The Synaptic Complex of RecA Protein Participates in Hybridization and Inverse Strand Exchange Reactions†

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ABSTRACT: RecA protein catalyzes strand exchange between homologous single-stranded and double-stranded DNAs. In the presence of ATPγS, the post-strand exchange synaptic complex is a stable end product that can be studied. Here we ask whether such complexes can hybridize to or exchange with DNA, 2′-OMe RNA, PNA, or LNA oligonucleotides. Using a gel mobility shift assay, we show that the displaced strand of a 45 bp synaptic complex can hybridize to complementary oligonucleotides with different backbones to form a four-stranded (double D-loop) joint that survives removal of the RecA protein. This hybridization reaction, which confirms the single-stranded character of the displaced strand in a synaptic complex, might initiate recombination-dependent DNA replication if it occurs in vivo. We also show that either strand of the heteroduplex in a 30 bp synaptic complex can be replaced with a homologous DNA oligonucleotide in a strand exchange reaction that is mediated by the RecA filament. Consistent with the important role that deoxyribose plays in strand exchange, oligonucleotides with non-DNA backbones did not participate in this reaction. The hybridization and strand exchange reactions reported here demonstrate that short synaptic complexes are dynamic structures even in the presence of ATPγS.

Genetic recombination is usually initiated as a three-strand event catalyzed within a RecA or Rad51 nucleoprotein filament. In the conventional pathway, single-stranded DNA (ssDNA)[1] first associates with recombinase to form a presynaptic filament (*1*, *2*). Within this filament, ssDNA occupies the primary binding site of the recombinase. A secondary binding site accommodates double-stranded DNA (dsDNA) in such a way that its sequence can be rapidly scanned for homology by the resident single strand (*3*−*8*). The recombinase energetically activates the dsDNA by extending its backbone 1.5-fold relative to B-form DNA (*4*, *9*−*11*). Homologous alignment of the two DNA molecules and subsequent strand exchange are believed to occur through a concerted rotation of base triples within a common plane (*12*, *13*). At the conclusion of strand exchange, the heteroduplex and displaced strand of the synaptic complex are located in the primary and secondary binding sites of the RecA filament, respectively (*8*, *14*−*18*).

The extended structure of dsDNA that is bound to a recombinase facilitates several unconventional strand exchange reactions. For example, the inverse of regular strand exchange occurs when dsDNA occupies the primary binding site and ssDNA or ssRNA occupies the secondary binding site of a RecA or Rad51 filament (*19*−*21*). If dsDNAs occupy both binding sites of the filament, reciprocal four-strand exchange between the two duplexes can sometimes be observed (*22*, *23*). However, for reasons not entirely understood, four-strand exchange is usually more robust when a DNA−RNA duplex resides in the primary binding site. Exchange in trans occurs when ssDNA in the primary binding site is heterologous to dsDNA in the secondary binding site of the filament (*24*). Under these conditions, the dsDNA can exchange with a homologous ssDNA that is free in solution.

Analysis of joint molecules formed by RecA protein in the presence of ATPγS has provided insight into the structure of the post-strand exchange synaptic complex. ATPγS is a slowly hydrolyzable analogue of ATP. Joint molecules formed in its presence are not released from the RecA filament (*16*, *25*, *26*). Under these conditions, joints formed in the middle of a dsDNA substrate by a homologous ssDNA appear to have a novel D-loop-like structure in which the heteroduplex is separated from the displaced strand (*27*−*31*). The RecA protein remains tightly associated with the heteroduplex, stabilizing what would otherwise be a labile intermediate. This has led to the perception that synaptic complexes formed in the presence of ATPγS are stable, relatively static structures despite the high-energy state of the heteroduplex product and the single-stranded character of the displaced strand. The results presented here show otherwise.

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[1] Abbreviations: ON, oligonucleotide; ODN, oligodeoxynucleotide; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; LNA, locked nucleic acid; PNA, peptide nucleic acid; ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate.

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We investigated whether the displaced strand in a RecA synaptic complex can hybridize to complementary oligonucleotides (ONs) synthesized with different backbones and found that hybridization occurred as long as certain restrictions on length and type of backbone were avoided. ONs with an RNA-like backbone were the most efficient, yielding complement-stabilized joints that might function as origins of replication. The shortest synaptic complex in this study was also susceptible to novel inverse strand exchange reactions in which either strand of the heteroduplex could be replaced with a homologous oligodeoxynucleotide (ODN). Noting that every ATPγS-dependent activity of RecA protein also occurs in the presence of ATP (*24*), it is likely that the hybridization and strand exchange reactions described here also take place in the presence of ATP and with other recombinases.

