Evidence for a Four-Strand Exchange Catalyzed by the RecA Protein†

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ABSTRACT: Strand exchange between two duplexes is usually initiated as a three-strand event that requires the presence of a single-stranded overhang or gap in one of the two molecules. Here we show that the RecA protein can catalyze a four-strand exchange. Specifically, it can recombine short hairpin substrates with homologous stems provided that one of the hairpins possesses a chimeric DNA/RNA backbone. This four-strand exchange reaction goes to completion in the presence of ATPγS and releases a stable heteroduplex upon removal of the RecA protein. Under identical conditions, strand exchange between two DNA hairpins is incomplete and generates a nascent heteroduplex that rapidly dissociates when the RecA protein is denatured. Since presynaptic filament formation does not appear to melt either type of hairpin, we propose that exchange occurs between homologously aligned duplexes that are extended and unwound within a RecA filament. The first reaction provides a mechanism for gene targeting by chimeric double-hairpin oligonucleotides while the second reaction explains the ability of the RecA protein to transiently align double-stranded DNA molecules.

A hallmark of the RecA protein is its ability to carry out strand exchange between single-stranded DNA and double-stranded DNA of homologous sequence (for reviews, see *1*−*3*). Several studies (*4*−*10*) suggest that the RecA protein might also initiate strand exchange between two double-stranded molecules as a four-strand event. However, unequivocal demonstration of such a reaction has not been reported. Here we test the RecA protein for four-strand exchange activity in a simple model system.

RecA-catalyzed strand exchange between two dsDNAs[1] usually does not take place unless one of the molecules contains a single-stranded overhang or gap (*11*−*13*). The single strand readily forms a presynaptic filament with the RecA protein, and this filament initiates strand exchange with the homologous target as a three-strand event. Propagation of the heteroduplex into the flanking dsDNA creates a Holliday junction characteristic of four-strand exchange. Unidirectional movement of this RecA-associated junction in the presence of ATP leads to a switch of strands between two parental duplexes (*13*−*15*).

While the above pathway has been well characterized, the RecA protein might also be able to initiate strand exchange between two homologous duplexes without the involvement of a single strand. For instance, earlier work showed that the RecA protein could align portions of two homologous dsDNAs when one molecule was a supercoiled plasmid (*4*, *5*, *7*). As a consequence of this interaction, partial unwinding of the plasmid DNA was detected. Also, studies on recombination between partially single-stranded and fully duplex DNA molecules supported a role for duplex-by-duplex pairing in facilitating the strand exchange reaction (*6*, *8*). More recently, Zaitsev and Kowalczykowski (*9*) demonstrated that the RecA protein could bind two dsDNA molecules in the presence of ATPγS and a high concentration of $MgCl_2$. They suggested that the two molecules might be capable of homologous recognition and strand exchange although direct evidence for a four-strand reaction was not obtained.

Even if strand exchange is observed between two duplexes, a three-strand mechanism could still be operative if the two strands of the incoming dsDNA are separated prior to synapsis. In this regard, the RecA protein was shown to melt dsDNA less than 30 base pairs long provided that the DNA had a single-stranded overhang (*16*). Strand separation was much more efficient with ATP than with ATPγS, implying that hydrolysis of ATP was linked to disruption of base pairing. Incorporation of each arm of a hairpin into a continuous presynaptic filament would preclude reannealing but promote strand exchange with a homologous dsDNA target.

Here we investigate strand exchange between hairpin oligonucleotides that possess homologous stems of 25 bp in length. Our interest in these molecules stems from the successful use of related hairpin constructs as gene targeting agents (*17*). In particular, double hairpin oligonucleotides with a chimeric DNA/2′-*O*-methyl-RNA backbone can introduce specific point mutations into DNA that are specified by the sequence of the oligonucleotide (*18*). This activity naturally raises the question of how such oligonucleotides

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; bp, base pair(s); ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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might complex with DNA. In a recent study, we reported that the RecA protein could carry out strand exchange between a limited number of chimeric hairpins and a homologous dsDNA target (*10*). However, the low level of joint molecule formation in that study and the presence of contaminating ssDNA in the reaction mixtures prevented us from differentiating between a three-strand and a four-strand mechanism for recombination.

The hairpin substrates used in this study were expected to form stable heteroduplexes not subject to dissociation by branch migration and not contaminated with single-stranded nucleic acid. We show that the RecA protein promoted efficient strand exchange between some of these substrates especially when one of the hairpins had a chimeric DNA/RNA backbone. By contrast, recombination between two DNA hairpins generated a labile joint that was indicative of partial strand exchange. We could not detect strand separation of the incoming hairpin in either reaction. As such, this suggests that exchange between these model double-stranded substrates occurred by a four-strand mechanism.

MATERIALS AND METHODS

Nucleic Acid Substrates. Oligonucleotides were synthesized using commercial phosphoramidites (Glen Research, Sterling, VA). Following deprotection and precipitation, each oligomer was purified by electrophoresis on a 7 M urea/12% polyacrylamide gel. In the case of hairpin oligonucleotides, the gels were run at 70 °C using a DCode system (Bio-Rad, Hercules, CA). Hairpin concentrations were determined by assuming that 40 μg/mL is equivalent to 1 optical density unit at 260 nm. Oligonucleotide stocks in water were stored at −20 °C. End-labeling reactions were carried out by incubating 100 pmol of an oligonucleotide with a slight excess of [γ-^{32}P]ATP (6000 Ci/mmol; NEN, Boston, MA) for 1−3 h at 37 °C in the presence of 10 units of T4 polynucleotide kinase. Labeled oligonucleotides were separated from free counts by centrifugation through a spin gel filtration column (Centri-Spin 20; Princeton Separation, Adelphia, NJ) hydrated in water. Recovered oligonucleotides were diluted 6-fold with cold carrier and adjusted to 100 nM concentration. Prior to use, each labeled hairpin was heated to 92 °C for 2 min in water and cooled to ambient temperature in a thermocycler. This heat−cool cycle disrupted the small amount of hairpin dimer present in every hairpin preparation.

Strand Exchange Reactions. A two-step reaction protocol was carried out to form joint molecules (*19*). In the first step (presynapsis), an incoming oligonucleotide (final concentration 160 nM in molecules) was incubated for 10−20 min at 37 °C with the RecA protein (final concentration 6.8 μM, nuclease free; USB, Cleveland, OH) in 25 mM Tris−OAc, pH 6.8, 1 mM DTT, 1 mM $Mg(OAc)_2$, and 1 mM ATPγS to form a presynaptic filament. In the second step (synapsis), strand exchange was carried out by increasing the [$Mg(OAc)_2$] to 10 mM, adding radiolabeled target hairpin (final concentration 20 nM in molecules), and continuing the incubation at 37 °C for 10−20 min. Reactions of a final volume of 10 μL were terminated using two protocols. In the first, a 0.1 volume of 10% SDS at 37 °C was added to the reactions which were left at room temperature for approximately 45 s before quickly freezing in dry ice. In the second protocol, reactions were cooled to 4 °C in a thermocycler prior to adding the SDS. After 1−5 min at this temperature, the reactions were quickly frozen in dry ice and stored at −20 °C until analyzed. For strand exchange reactions with an incoming single-stranded oligonucleotide, the SDS solution usually contained a 25-mer complementary to the outgoing strand of the target hairpin. The final concentration of this 25-mer was 4 times that of the incoming oligonucleotide.

