerol, and 1 mM Mg(OAc)₂ at 38 °C. 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, 27.8 A₂₆₀ mg⁻¹ 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 MgCl₂ 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 ATP-S for 10 min. Additional reaction components were added to the preincubation as indicated. The reactions were initiated by the addition of an equal amount of ATP-S to the preincubation mixture to 14.9 mM. Reactions were incubated at 38 °C for 10 min, followed by another 5 min at 38 °C. Aliquots of 9 μl were removed at the indicated times and dialyzed, electrophoresed, and analyzed as described for the reaction series used for 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 dX174 DNA Substrates—Three strand DNA exchange reactions with dX174 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 dX174 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 DNA substrates. Reactions were performed in 15 μl. Reactions were incubated at 38 °C for 10 min, followed by another 5 min of incubation at 38 °C. Aliquots of 9 μl were removed at the indicated times and subjected to electrophoresis and quantitated 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 reactions. 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 dX174. 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 (11). Most of this work focuses on the RecA proteins for the reason that, 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.
Experimental Procedures

RecA and Rad51-promoted DNA Pairing

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 18.1 μM RecA protein, 56 μM 70-mer DNA, 12.8 μM 39-mer duplex, 10 mM Mg(OAc)₂, and 3 mM ATP. S. 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 substrates 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 adenine in the pairing region in all three oligonucleotides. Thus, 2AP appears at least 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 (nickd duplex circles) appear first after a lag of somewhat more than 10 min and then increase steadily 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. 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-
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. 3C.

The differences between RecA and Rad51 are even more dramatic when the DNA strand exchange with \(\phi X174\) 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. 5B. Higher levels of Mg\(^{2+}\) slightly inhibit the reaction when spermidine is present (Fig. 5C). 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 \(\phi X174\)-derived substrates (Fig. 6). Low levels of Mg\(^{2+}\) alone do not allow for product formation, as already shown. 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 limited at only 1 mM (data not shown). At normal (11 mM) concentrations of Mg\(^{2+}\), the DNA strand exchange with \(\phi X174\) 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 \(\phi X174\) DNA reaction without ATP hydrolysis (9). We therefore tried to see if spermidine would enhance this reaction. With ATP-S 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 \(\phi X174\)-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. 9B).

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. 3. Effects of Mg\(^{2+}\) on the DNA strand exchange with oligonucleotide substrates.](image-url) 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 \(\mu\)M 70-mer ssDNA substrate (0.45 \(\mu\)M molecules), 12 \(\mu\)M \(^{32}\)P-labeled 40-mer dsDNA substrate (0.15 \(\mu\)M molecules), 10.5 \(\mu\)M RecA protein, and 3 mM ATP-S. Rad51 reactions (B) contained the same concentrations of DNA substrates, 9.55 \(\mu\)M 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.
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. 1A). The reaction promoted by Rad51 protein, was reduced by 1/4 to 1/3 by an...
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 adenine 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 transition 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. 13B). 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. 8. Effects of spermidine on RecA protein-promoted DNA strand exchange with bacteriophage X174-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 ATP-S, 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.](image-url)
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 heterologus base substitutions and then incorporated into the strand exchange assay (Fig. 13C). All seven Gs in the pairing region of the 70-mer ssDNA substrate (Oligo A<sub>P</sub> 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. 13C).

**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.

**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)<sub>2</sub>, 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)<sub>2</sub>, 2.13 mM 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.
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. 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 32P-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 ATP·S. 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 32P-labeled 40-mer dsDNA substrate (0.15 μM molecules), 10.5 μM RecA protein, and 3 mM ATP·S. 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.
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 pair to form a plausible base triple (with one hydrogen bond) that would 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. During a sampling process of homologous alignment via an R-form triplex, a 2AP should in principle align with a G:C pair as well as with a 2AP:T pair. A 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.

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-substituted 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 32P-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 2AP-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.
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. 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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