MATERIALS AND METHODS

Nucleic Acid Substrates. Oligonucleotides (ONs) with DNA, 2′-OMe RNA, or phosphorothioate backbones were purchased from Integrated DNA Technology (Coralville, IA). Locked nucleic acids (LNAs) were provided by Proligo (Boulder, CO). Peptide nucleic acids (PNAs) were synthesized on an Expedite 8909 synthesizer (PE Biosystems, Foster City, CA) using PNA monomers and other reagents obtained from PE Biosystems. Radiolabeled duplexes were prepared by annealing complementary ODNs, one of which was 5′-labeled with ^{32}P. After electrophoresis in a nondenaturing 12% polyacrylamide gel, the double-stranded product was recovered from a gel slice by shaking overnight at 4 °C in Tris-EDTA buffer and then further purified by being passed through a Sep-Pak light C18 cartridge (Waters Corp.). After being dried in a Speed-Vac (ThermoSavant, Holbrook, NY), the dsDNA was dissolved in water and stored at −20 °C. Prior to use, a fresh aliquot of the dsDNA stock was diluted in strand exchange buffer (see below) and incubated for at least 15 min at room temperature. Under these conditions, electrophoretic analysis confirmed that the DNA was double-stranded.

Strand Exchange and Hybridization Reactions. Unless otherwise noted, the following protocol was followed. A single-stranded "incoming" ODN (0.8 pmol in molecules, 16−46 bases in length) was incubated for 10 min at 37 °C with 25 pmol of RecA protein (nuclease free; U.S. Biochemical, Cleveland, OH) in the presence of strand exchange buffer [1 mM ATPγS, 1 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 25 mM Tris-OAc buffer (pH 7.15)]. Exchange was initiated by adding homologous radiolabeled dsDNA (67−70 bp in length, 0.2 pmol in molecules) in strand exchange buffer with extra $Mg(OAc)_2$ so that once the contents were mixed the concentration was increased to 10 mM. RecA-stabilized D-loop or Y-arm joints were formed by using incoming ODNs that were homologous to the middle or the end the dsDNA substrate. After 10 min at 37 °C, 6.4 pmol of an "annealing" ON was added. This ON was hybridized to the displaced "outgoing" strand of the synaptic complex for 10 min at 37 °C, after which the reaction mixture (10−12 μL in volume) was cooled in an ice bath, mixed with 0.1 volume of 10% SDS, and stored at −20 °C. If the amount of an annealing ODN was reduced to 0.8 pmol, the yield of the complement-stabilized joint dropped by 28%.

Immediately prior to electrophoretic analysis, samples were thawed in an ice bath and spiked with tracking dyes and ficoll. Aliquots were loaded onto a 12% polyacrylamide gel that contained 1 mM $MgCl_2$. After electrophoresis in a cold room, the gel was vacuum-dried onto heated filter paper. Radioactive bands were detected by autoradiography. Images acquired on a Storm phosphorimager (Molecular Dynamics, Sunnyvale, CA) were analyzed using ImageQuant software.

The concentrations of reactants described here were optimized for complement-stabilized joint molecule formation. A 2−3-fold molar excess of RecA protein was used to drive presynaptic filament formation and strand exchange by short incoming ODNs. Hybridization to the displaced strand of a synaptic complex was also driven by employing a high concentration of annealing ON. This concentration precluded interference by free RecA protein when using annealing ONs with a DNA backbone.

RESULTS

Model Systems. In the presence of ATPγS, RecA protein catalyzes exchange between homologous single-stranded and double-stranded ODNs but is unable to resolve the resulting synaptic complexes. We have taken advantage of this fact to study the interaction of short synaptic complexes with single-stranded ONs. A major advantage in working with oligomeric substrates is the ability to use polyacrylamide gel electrophoresis to resolve deproteinized intermediates. By analyzing such joints at low temperatures in the presence of $MgCl_2$, we have been able to characterize novel hybridization and strand exchange reactions that take place within the RecA synaptic complex.

The two model systems we have used are described in Figure 1. The first panel summarizes the ON substrates used to form internal joints. The sequence of the 70 bp target duplex was taken from the neomycin phosphotransferase gene. Aligned below it are the single-stranded ONs used in forming three-stranded and four-stranded joints. Similar information is presented in the second panel for substrates used in forming joints at the end of a dsDNA. In this case, the sequence of the 67 bp target was taken from the human β-globin gene.

We have extended the nomenclature first used by Adzuma (*27*) to functionally identify the strands involved in recombination and hybridization. Incoming ODNs initiate homologous pairing and strand exchange with a double-stranded substrate after presynaptic filament formation with RecA protein. The "recipient" and outgoing strands of the duplex are, respectively, complementary and homologous to the incoming ODN. Upon strand exchange, the RecA filament stabilizes a nascent joint (the synaptic complex) in which the incoming ODN is hybridized to the recipient strand of the target duplex and the outgoing (or displaced) strand is unpaired. RecA protein does not catalyze strand exchange by ONs with highly modified backbones (data not shown).