Immediately prior to electrophoretic analysis, samples were thawed in an ice bath and spiked with a weighting solution containing ficoll, bromphenol blue, and xylene cyanol. Half of each sample was loaded onto a precooled 12% polyacrylamide gel containing 1 mM $MgCl_2$. After electrophoresis for 2.5 h in a cold room, the gel was dried on filter paper, and bands were detected by autoradiography. Quantitation of the data was achieved using a phosphorimager (Molecular Devices, Sunnyvale, CA).

RESULTS

Model System Used To Study Strand Exchange. We have adapted the nomenclature of Adzuma (*20*) to identify the four strands involved in an exchange between two hairpins. The incoming hairpin is complexed with the RecA protein to form a presynaptic filament and so occupies the primary binding site of the protein. The target hairpin, which is added to initiate strand exchange, occupies the weaker secondary binding site. The two arms of the first hairpin are defined as the incoming and donor strands, respectively, while the two arms of the second hairpin are defined as the recipient and outgoing strands, respectively. The incoming strand initiates exchange by forming a heteroduplex with the complementary recipient strand. Subsequent hybridization of the complementary donor and outgoing strands completes the strand exchange reaction.

Reactions with the RecA protein were carried out at 37 °C in the presence of ATPγS. In the first step, the incoming single-stranded or hairpin oligonucleotide was incubated with excess RecA protein to form a nucleoprotein filament in the presence of 1 mM $Mg(OAc)_2$. Such filaments contain one RecA protein per 3 bases of single-stranded or 3 base pairs of double-stranded DNA (*21*, *22*). Strand exchange was initiated by adding radiolabeled target hairpin and increasing the $Mg(OAc)_2$ concentration to 10 mM. At this concentration of magnesium ion, the RecA filament can accommodate two double-stranded DNA molecules in a high-energy state (*9*). To drive exchange of the target hairpin into a joint molecule, an 8-fold excess of incoming oligonucleotide was used. Reactions were terminated with SDS and analyzed for joint molecule formation by native polyacrylamide gel electrophoresis.

The reactions utilized one of two hairpin oligonucleotides as a target for strand exchange. Figure 1A shows the structure of the HP1 hairpin. This hairpin has a 25 bp long DNA stem connected at one end by a 4 nucleotide long loop. By synthesizing the loop with a 2′-*O*-methyl-RNA backbone, we expected to decrease the possibility of nucleation of the RecA protein at the loop. The HP2 hairpin is identical in structure to HP1 except that the RNA loop is attached to the opposite end of the stem. Since these hairpins interacted weakly with the RecA protein (see below), strand

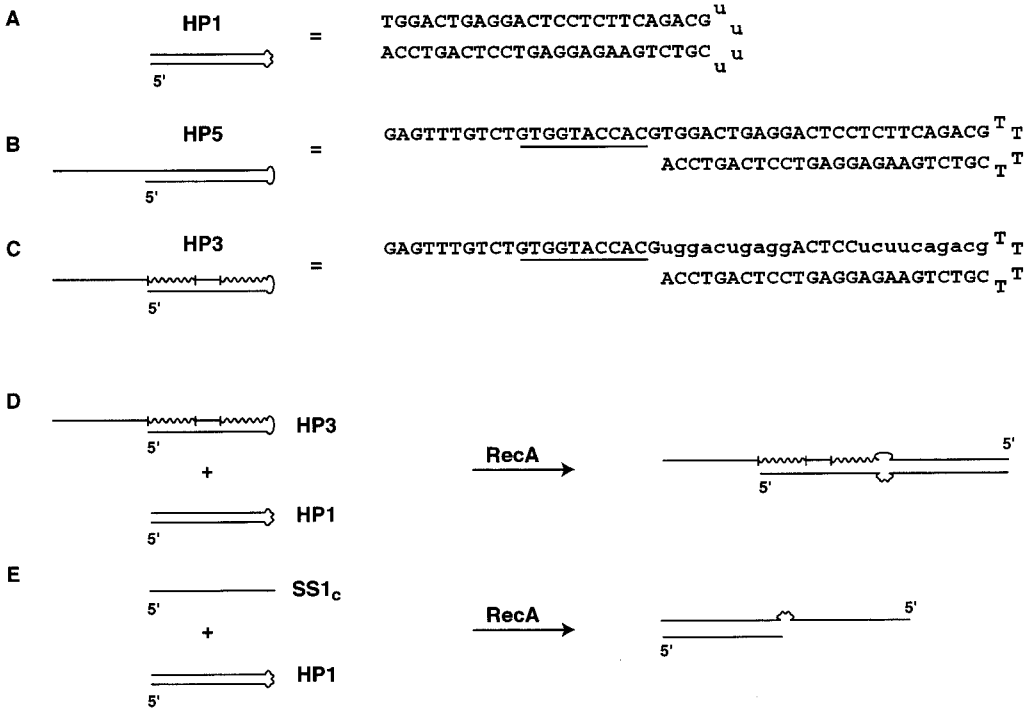

FIGURE 1: Representative oligonucleotides used as substrates for strand exchange and the resulting heteroduplex joints. (A) Target hairpin HP1; (B) incoming DNA hairpin HP5; (C) incoming chimeric DNA/2′-*O*-methyl-RNA hairpin HP3; (D) schematic of the strand exchange reaction between HP5 and HP1; and (E) schematic of the strand exchange reaction between SSl_c and HP1. In the schematic representations, a smooth line denotes a DNA backbone while a wavy line denotes a 2′-*O*-methyl-RNA backbone. In the lettered representations, these backbones are represented by upper and lower case letters, respectively. All of the oligonucleotides used as substrates for strand exchange in this study have sequences derived from that portion of the human β-globin gene to which the sickle mutation is mapped. The overhangs of HP3 and HP5 contain a 10 base long palindrome (underlined). Hairpins with this sequence could hybridize to each other.

exchange reactions were carried out in the presence of excess enzyme.

Incoming hairpins had stem−loop structures identical in size and sequence to the target hairpins. In addition, these hairpins had a short single-stranded or stem−loop extension designed to promote nucleation of the RecA protein during presynaptic filament formation. The incoming hairpins had either DNA or chimeric DNA/2′-*O*-methyl-RNA backbones. In the latter case, one arm of the hairpin had 5 nucleotides of DNA flanked on both sides by 10 nucleotides of 2′-*O*-methyl-RNA. The structures of two representative incoming hairpins are shown in Figure 1B,C. Schematic drawings of all the hairpins are presented in Table 1.

Strand exchange between two hairpins was expected to generate a stable 50 bp long heteroduplex interrupted by a 4 base long bulge in the middle (Figure 1D). This joint is distinct from the product formed between a single-stranded oligonucleotide and a hairpin target (Figure 1E). The stable 50 bp long joint is formally equivalent to the complement-stabilized D-loop (*23*, *24*) that would arise from a strand exchange reaction between a hairpin and a long dsDNA. Unlike a complement-stabilized D-loop, however, the joints formed here lacked junctions with parental DNA, thereby eliminating extrusion of the incoming hairpin due to branch migration. We anticipated that this modification would improve the yield of recombinant molecules. From a mechanistic perspective, the use of hairpins guaranteed that all four strands would remain linked together during the strand exchange reaction, thus permitting us to follow the fate of strands that might otherwise dissociate from the joint. Finally, by using hairpins in place of intermolecular DNA hybrids, we eliminated contamination of the reaction mixture with single-stranded nucleic acid. Alternative reaction schemes utilizing single strands could thus be discounted.