We have used both sense and antisense ONs to probe synaptic complexes. In these experiments, annealing ONs are complementary to both the incoming ODN and the outgoing (or displaced) strand of a synaptic complex. These ONs could possibly hybridize to the outgoing strand or exchange with the recipient strand of a synaptic complex. "Complementary" ODNs are homologous in sequence but

A. NEOMYCIN PHOSPHOTRANSFERASE

N_{12}GGGTGGAGAGGCTATTCGGCTACGACTGGGCACAACAGACAATCGGN_{12}-3' dsDNA$_{70}$
N_{12}CCCACCTCTCCGATAAGCCGATGCTGACCCGTGTTGTCTGTTAGCCN_{12}-5'
ATTCGGCTACGACTGGGCAC kDNA$_{20w}$
GCTATTCGGCTACGACTGGGCACAA kDNA$_{25w}$
AGGCTATTCGGCTACGACTGGGCACAACAG kDNA$_{30w}$
GGTGGAGAGGCTATTCGGCTACGACTGGGCACAACAGACAATCGG kDNA$_{45w}$
GATGCTGAC kLNA$_{9c}$
CGATGCTGACC kLNA$_{11c}$
CCGATGCTGACCC kLNA$_{13c}$
GCCGATGCTGACCCG kDNA$_{15c}$,kDNAS$_{15c}$,kLNA$_{15c}$,kPNA$_{15c}$
GCCGAUGCUGACCCG kRNA$_{15c}$
AAGCCGATGCTGACCCGT kPNA$_{18c}$
TAAGCCGATGCTGACCCGTG kDNA$_{20c}$
CGATAAGCCGATGCTGACCCGTGTT kDNA$_{25c}$
CGAUAAGCCGAUGCUGACCCGUGUU kRNA$_{25c}$
TCCGATAAGCCGATGCTGACCCGTGTTGTC kDNA$_{30c}$
UCCGAUAAGCCGAUGCUGACCCGUGUUGUC kRNA$_{30c}$
CCTCTCCGATAAGCCGATGCTGACCCGTGTTGTCTGTTAG kDNA$_{40c}$
CCUCUCCGAUAAGCCGAUGCUGACCCGUGUUGUCUGUUAG kRNA$_{40c}$
N_2CCCACCTCTCCGATAAGCCGATGCTGACCCGTGTTGTCTGTTAGCCN_2 kDNA$_{50c}$
N_2CCCACCUCUCCGAUAAGCCGAUGCUGACCCGUGUUGUCUGUUAGCCN_2 kRNA$_{50c}$
N_{12}CCCACCTCTCCGATAAGCCGATGCTGACCCGTGTTGTCTGTTAGCCN_{12} kDNA$_{70c}$
N_{12}CCCACCUCUCCGAUAAGCCGAUGCUGACCCGUGUUGUCUGUUAGCCN_{12} kRNA$_{70c}$

B. GLOBIN

N_{21}CTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGC-3' dsDNA$_{67}$
N_{21}GAGTTTGTCTGTGGTACCACGTGGACTGAGGACTCCTCTTCAGACG-5'
TGAGGAGAAGTCTGC gDNA$_{15w}$
ACTCCTGAGGAGAAGTCTGC gDNA$_{20w}$
ACCTGACTCCTGAGGAGAAGTCTGC gDNA$_{25w}$
ACCUGACUCCUGAGGAGAAGUCUGC gRNA$_{25w}$
GGTGCACCTGACTCCTGAGGAGAAGTCTGC gDNA$_{30w}$
ACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGC gDNA35w
CAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGC gDNA$_{40w}$
CTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGC gDNA$_{46w}$
CTCAAACAGACACCATGGTGCACCTGACTCCTGAGGAGAAGTCTGCN$_{21}$ gDNA$_{67w}$
ACTCCTCTTCAGACG gDNA$_{15c}$
TGAGGACTCCTCTTCAGACG gDMA$_{20c}$
TGGACTGAGGACTCCTCTTCAGACG gDNA$_{25c}$
UGGACUGAGGACUCCUCUUCAGACG gRNA$_{25c}$
CCACGTGGACTGAGGACTCCTCTTCAGACG gDNA$_{30c}$
TGGTACCACGTGGACTGAGGACTCCTCTTCAGACG gDNA$_{35c}$
GTCTGTGGTACCACGTGGACTGAGGACTCCTCTTCAGACG gDNA$_{40c}$
GAGTTTGTCTGTGGTACCACGTGGACTGAGGACTCCTCTTCAGACG gDNA$_{46c}$