In Figure 2 several control and complete strand exchange reactions were analyzed for joint molecule formation by nondenaturing polyacrylamide gel electrophoresis. Using HP1 as the target for strand exchange, joint molecules were formed by HP3 (a chimeric DNA/RNA hairpin), HP5 (a DNA hairpin otherwise identical to HP3), and $SS1_c$ (a single-stranded DNA 25-mer). The most efficient reaction was observed with HP3 (26% yield of joint molecule). HP5 was less active (16% yield). Although these hairpins should have formed identical joints with HP1, the HP3/HP1 joint ran slower than the HP5/HP1 joint. As expected, both joints ran slower than a 50 bp DNA standard that contained a central 4 nucleotide long hinge. The single-stranded 25-mer $SS1_c$ formed two complexes with HP1 (19% total yield). The slower moving complex was identified as a heteroduplex based upon its comigration with a 25 bp long DNA standard that contained a 29 nucleotide long overhang. The faster moving complex that ran as a smear was tentatively identified as a triple-stranded intermediate (data not shown). Control reactions that were carried out with each of these substrate pairs confirmed that the RecA protein and ATPγS were required for strand exchange activity. When ATPγS was replaced with ATP, no recombinant joints were detected when using two hairpin oligonucleotides as the substrates (data not shown). The inability of HP1 to function as an incoming hairpin suggests that the DNA overhang played a role in presynaptic filament formation.

All of the strand exchange reactions were terminated by addition of SDS to a final concentration of 1%. To verify the effectiveness of this protein denaturant, a strand exchange

Table 1: Percent Joint Molecule Formation between Two Hairpin Oligonucleotides[a]

Incoming Oligonucleotide	Target Oligonucleotide HP1	Target Oligonucleotide HP2
HP3	34.2%	17.0%
HP4	24.4	12.7
HP5	14.5	10.8
HP6	27.0	22.7
HP7	11.6	10.0
HP8	8.6	13.1
HP9	18.3	75.4
HP10	7.0	55.8
HP11	3.2	26.3
HP12	0	6.8
HP13	3.1	34.2
HP14	0	20.7

[a] Using the protocol described under Materials and Methods, strand exchange reactions were carried out between the listed hairpin oligonucleotides. Reactions were terminated by addition of SDS at 37 °C. Incoming and target hairpin oligonucleotides had homologous stems 25 bp in length. In addition, each incoming hairpin had a DNA extension consisting of a 21 nucleotide long single strand or a 5−6 bp long stem with a 4 nucleotide long loop. Strand identity is arbitrarily designated by the letters w (Watson) and c (Crick). Smooth lines denote DNA, and wavy lines denote 2′-*O*-methyl-RNA.

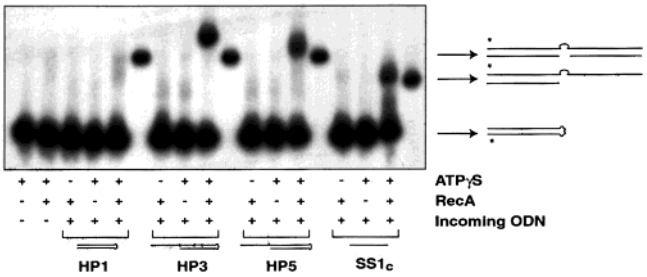

FIGURE 2: Requirements for joint molecule formation with HP1. Incoming oligonucleotides were reacted with HP1 under standard conditions or in the absence of the RecA protein or ATPγS. Reactions were stopped with SDS at 37 °C and assayed for joint molecule formation by native PAGE. The structures of immobile joints used as standards are shown to the right of the gel. These joints were formed by hybridization of non-self-complementary oligonucleotides.

reaction between HP3 and HP1 was analyzed on a nondenaturing gel after aliquots had been treated with water, SDS, or SDS plus proteinase K. Representative oligonucleotides that had been incubated with the RecA protein were similarly treated and analyzed on the same gel (Figure 3A). Stable presynaptic filaments were formed by HP3 and $SS3_w$ (a single-stranded 46-mer with a DNA backbone) but not by HP1. The RecA protein was stripped from these oligonucleotides by treatment with SDS at 4 °C or with SDS plus proteinase K at 37 °C. While a discrete reaction product was not detected for the HP3 + HP1 reaction in the absence of SDS, much of the labeled HP1 was contained in a high molecular weight nucleoprotein aggregate that did not enter the gel. Each of the three denaturation protocols tested liberated the expected protein-free joint from this aggregate. The presence of two additional weak bands following treatment of the HP3 + HP1 reaction with SDS at 4 °C probably represents labile reaction intermediates given that these bands were not observed when the incubation time at 4 °C was extended or when SDS was added at 37 °C. Of note is the slow moving band present in the control lane for HP3. We believe that this band represents two hairpins held together by hybridization of the overhangs at the site of a 10 base long palindromic sequence (see Figure 1B for location of the palindrome). This complex, which was occasionally formed by some of the hairpin substrates, did not usually survive the strand exchange reaction.

If recombination between two hairpins is to serve as a model for reciprocal exchange between four strands, it is important to verify that the hairpins have intact stem−loops at the conclusion of the reaction. If one of the hairpin stocks is contaminated with partially degraded molecules or if the

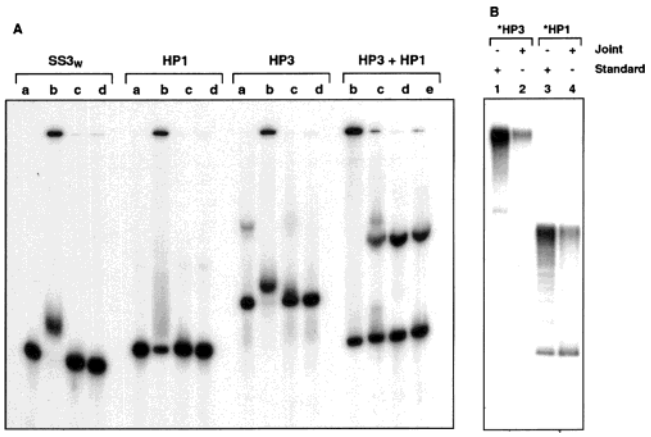

FIGURE 3: Qualification of the strand exchange assay. (A) Denaturation of the RecA protein in presynaptic filaments and synaptic complexes. Presynaptic filaments containing radiolabeled $SS3_w$, HP1, or HP3 and a synaptic complex formed between HP3 and radiolabeled HP1 were subjected to different protein denaturation protocols prior to analysis by native PAGE. Control reactions (a) lacked the RecA protein or (b) were not deproteinized. The RecA protein was denatured by treatment with (c) 1% SDS for 1 min at 4 °C, (d) 0.5% SDS and 100 μg/mL proteinase K for 10 min at 37 °C, and (e) 1% SDS for approximately 45 s at room temperature. All reactions were frozen in dry ice and stored at −20 °C until electrophoretic analysis. (B) Integrity of the HP1 and HP3 hairpins isolated from a joint molecule. The RecA protein was used to form a recombinant joint between HP3 and HP1. In one joint, HP3 was labeled, and in the other, HP1 was labeled. Each joint was isolated by native PAGE. After recovery from the gel, each joint was denatured for 2 min at 90 °C in 90% formamide and electrophoresed in a 7 M urea/12% polyacrylamide gel next to labeled hairpin standards. In each lane, the slower moving band represents fully denatured hairpin, and the faster moving band represents reannealed hairpin.

RecA preparation has contaminating nuclease activity, the formation of joint molecules could be initiated by a three-strand exchange mechanism or could occur by simple reannealing of unpaired Watson and Crick strands. This possibility was evaluated for the joint formed between HP1 and HP3. Figure 3B shows an analysis of the radiolabeled oligonucleotides on a denaturing gel before and after joint molecule formation. In the gel, a majority of each hairpin electrophoresed as a full-length single-stranded oligonucleotide. Faster moving material was assumed to represent hairpin that had partially or completely reannealed prior to the start of electrophoresis. We verified this assumption by showing that upon reanalysis material derived from the faster moving band reproduced the original electrophoretic profile. Since the hairpins gave similar profiles before and after strand exchange, we conclude that they were intact and that the RecA protein was free of nucleases. For the HP1/HP3 joint, this conclusion was corroborated by gel shift experiments which showed that the overhang contributed by HP3 was present and available for hybridization but that the complementary arms of the two hairpins were fully hybridized (see Figure 7).

Structure−Activity Relationships for Strand Exchange. A series of hairpin oligonucleotides were tested for joint molecule formation with the HP1 and HP2 target hairpins (Table 1). The incoming hairpins differed with respect to composition of the backbone (DNA versus DNA/RNA), placement of the loop (left versus right), orientation of the overhang (5′ versus 3′), and structure of the overhang (single strand versus stem−loop). The extent of strand exchange varied widely, with 0−75% of the limiting target oligonucleotide incorporated into a joint (Table 1). Certain trends were clearly evident in the results. Chimeric hairpins were usually more active than DNA hairpins (e.g., compare HP9 with HP11), and hairpins with a single-stranded overhang were usually more active than hairpins with a stem−loop overhang (e.g., compare HP9 with HP10). Participation of double-hairpin oligonucleotides in strand exchange can be rationalized by assuming that the smaller hairpin (with a 5−6 bp long stem) in such molecules exists in equilibrium between folded and unfolded conformations. The RecA protein should readily nucleate onto the unfolded conformation to initiate presynaptic filament formation. Among the chimeric hairpins, linkage of the single-stranded or stem−loop overhang to the chimeric strand was preferable to linkage to the DNA strand (e.g., compare HP9 with HP13 and HP10 with HP14). Exchange of strands between hairpins with apposed loops was usually more efficient than between hairpins with opposed loops (e.g., compare HP3 + HP1 with HP3 + HP2). The best pair of reactants was HP9 + HP2, which recombined with 75% efficiency. With a 5′ single-stranded overhang, HP9 can accommodate the preferred 5′ to 3′ loading of the RecA protein during filament extension (*25*−*27*). The relationships in Table 1 confirm and extend earlier results reported by us using a short dsDNA as a target for strand exchange with a limited number of hairpin oligonucleotides (*10*).

Single-stranded oligonucleotides complementary to an arm of the HP1 target were also tested for joint molecule formation. The protocol was identical to that used in the hairpin reactions except that denaturation of the RecA protein was conducted in the presence of a complement to the outgoing strand of HP1. This modification improved yields by converting the products of strand exchange to stable heteroduplexes. All of the oligonucleotides with a DNA backbone were effective strand exchange agents regardless of their length (25-mers and 46-mers giving 36−62% joint molecule formation). In contrast, none of the oligonucleotides with a 2′-*O*-methyl-RNA or a chimeric DNA/2′-*O*-methyl-RNA backbone in the region of homology exhibited competency in strand exchange (data not shown). The introduction of flanking DNA sequences on one or both sides of the chimeric domain did not enhance joint molecule formation. These results are consistent with previous observations that the RecA protein has a low affinity for RNA (*28, 29*) and that the 2′-hydroxyl group interferes with strand exchange (*30*). The inability of RNA to form a competent presynaptic filament with the RecA protein suggests that it is the DNA strand of the incoming chimeric hairpin that is likely to initiate strand exchange with a target duplex.

Kinetics of Strand Exchange. As a preliminary determination of whether strand exchange between two hairpins occurs by a three-strand or four-strand mechanism, we compared the time course of exchange between HP3 + HP1 and $SS1_c$ + HP1. If the incoming hairpin is melted within the presynaptic filament, we reasoned that the subsequent strand exchange step should be similar in rate to that observed for an authentic three-strand reaction. In contrast, if the two hairpins synapse as unwound but intact duplexes, one would expect the rate of exchange to be different. We therefore prepared presynaptic filaments of the chimeric

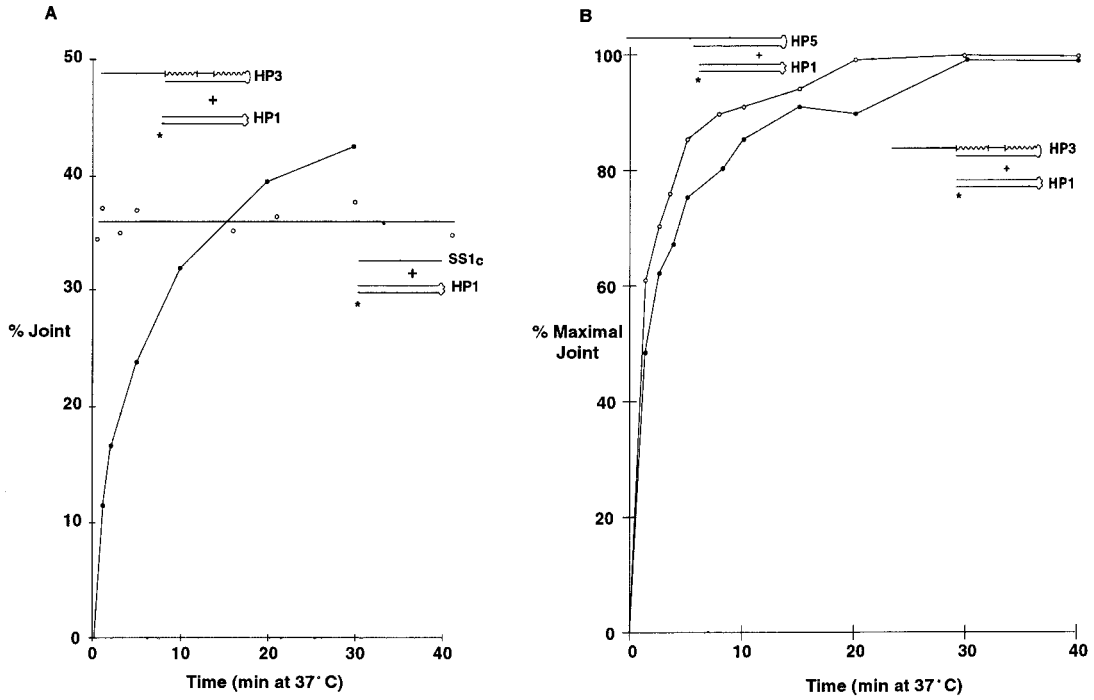

FIGURE 4: Kinetics of strand exchange. Presynaptic filaments were prepared as usual. Following addition of the HP1 target, aliquots of each reaction were removed over time for determination of joint molecule formation. (A) Comparison of reactions between $SS1_c$ + HP1 and HP3 + HP1. Reactions were terminated with SDS at 37 °C. (B) Comparison of reactions between HP5 + HP1 and HP3 + HP1. Reactions were terminated by rapid cooling to 4 °C prior to adding SDS. After 40 min incubation, 40% or 71% of the HP1 target was complexed with HP5 or HP3, respectively.