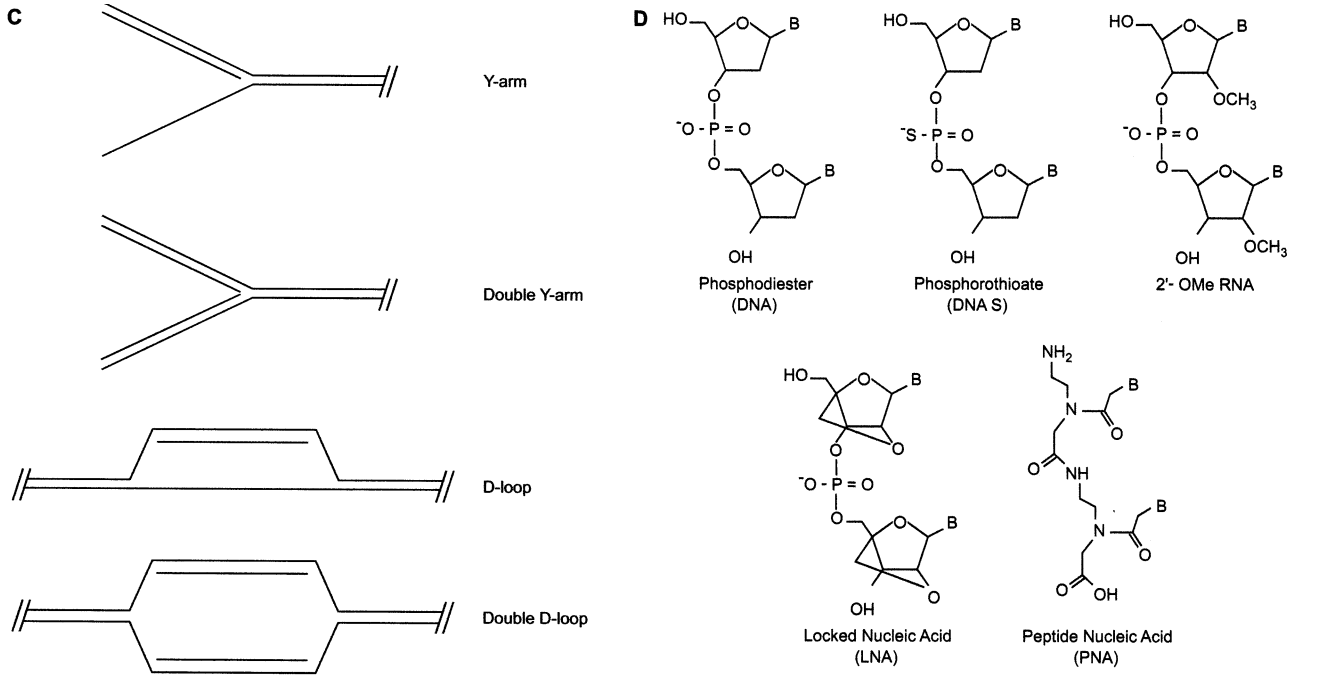

FIGURE 1: Model systems for studying joint molecule formation. (A and B) Sequences of double-stranded DNA targets and single-stranded incoming, annealing, and complementary ONs. The neomycin phosphotransferase system (A) was used to form D-loop and double D-loop joints. The globin system (B) was used to form Y-arm and double Y-arm joints. Watson (w) and Crick (c) strands correspond to the sense and antisense sequences of the parental genes, respectively. The length of each ON is indicated with a subscript. DNAS is DNA with phosphorothioate linkages. RNA is 2′-OMe RNA. N is a nucleotide. The two sets of ONs are differentiated by the prefixes k (kanamycin) and g (globin). (C) Schematic representations of Y-arm, double (complement-stabilized) Y-arm, D-loop, and double (complement-stabilized) D-loop joints. (D) Structures of DNA, DNAS, 2′-OMe RNA, LNA, and PNA backbones.

usually different in length from the incoming ODN. These ODNs could possibly exchange with the incoming ODN that is present in a synaptic complex. Annealing and complementary ONs are not complexed with RecA protein prior to use.

The last two panels of Figure 1 depict the three-stranded and four-stranded joints formed in this study as well as structures of the modified backbones present in some of the annealing ONs. We have used ONs with modified backbones to differentiate between reactions in which the RecA filament plays an active role (such as strand exchange) and those in which it might not (such as hybridization to the displaced strand). Whereas ONs with a DNA backbone should participate in both types of reactions, ONs with a completely modified backbone should only take part in reactions that are not catalyzed by the recombinase. Of the modified ONs used in this study, PNAs contain a neutral peptide backbone while 2′-OMe RNA and LNA ONs possess an RNA-like backbone. In LNA, each sugar residue is locked into the 3′-endo conformation due to a methylene bridge that connects the 2′-oxygen of ribose with the 4′-carbon. This sugar conformation promotes the formation of extremely stable hybrids with complementary DNA or RNA, conferring T_m increases of up to 10 °C per base (*32*, *33*).

Hybridization between Annealing ONs and Synaptic Complexes. To determine whether the displaced strand of a short synaptic complex can hybridize to a complementary annealing ON, we screened multiple reaction protocols using four sets of substrates. The results of this analysis are presented in Figure 2, where nondenaturing polyacrylamide gel electrophoresis was used to determine the yield of three-stranded and four-stranded joints for each protocol and combination of substrates. In the first panel, the synaptic complex stabilized a 30 bp long Y-arm joint, while in the last three panels, it stabilized a D-loop joint either 30 or 45 bp long. Annealing ONs differed with respect to both length and backbone. By comparing the reactions in these panels, we have been able to characterize some of the parameters that influence complement-stabilized joint formation.

Formation of Y-Arm and D-Loop Joints. When an incoming ODN is shorter than the dsDNA target, RecA-mediated strand exchange generates a Y-arm- or D-loop-like structure in which the recipient strand of the dsDNA is hybridized to the ODN and the outgoing strand is left without a partner (see Figure 1C). In the presence of ATPγS, the product of strand exchange remains associated with the RecA filament as a stable synaptic complex (*34*). Upon deproteinization, however, these structures usually resolve by branch migration to release dsDNA and free ODN. As a consequence, SDS treatment of a short synaptic complex is usually accompanied by dissociation of the underlying joint unless it is stabilized by the torsional strain of a supercoiled plasmid (*35*, *36*).

We deproteinized the synaptic complexes of Figure 2 to ascertain whether any three-stranded joint molecules could be detected by a gel mobility shift assay. While none were observed when the synaptic complex contained an incoming DNA 30-mer, two products were obtained when the synaptic complex contained an incoming DNA 45-mer (compare lane 1 in Figure 2A−C with lane 1 in Figure 2D). In the latter case, addition of SDS at 4 °C generated two bands. The faster moving band has not been identified, but the slower moving product (with a yield of 4%) was assigned a D-loop structure on the basis of its comigration with a stable D-loop of similar size. The reference joint was formed by sequential hybridization of three ODNs to give a D-loop joint with heterologous arms (data not shown). We surmise that secondary structure in

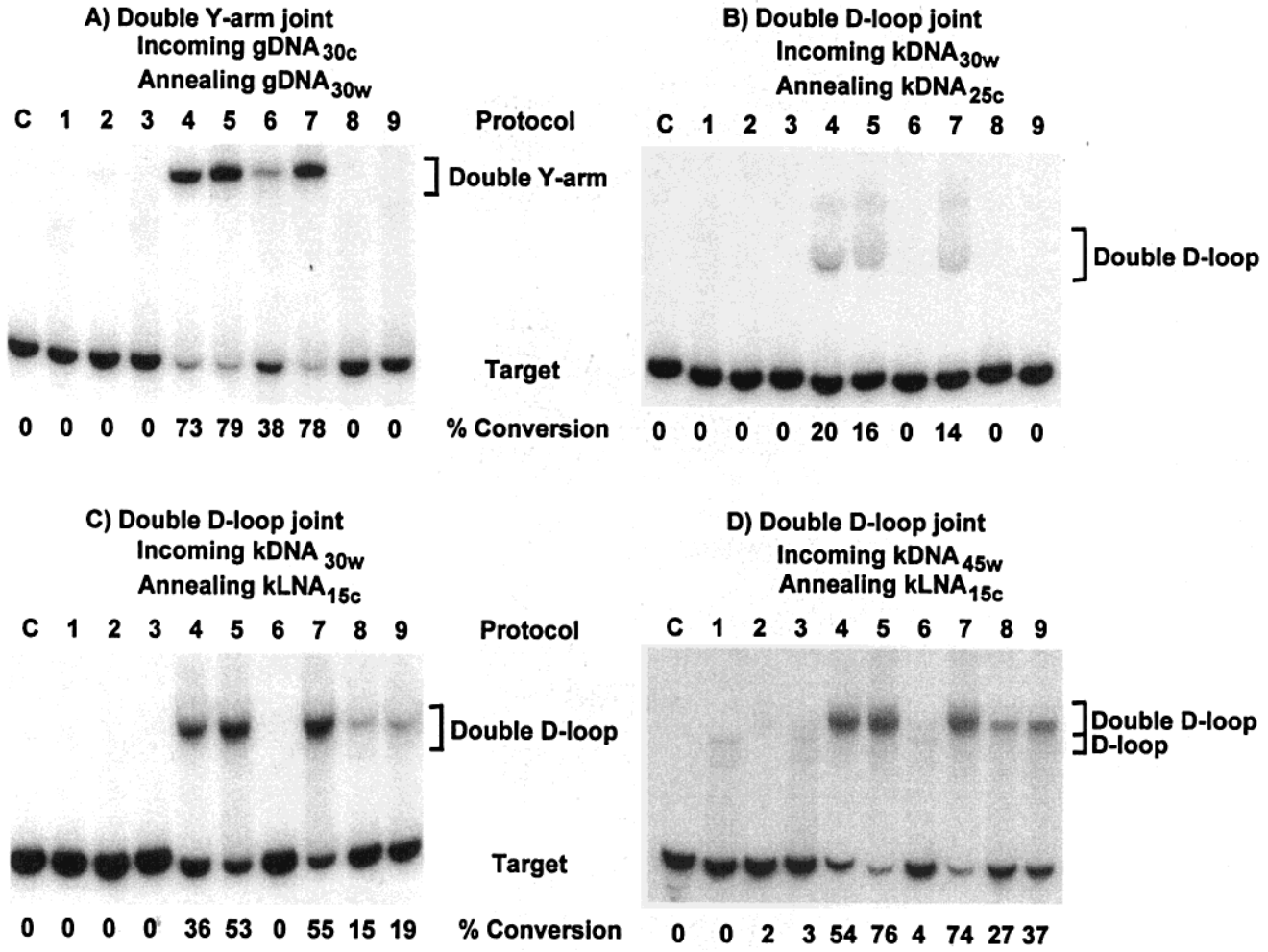

FIGURE 2: Hybridization of ONs to the displaced strand of dsDNA in different post-strand exchange synaptic complexes. RecA-stabilized joints were formed in the middle or at the end of a radiolabeled 70 or 67 bp DNA target using a homologous incoming ODN. The outgoing strand of these complexes was hybridized to a complementary annealing ON to form a complement-stabilized D-loop or Y-arm joint. After denaturation of RecA protein with SDS, an aliquot of each reaction mixture was analyzed by electrophoresis on a nondenaturing 12% polyacrylamide gel run at 4 °C in the presence of 1 mM $MgCl_2$. ONs are denoted by type of backbone and length: (A) double Y-arm joint with incoming $gDNA_{30c}$ and annealing $gDNA_{30w}$, (B) double D-loop joint with incoming $kDNA_{30w}$ and annealing $kDNA_{25c}$, (C) double D-loop joint with incoming $kDNA_{30w}$ and annealing $kLNA_{15c}$, and (D) double D-loop joint with incoming $kDNA_{45w}$ and annealing $kLNA_{15c}$. Processing of three-stranded synaptic complexes: lane 1, addition of SDS at 4 °C; lane 2, addition of annealing ON and SDS at 37 °C; lane 3, addition of annealing ON and SDS at 4 °C; lane 4, incubation for 10 min with annealing ON at 37 °C and then addition of SDS at 37 °C; lane 5, incubation for 10 min with annealing ON at 37 °C and then addition of SDS at 4 °C ("standard protocol"); lane 6, standard protocol, only incubation with annealing ON preceded by the addition of a single-stranded complement (19 pmol; $gDNA_{30c}$ in panel A, $kDNA_{25w}$ in panel B, and $kDNA_{20w}$ panels in C and D); and lane 7, standard protocol, only with the SDS solution containing 19 pmol of the same ODNs listed in the preceding lane. In the reactions analyzed in lanes 8 and 9, equimolar amounts of incoming and annealing ONs were incubated either together or separately with RecA protein prior to mixing with target DNA. After 10 min at 37 °C, these reactions were terminated by addition of SDS at 4 °C. All reaction mixtures were quickly frozen in dry ice and stored at −20 °C while awaiting analysis. Lane C shows the target duplex only. Percent conversion refers to the efficiency of complement-stabilized joint molecule formation.

the displaced strand of a long D-loop joint may retard branch migration. Our failure to detect either three-stranded joint when SDS treatment was carried out at 37 °C (lane 2 of Figure 2D) reflects the instability of these complexes and underscores the lability of Y-arm and D-loop joints.