hairpin HP3 and the single-stranded 25-mer $SS1_c$ according to the standard protocol. Following the addition of labeled HP1 target together with more $Mg(OAc)_2$, aliquots were removed over time, and the extent of joint molecule formation was determined (Figure 4A). The kinetics of the three-strand exchange were too rapid to follow. Homogeneous assays have shown that these reactions are complete within 1−2 min under optimal conditions (*31*, *32*). By contrast, exchange between two hairpins proceeded at a much slower rate and was not complete after 30 min incubation. To ascertain whether the slow kinetics exhibited by HP3 were attributable to the presence of a chimeric DNA/2′-*O*-methyl-RNA backbone in the hairpin, we repeated the experiment using the DNA hairpin HP5. Given that HP5 forms half as many joints as HP3 (see Table 1), we plotted the results as a percent of maximal joint formation to facilitate comparison (Figure 4B). The time courses show that the rate of strand exchange was somewhat reduced for HP3 relative to that for HP5. From this analysis, it follows that the kinetics of strand exchange are sensitive to the number of strands in the recombining molecules. It is therefore unlikely that exchange between two duplexes occurs by a three-strand mechanism.

Hybridization of Oligonucleotides to Presynaptic Filaments Containing HP3 or HP5. $SS1_w$ and $SS1_c$ are complementary 25-mers homologous to the arms of the recombining hairpins. A high concentration of each was added to RecA filaments containing a chimeric (HP3) or DNA (HP5) hairpin to determine whether the hairpins had single-stranded character. If the RecA protein separates strands of these molecules during filament formation, the separated strands should hybridize to both 25-mers. Since the resulting hybrids would be similar to those formed by a strand exchange between an incoming 25-mer and the HP1 hairpin, the joints

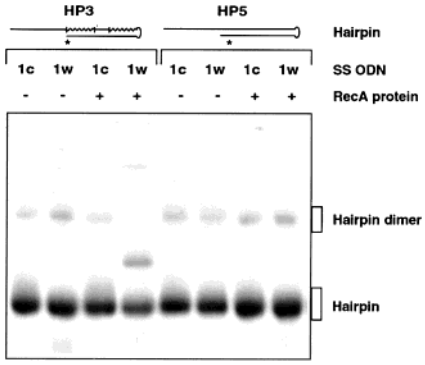

FIGURE 5: Hybridization of presynaptic filaments containing HP3 or HP5 to complementary single-stranded oligonucleotides. Presynaptic filament formation was carried out as usual with 10 nM HP3 or HP5 that had been end-labeled with ^{32}P. One minute prior to termination of the incubation, $SS1_c$ or $SS1_w$ was added to give a final concentration of 5 μM. The RecA protein was denatured by addition of SDS at 37 °C. After 45 s, the reactions were quick-frozen in dry ice and stored at −20 °C until analyzed by gel electrophoresis. Control reactions lacking the RecA protein were similarly processed.

were expected to survive the addition of SDS and electrophoretic analysis. As shown in Figure 5, neither hairpin hybridized to both single-stranded oligonucleotides. Instead, the HP5 filament did not hybridize to either oligonucleotide while the HP3 filament hybridized to only one of the two oligonucleotides. This argues against a mechanism in which the RecA protein fully unwinds the hairpins. The results were duplicated using 25-mers with a 2′-*O*-methyl-RNA backbone, thus ruling out the possibility that binding of the RecA protein to the DNA oligomers might have blocked their interaction with the target filament (data not shown). Hybridization of $SS1_w$ to the DNA strand of the HP3 filament indicates that the presence of a chimeric strand in a hairpin

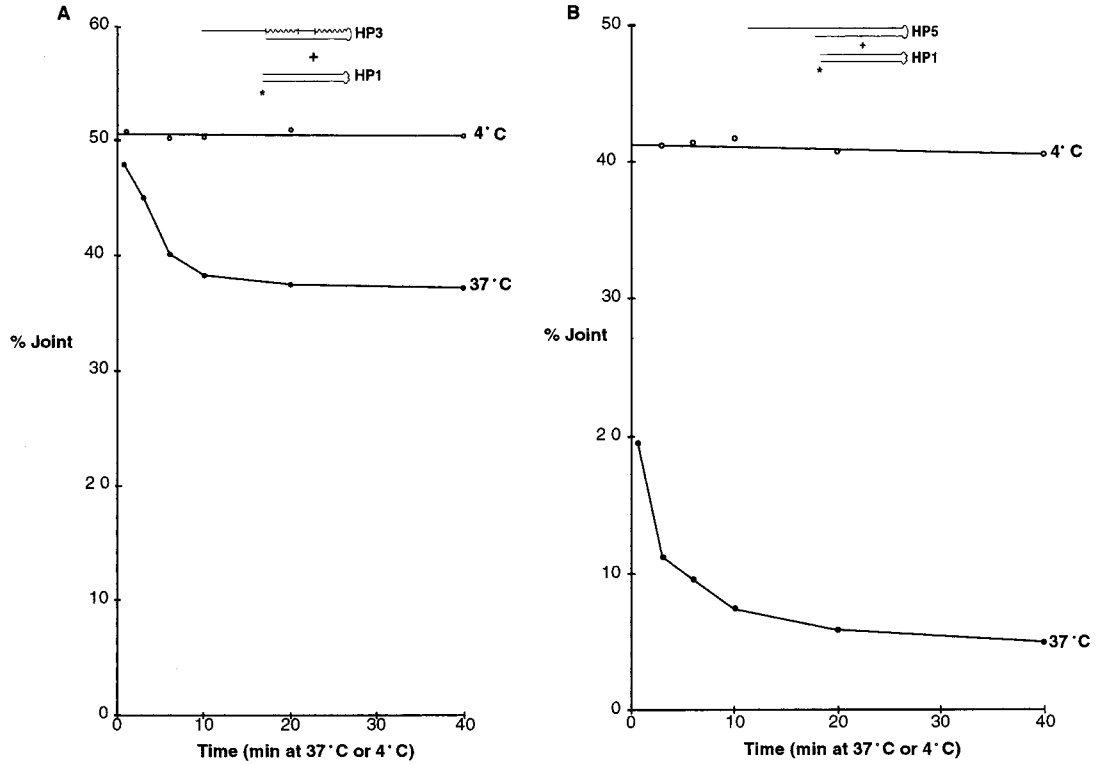

FIGURE 6: Stability of protein-free joints formed between (A) HP3 + HP1 and (B) HP5 + HP1. Recombinant joints were formed using the RecA protein according to the standard protocol. SDS was then added to the reaction mixtures at 4 or 37 °C. Incubation was continued at these temperatures as aliquots were withdrawn for quick-freezing in dry ice. The percent of labeled HP1 in a recombinant joint is plotted as function of time following addition of SDS.

enhances the single-stranded character of the complementary DNA strand and may partly explain why HP3 is more efficient than HP5 in strand exchange.

Stability of Recombinant Joints. Since we had confirmed that SDS could effectively strip the RecA protein from oligonucleotides at temperatures as low as 4 °C, we compared the stabilities of protein-free joints formed by the RecA protein between the incoming oligonucleotides HP3 and HP5 and the hairpin target HP1 at 4 and 37 °C. The results in Figure 6 show that while both protein-free joints were stable at 4 °C, each contained a component that rapidly dissociated at 37 °C. Joints that survived incubation at the higher temperature were assumed to represent stable heteroduplex products while joints that dissociated were assumed to represent labile intermediates in which strand exchange had probably not gone to completion. The instability of some of the joints at 37 °C implies that strand exchange reactions terminated at this temperature underestimate synaptic complex formation.

The difference in stability between the two joints is striking. Whereas most of the HP3/HP1 joint survived incubation at 37 °C (Figure 6A), the majority of the HP5/HP1 joint fell apart (Figure 6B). It appears that the RecA protein can mediate complete strand exchange when the incoming hairpin has a chimeric DNA/RNA backbone but is unable to do so when the same hairpin has a DNA backbone. In the latter case, strand exchange may only take place between two of the four strands. We estimate that 75% of the HP3/HP1 joint is a stable heteroduplex while 90% of the HP5/HP1 joint is an unstable intermediate with an indeterminate structure. As predicted by this model, the two joints exhibited different electrophoretic mobility (Figure 2). In multiple experiments, we have not observed rearrangement of the HP5/HP1 joint to a stable heteroduplex with a mobility equivalent to that observed for the HP3/HP1 joint. We therefore suspect that background counts in the gel may account for why the HP5/HP1 plot in Figure 6B does not drop off to zero.

Gel Mobility Shift Experiments. HP3/HP1 and HP5/HP1 joints were surveyed for hybridization with single-stranded oligonucleotides complementary to the Watson or Crick arms of the recombining hairpins (see Table 1 for assignments). In this experiment, the synaptic complexes were cooled to 4 °C prior to addition of SDS or SDS plus a 25-mer or 46-mer oligonucleotide. After 5 min at 4 °C, the joints were electrophoresed to detect a shift in mobility attributable to hybridization. The results are presented in Figure 7. The HP3/HP1 joint ran as a sharp band and only hybridized to one oligonucleotide (Figure 7A). This oligonucleotide ($SS2_c$) was complementary to the entire Watson strand of HP3 including the overhang and therefore was expected to interact with the joint. Upon addition of $SS2_c$, most of the surviving joint was shifted into a ternary complex (indicated by an arrow). The inability of the other oligonucleotides to hybridize to the HP3/HP1 joint strengthens our supposition that HP3 and HP1 had formed a stable heteroduplex involving all four arms of the two hairpins.

Analysis of the HP5/HP1 joint gave different results (Figure 7B). The majority of this joint reacted with each of the Crick oligonucleotides. Treatment with $SS1_c$ and $SS3_c$ converted the joint to a ternary complex whereas treatment with $SS2_c$ dissociated the joint and released free HP1. With the exception of a small amount of ternary complex formed by $SS2_w$, the three Watson oligonucleotides ($SS1_w$, $SS2_w$,

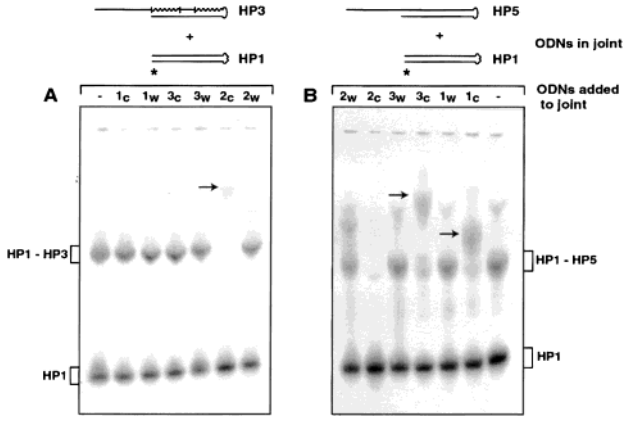

FIGURE 7: Hybridization of complementary single-stranded oligonucleotides to joints formed between (A) HP3 + HP1 and (B) HP5 + HP1. Recombinant joints were formed by the RecA protein using the standard protocol. The resulting strand exchange reactions were cooled to 4 °C and quenched with SDS or SDS plus the indicated oligonucleotide (final concentration 4-fold greater than the incoming hairpin). After 5 min at 4 °C, the reactions were quick-cooled in dry ice and stored at −20 °C while awaiting electrophoretic analysis. Ternary complexes are denoted by arrows. $SS1_w$ and $SS1_c$ are single-stranded 25-mers identical to the upper and lower strands of HP1. $SS2_w$, $SS2_c$, $SS3_w$, and $SS3_c$ are single-stranded 46-mers identical to the long arms of HP5, HP7, HP11, and HP13, respectively.

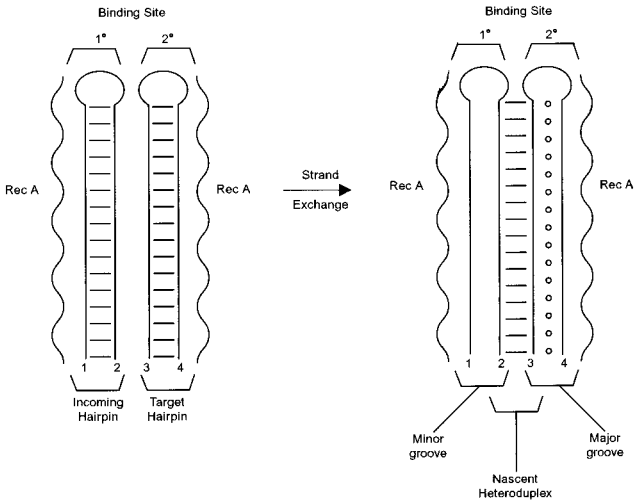

FIGURE 8: Model for RecA-mediated heteroduplex formation between two homologous DNA hairpins. A lateral view looking into the deep groove of a fully unwound RecA filament is presented. In the pre-strand exchange complex, the primary binding site of the RecA filament holds the incoming hairpin while the secondary binding site holds the target hairpin. We postulate that the major groove of the incoming hairpin faces the minor groove of the target hairpin. Hairpin arms 1, 2, 3, and 4 correspond to the donor, incoming, recipient, and outgoing strands, respectively. Hybridization of strands 2 and 3 displaces the outgoing strand into the major groove and the donor strand into the minor groove of the nascent heteroduplex. Hydrogen bonding may take place between the outgoing strand and the heteroduplex.

and $SS3_w$) did not interact with the HP5/HP1 joint. These observations are consistent with the idea that the RecA protein had catalyzed the formation of a labile intermediate between HP5 and HP1. Upon addition of SDS, a Watson strand in this intermediate was available for hybridization. When incubated for 20 min at 4 °C, the protein-free HP5/HP1 joint did not rearrange to a stable heteroduplex (data not shown). We point out that the slow moving band observed in the untreated control lane of Figure 7B probably represents two joint molecules held together by hybridization of the free HP5 overhangs (see Figure 1B for the palindromic sequence). A similar band was occasionally observed in strand exchange reactions between HP3 and HP1.