Formation of Complement-Stabilized Y-Arm and D-Loop Joints. We used the same gel mobility shift assay to ascertain whether the outgoing DNA strand in short synaptic complexes could hybridize to complementary ONs. Hybridization was expected to generate four-stranded structures (i.e., complement-stabilized Y-arm and D-loop joints) of sufficient stability to survive detection by a gel mobility shift assay in the absence of RecA protein. Annealing ONs with different backbones were tested in this assay to determine whether the RecA filament of a synaptic complex influenced their interaction with the displaced strand. In the special case of annealing ONs with a DNA backbone, possible complications due to presynaptic filament formation were considered unlikely due to the high ratio of annealing ON to free RecA protein. Alternative hybridization protocols were tested using synaptic complexes that had been formed at the end (Figure 2A) or in the middle (Figure 2B,C) of a dsDNA substrate. If hybridization occurred, it would provide independent confirmation of the single-stranded character of the outgoing strand in a synaptic complex. The annealing reaction would also provide a way to trap unstable recombination intermediates for subsequent analysis.

Synaptic complexes that had been incubated with an annealing ON were analyzed after treatment at 37 or 4 °C with SDS (lanes 4 and 5 in Figure 2A−D). In every

experiment, we observed a band shift consistent with hybridization of the annealing ON to the outgoing strand of the complex. In Figure 2D, the putative double D-loop joint exhibited reduced mobility relative to the D-loop joint. Confirmation of the four-stranded structure of these products was based on co-electrophoresis of each with a standard joint prepared by sequential hybridization of the same four constituent ONs (data not shown). With the exception of the experiment in Figure 2B, the yield of the complement-stabilized joint was greater when SDS was added at 4 °C. It may be that at the instant of deproteinization the joints are fluid structures prone to dissociation. If the temperature of this step is reduced, more of the joint survives intact.

A comparison of the four experiments in Figure 2 shows great disparity in the yield of the complement-stabilized joint. Employing incoming and annealing ONs of similar length, we formed a double Y-arm joint more readily than a double D-loop joint. With one end of the outgoing strand free to bend outward, the double Y-arm joint electrophoresed more slowly than the double D-loop joint. Formation of complement-stabilized D-loops was improved by forming the synaptic complex with a longer incoming ODN (compare lane 5 of panels C and D of Figure 2) and by switching to an annealing ON with a LNA instead of a DNA backbone (compare lane 5 of panels B and C of Figure 2).

The Displaced Strand of a Synaptic Complex Is Accessible to Annealing ONs. Detection of complement-stabilized Y-arm and D-loop joints raised the question of whether the hybridization reaction occurred within the synaptic complex or at the instant RecA was denatured by SDS. To distinguish between these two possibilities, control experiments were carried out. In the reactions analyzed in lanes 2 and 3 of Figure 2A−D, the synaptic complex was treated with a mixture of annealing ON and SDS at 37 and 4 °C, respectively. Under these conditions, hybridization was either nonexistent (Figure 2A−C) or barely detectable (Figure 2D). It is likely that deproteinization was accompanied by rapid branch migration leading to resolution of the nascent Y-arm or D-loop joint and that the time scale of the intramolecular branch migration reaction was much shorter than that required for an annealing ON to hybridize to the outgoing strand of the joint. Thus, the hybridization observed using the standard protocol (lane 5 of Figure 2A−D) must have taken place within the synaptic complex. This conclusion is supported by experiments in which the standard reaction was terminated by addition of a SDS stop solution fortified with a 3-fold molar excess of an ODN that was complementary to the annealing ON. The modified protocol did not reduce the yield of complement-stabilized joint (compare lanes 5 and 7 of Figure 2A−D).