DISCUSSION

This study shows that the RecA protein can initiate strand exchange between two short hairpins using ATPγS as a cofactor. Exchange appears to take place between intact hairpins that are homologously aligned within the RecA filament. The immediate product of the exchange is probably a nascent heteroduplex in which only one strand from each hairpin has recombined (Figure 8). If each hairpin has a DNA stem, we postulate that further strand exchange is arrested due to stable interaction of the two remaining single strands with the RecA filament. This model explains why the nascent joint rapidly dissociates when the RecA protein is denatured.

Strand exchange between two hairpins can be significantly enhanced if one arm of the incoming hairpin has a chimeric DNA/RNA backbone. In this case, strand exchange proceeds to completion, and addition of SDS releases a stable heteroduplex. The extent of this four-strand exchange reaction is comparable to that observed for a three-strand exchange. In modeling this reaction, we presume that the presence of a chimeric strand does not interfere with nascent heteroduplex formation between the two hairpins. However, in the resulting synaptic complex, the chimeric strand (unlike a DNA strand) is weakly bound by the RecA protein and can hybridize to its complement, thus forming a stable joint molecule.

We evaluated the possibility of a three-strand exchange mechanism wherein the RecA protein separates the strands of the incoming hairpin during presynaptic filament formation. The three-strand exchange mechanism suggests that subsequent pairing and initial strand exchange could follow the traditional pathway. The three-strand mechanism is supported by the ability of the RecA protein to melt DNA hybrids up to 30 bp in length in the presence of ATP and to a lesser extent in the presence of ATPγS (*16*). Elements of the RecA protein share structural similarity to the helicases (*33*, *34*) and could be involved in this activity. A three-strand exchange mechanism might also explain how the RecA protein is able to bypass a double strand break during strand exchange between two dsDNAs (*35*). The results presented in this paper, however, are not consistent with such a mechanism for strand exchange between two hairpins. If the incoming hairpin was converted to a single strand within the presynaptic filament, then each arm of the hairpin should have hybridized to a single-stranded complement. This, however, was not observed for either DNA or chimeric hairpins. In a similar vein, a melted out DNA hairpin should have recombined with a double-stranded target at the same rate as observed for an incoming single-stranded DNA molecule. Instead, we observed that synapsis was much slower when the incoming molecule was a hairpin.

We propose an alternative model in which homologous pairing and strand exchange take place between two partially unwound DNA hairpins that are aligned within a RecA filament (Figure 8). Previous studies have shown that the

RecA protein can align (*4, 5, 7*) and bind (*9*) two homologous dsDNAs. These studies support a four-strand exchange model. Drawing upon recent observations on the three-strand exchange reaction (*36*), we suggest that base pair switching between complementary strands of the two hairpins mediates homologous alignment and strand exchange in a process that involves transient perturbation of both helices during the search process (Figure 8). The highly extended and unwound state of both DNA molecules in the RecA filament (*37*−*39*) facilitates this reaction. Once the hairpins are aligned, one strand from each hairpin hybridizes to its complement through a mutual rotation of bases (*40, 41*). The resulting heteroduplex is flanked on one side by the outgoing strand of the target hairpin and on the other side by the donor strand of the incoming hairpin. If the outgoing strand occupies the major groove side of the heteroduplex as it does in the three-strand exchange reaction (*42*−*45*), then bases of this strand could form hydrogen bonds to the duplex. In our model, prior to strand exchange the primary and secondary DNA binding sites of the RecA filament (*22, 46, 47*) accommodate the incoming and target hairpins, respectively. Following strand exchange, the donor strand remains bound to the primary site while the outgoing strand remains bound to the secondary site. As a consequence, the nascent heteroduplex spans both sites. In the presence of ATPγS, we postulate that this four-stranded intermediate is stable and does not undergo further strand exchange.

Our four-strand exchange model predicts that upon denaturation of the RecA protein the synaptic complex should release a triple-stranded joint with an attached donor strand. Since the donor strand is complementary to the outgoing strand of the triplex, one might expect these two strands to hybridize, thus completing the process of strand exchange. This was not observed experimentally. Instead, the deproteinized joint rapidly dissociated at 37 °C. Despite enhanced stability at 4 °C, the majority of the protein-free joint hybridized to single-stranded Crick oligonucleotides. We propose that the Watson arm of the incoming hairpin is the donor strand and that upon deproteinization of the joint this strand can hybridize with an external complement but not with the internal complement. The outgoing strand, presumably hydrogen bonded to the major groove of the heteroduplex, is not accessible. Chiu et al. (*42*) have previously shown that short triple-stranded DNA molecules formed with the assistance of the RecA protein are very stable when two of the three strands are part of a hairpin oligonucleotide.

We had previously demonstrated that the RecA protein could mediate low levels of joint molecule formation between a chimeric hairpin and a DNA duplex. In these reactions, the equivalent of a complement-stabilized D-loop had been formed. Our study of the HP3/HP1 reaction is consistent with complete strand exchange occurring to give a stable heteroduplex product. While this reaction is accommodated by either model, we favor one in which the incoming hairpin is extended and unwound but not strand-separated in the presynaptic filament. The DNA arm of the incoming hairpin is presumed to initiate strand exchange since RNA is not an appropriate substrate. Following heteroduplex formation, hybridization of the chimeric arm of the incoming hairpin (i.e., the donor strand) with the complementary strand of the target hairpin (i.e., the outgoing strand) would complete the exchange reaction. We propose that any interaction between the RecA protein and the chimeric strand is weak, thereby permitting hybridization to occur within the synaptic complex.

Several examples exist of RNA participating in the RecA-mediated strand exchange reactions even though the protein has a low affinity for RNA. The results obtained here with single-stranded RNA or chimeric DNA/RNA oligonucleotides support the supposition that the RecA protein does not form a productive presynaptic filament with RNA. Nonetheless, when RNA is part of a double-stranded nucleic acid, it can participate in strand exchange reactions. This was first shown by Kotani et al. (*48*), who demonstrated that a single-stranded DNA can form a joint molecule with a short chimeric DNA/RNA hairpin. More recently, two groups have shown that RecA-coated dsDNA can exchange with single-stranded DNA or RNA (*49, 50*). The chimeric hairpins studied here provide the first example of strand exchange initiated by an RNA-containing duplex.

The ability of the RecA protein to initiate and complete stable four-strand exchange when the incoming duplex has a chimeric DNA/RNA composition is unexpected. We must therefore consider the possibility that DNA−RNA hybrids may enter into recombination with double-stranded and single-stranded DNA molecules. The reactions we observed with the RecA protein may also be catalyzed by eukaryotic recombinases within mammalian cells, thus providing an explanation for how chimeric double hairpin oligonucleotides can complex with dsDNA. Recent experiments in our laboratory have shown that the yeast Rad51 protein can utilize chimeric DNA/RNA hairpins as substrates for joint molecule formation with dsDNA as long as the hairpin contains a single-stranded DNA overhang and the buffer contains ATP or ATPγS (data not shown).