The Displaced Strand of a Terminal Synaptic Complex Is Not Encumbered by the RecA Filament. Synaptic complexes formed at the end of a dsDNA substrate are different from those formed at an internal site. One major difference appears to be the accessibility of the displaced strand to complementary ONs. Hence, annealing ODNs of similar length hybridized to 30 bp long internal or terminal synaptic complexes with 16 or 79% efficiency (compare lane 5 of panels A and B of Figure 2). In a RecA-stabilized D-loop joint, the outgoing strand is necessarily constrained within the vicinity of the RecA filament, and this probably reduces its accessibility to a complementary ON. In contrast, the free end of the outgoing strand in a RecA-stabilized Y-arm joint could unravel about the nucleoprotein filament, thereby exhibiting properties more characteristic of a free single-stranded DNA. This supposition is supported by the work of Gumbs and Shaner (*35*), who showed that a synaptic complex formed using 30-mer substrates in the presence of ATPγS released the outgoing strand.

Simultaneous Addition of Incoming and Annealing ONs. Jayasena and Johnston (*38*) and Sena and Zarling (*39*) first described RecA-assisted complement-stabilized D-loop formation. In their work, incoming and annealing DNA strands were first complexed with RecA protein and then added together or one after the other to the dsDNA target. The yield of complement-stabilized product was negligible for joints of the length described here. In lanes 8 and 9 of Figure 2A−D, we duplicated their protocol using equimolar amounts of incoming and annealing ONs. In one reaction, the ONs were incubated together with RecA protein (lane 8), and in the other, they were incubated separately with the recombinase (lane 9) prior to adding both to the dsDNA target. Neither protocol yielded a complement-stabilized joint when ODNs were employed (Figure 2A,B). We presume that hybrid formation between incoming and annealing ODNs or uptake of both ODNs into separate RecA filaments interfered with joint molecule formation (*40*, *41*). The simultaneous addition of an incoming ODN and annealing LNA yielded some double D-loop joint by an unknown pathway (Figure 2C,D). In what may be a related reaction, Belotserkovskii and Zarling (*42*) recently reported that simultaneous addition of PNA can augment double D-loop formation by RecA-coated complementary single-stranded DNA probes.

Annealing ONs with Different Backbones. The 3-fold increase in the yield of the complement-stabilized D-loop when using an annealing LNA 15-mer instead of a DNA 25-mer to hybridize to a 30 bp long internal synaptic complex was unexpected (compare lane 5 of panels B and C of Figure 2). While it is known that LNA forms hybrids of unprecedented stability with complementary RNA and DNA (*32*, *33*), the DNA 25-mer that we had used as an annealing agent should also have formed a stable hybrid with the displaced strand of the synaptic complex. Curious about what affect other backbones might have on hybridization efficiency, we synthesized several 15-mer ONs that differed only in the type of backbone. These oligomers were used as annealing agents in reactions with two synaptic complexes, one 30 and the other 45 bp in length. The results are presented in Figure 3.

With the smaller synaptic complex, the type of backbone in the annealing ON greatly affected the yield of the double D-loop joint. Annealing ONs with a LNA or 2′-OMe RNA backbone were good hybridization agents, while ONs with a DNA, phosphorothioate, or PNA backbone were less so. The pattern of complement-stabilized D-loop formation could not be explained in terms of hybrid stability since, for example, a PNA−DNA hybrid is more stable than a 2′-OMe RNA−DNA hybrid. Contrary to our initial expectation, the proximity of the outgoing strand to the RecA filament may in some way favor its hybridization to annealing ONs with an RNA-like backbone.

A completely different pattern of reactivity was observed with the longer synaptic complex. Here all five of the annealing ONs gave improved yields of complement-stabilized D-loop such that the hybridization reaction was

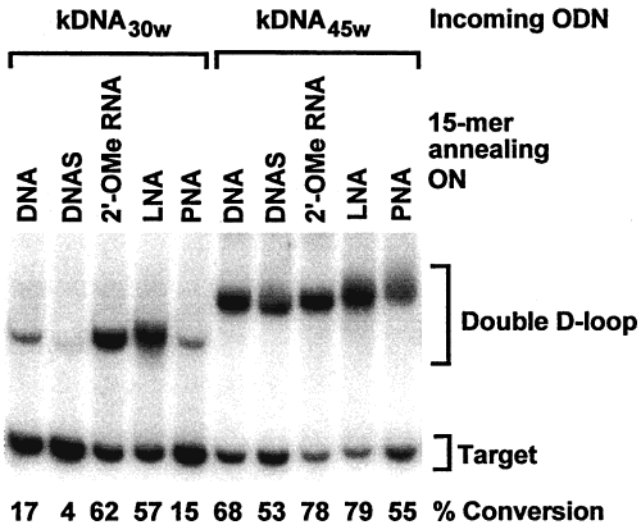

FIGURE 3: Complement-stabilized D-loop formation by annealing ONs with different backbones. Synaptic complexes were formed in the middle of the double-stranded neomycin phosphotransferase target with $kDNA_{30w}$ and $kDNA_{45w}$. The outgoing strand of each joint was hybridized to $kDNA_{15c}$, $kDNAS_{15c}$, $kRNA_{15c}$, $kLNA_{15c}$, or $kPNA_{15c}$.

less influenced by the type of backbone in the ON. In this case, the results were more in line with a simple hybridization reaction, suggesting that the outgoing strand in the longer synaptic complex may be more distant from the RecA filament.