Four-strand pairing reactions clearly have biological relevance. The partial strand exchange that we observe when using DNA hairpins provides a framework for understanding how RecA protein can homologously align two dsDNA molecules (*6, 8*). We postulate that in the presence of ATP the RecA filament can accommodate two dsDNA molecules, one with high affinity and the other with low affinity. Rapid and reversible binding of the second DNA molecule in conjunction with transient base pair switching rapidly aligns the two duplexes to form a metastable complex. With the molecules now in registry, a single-stranded overhang or gap in one of the molecules could initiate strand exchange. In our study, the RecA protein is unable to form mature joints between DNA hairpins even though these molecules have adjacent ends to permit plectonemic coiling. We conclude that stable recombination between DNA molecules must begin as a three-strand event.

It remains to be determined whether the four-strand exchange reaction described here can take place with a long dsDNA target. Stable accommodation of two duplexes by the RecA filament requires both high Mg^{2+} and ATPγS. Under these conditions, it has been suggested that the two duplexes may be held so tightly that the scanning required for homologous alignment might be extremely slow (*9*). This view, however, does not reconcile with our observation that four-strand exchange between two hairpins can occur in the presence of a large excess of heterologous single-stranded or double-stranded DNA (data not shown). Experiments are underway to test the suitability of plasmid DNA as a substrate

in the four-strand exchange reaction as well as to more fully delineate the mechanism of this novel strand exchange reaction.

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REFERENCES

1. Camerini-Otero, R. D., and Hsieh, P. (1995) *Annu. Rev. Genet. 29*, 509−592.
2. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol. 56*, 129−223.
3. Bianco, P. R., Tracy, R. B., and Kowalczykowski, S. C. (1998) *Front. Biosci. 3*, 570−603.
4. Conley, E. C., and West, S. C. (1989) *Cell 56*, 987−995.
5. Conley, E. C., and West, S. C. (1990) *J. Biol. Chem. 265*, 10156−10163.
6. Chiu, S. K., Wong, B. C., and Chow, S. A. (1990) *J. Biol. Chem. 265*, 21262−21268.
7. Lindsley, J. E., and Cox, M. M. (1990) *J. Biol. Chem. 265*, 10164−10171.
8. Chow, S. A., Chiu, S. K., and Wong, B. C. (1991) *Biochimie 73*, 157−161.
9. Zaitsev, E. N., and Kowalczykowski, S. C. (1999) *J. Mol. Biol. 287*, 21−31.
10. Gamper, H. B., Cole-Strauss, A., Metz, R., Parekh, H., Kumar, R., and Kmiec, E. B. (2000) *Biochemistry 39*, 3808−5816.
11. Cassuto, E., West, S. C., Mursalim, J., Conlon, S., and Howard-Flanders, P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3962−3966.
12. Cunningham, R. P., DasGupta, C., Shibata, T., and Radding, C. M. (1980) *Cell 20*, 223−235.
13. West, S. C., Cassuto, E., and Howard-Flanders, P. (1982) *Mol. Gen. Genet. 187*, 209−217.
14. DasGupta, C., Wu, A. M., Kahn, R., Cunningham, R. P., and Radding, C. M. (1981) *Cell 25*, 507−516.
15. West, S. C., Cassuto, E., and Howard-Flanders, P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2100−2104.
16. Bianchi, M., Riboli, B., and Magni, G. (1985) *EMBO J. 4*, 3025−3030.
17. Kmiec, E. B. (1999) *Gene Ther. 6*, 1−3.
18. Cole-Strauss, A., Yoon, K., Xiang, Y., Byrne, B. C., Rice, M. C., Gryn, J., Holloman, W. K., and Kmiec, E. B. (1996) *Science 273*, 1386−1389.
19. Rosselli, W., and Stasiak, A. (1991) *EMBO J. 10*, 4391−4396.
20. Adzuma, K. (1992) *Genes Dev. 6*, 1679−1694.
21. Di Capua, E., Engel, A., Stasiak, A., and Koller, T. (1982) *J. Mol. Biol. 157*, 87−103.
22. Zlotnick, A., Mitchell, R. S., Steed, R. K., and Brenner, S. L. (1993) *J. Biol. Chem. 268*, 22525−22530.
23. Jayasena, V. K., and Johnston, B. H. (1993) *J. Mol. Biol. 230*, 1015−1024.
24. Sena, E. P., and Zarling, D. A. (1993) *Nat. Genet. 3*, 365−372.
25. Register, J. C., and Griffith, J. (1985) *J. Biol. Chem. 260*, 12308−12312.
26. Shaner, S. L., and Radding, C. M. (1987) *J. Biol. Chem. 262*, 9211−9219.
27. Lindsley, J. E., and Cox, M. M. (1989) *J. Mol. Biol. 205*, 695−711.
28. Ogawa, T., Wabiko, H., Tsurimoto, T., Horii, T., Masukata, H., and Ogawa, H. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 909−915.
29. Kirkpatrick, D. P., Rao, B. J., and Radding, C. M. (1992) *Nucleic Acids Res. 20*, 4339−4346.
30. Nishinaka, T., Ito, Y., Yokoyama, S., and Shibata, T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 6623−6628.
31. Bazemore, L. R., Takahashi, M., and Radding, C. M. (1997) *J. Biol. Chem. 272*, 14672−14682.
32. Gumbs, O. H., and Shaner, S. L. (1998) *Biochemistry 37*, 11692−11706.
33. Yu, X., and Egelman, E. H. (1997) *Nat. Struct. Biol. 4*, 101−104.
34. Waksman, G., Lanka, E., and Carazo, J. M. (2000) *Nat. Struct. Biol. 7*, 20−22.
35. Shan, Q., and Cox, M. M. (1998) *Mol. Cell 1*, 309−317.
36. Gupta, R. C., Folta-Stogniew, E., O'Malley, S., Takahashi, M., and Radding, C. M. (1999) *Mol. Cell 4*, 705−714.
37. Egelman, E. H., and Stasiak, A. (1986) *J. Mol. Biol. 191*, 677−697.
38. Kiianitsa, K., and Stasiak, A. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 7837−7840.
39. Voloshin, O. N., and Camerini-Otero, R. D. (1997) *Genes Cells 2*, 303−314.
40. Nishinaka, T., Shinohara, A., Ito, Y., Yokoyama, S., and Shibata, T. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 11071−11076.
41. Bertucat, G., Lavery R., and Prevost, C. (1999) *Biophys. J. 77*, 1562−1576.
42. Chiu, S. K., Rao, B. J., Story, R. M., and Radding, C. M. (1993) *Biochemistry 32*, 13146−13155.
43. Baliga, R., Singleton, J. W., and Dervan, P. B. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10393−10397.
44. Podyminogin, M. A., Meyer, R. B., and Gamper, H. B. (1995) *Biochemistry 34*, 13098−13108.
45. Zhou, X., and Adzuma, K. (1997) *Biochemistry 36*, 4650−4661.
46. Takahashi, M., Kubista, M., and Norden, B. (1989) *J. Mol. Biol. 205*, 137−147.
47. Muller, B., Koller, T., and Stasiak, A. (1990) *J. Mol. Biol. 212*, 97−112.
48. Kotani, H., Germann, M. W., Andrus, A., Vinayak, R., Mulla, B., and Kmiec, E. B. (1996) *Mol. Gen. Genet. 250*, 626−634.
49. Kasahara, M., Clikeman, J. A., Bates, D. B., and Kogoma, T. (2000) *Genes Dev. 14*, 360−365.
50. Zaitsev, E. N., and Kowalczykowski, S. C. (2000) *Genes Dev. 14*, 740−749.

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