Effect of ON Length on Complement-Stabilized Joint Formation. Since the relative lengths of incoming and annealing ONs might influence the extent of complement-stabilized joint formation, we investigated these relationships by hybridizing annealing ONs of different lengths to synaptic complexes that contained Y-arm or D-loop joints. A good double Y-arm joint yield was obtained using every annealing ON that was tested (Figure 4A). Joints that were 20−46 bp long were formed efficiently using incoming and annealing ODNs of identical lengths. Equally good yields were obtained when the ODNs were of unequal length. An annealing ON with a 2′-OMe RNA backbone was equivalent in hybridization to one with a DNA backbone. Even a long annealing ODN that extended past both ends of the synaptic complex hybridized well to the outgoing strand. A decreased level of joint molecule formation was only observed when using incoming ODNs that were shorter than those required for efficient strand exchange (*36*, *43*). These results are consistent with a structure in which the displaced strand of a terminal synaptic complex is distant from the RecA filament and readily accessible to complementary ONs. Topological issues appear to be nonexistent since the outgoing strand has a free end.

In contrast, hybridization to synaptic complexes flanked on both sides by dsDNA was very dependent upon the length of the annealing ON relative to that of the incoming ODN. Panels B and C of Figure 4 summarize the yield of the double D-loop joint when hybridizing different annealing ONs to synaptic complexes that were either 30 or 45 bp long. The yield of the double D-loop joint was best when the annealing ON was shorter than the incoming ODN. This was true for annealing ONs with DNA, 2′-OMe RNA, or LNA backbones. The extent of hybridization to the outgoing strand of

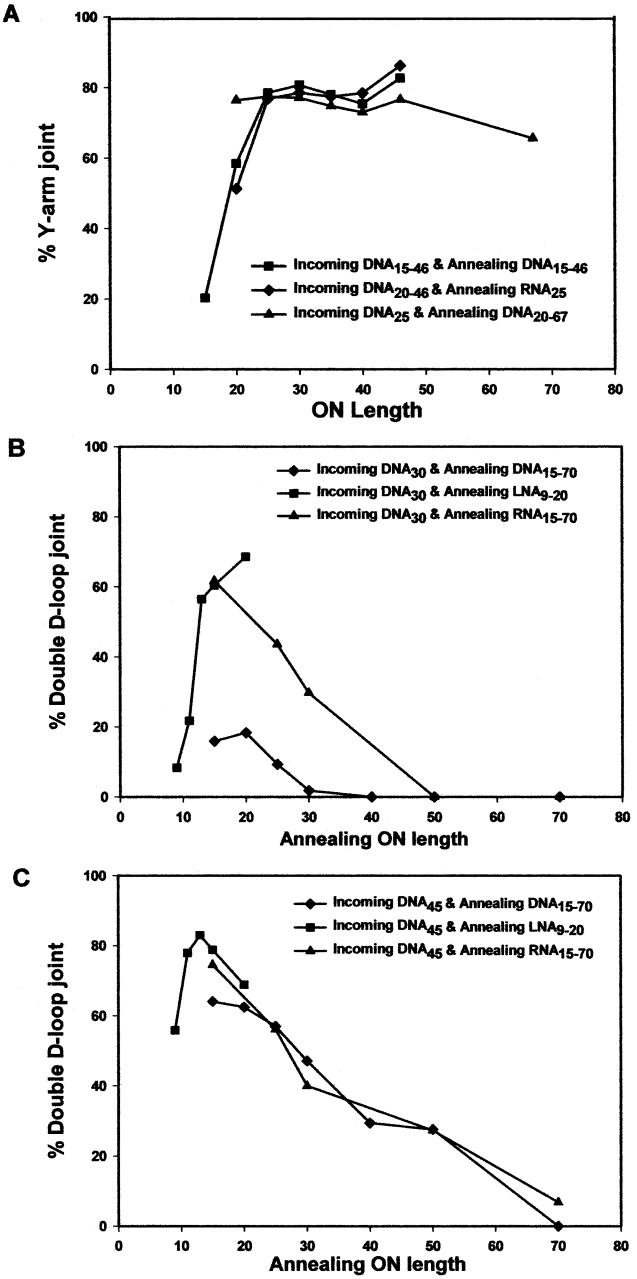

FIGURE 4: Complement-stabilized joint formation by incoming and annealing ONs with different lengths. (A) Synaptic complexes formed at the end of the globin dsDNA fragment were hybridized to annealing ONs as follows: (■) incoming and annealing ODNs identical in length (15−46 bases), (◆) annealing ON fixed ($gRNA_{25w}$) and incoming ODN varied (20−46 bases), and (▲) incoming ODN fixed ($gDNA_{25c}$) and annealing ODN varied (20−67 bases). (B) Synaptic complexes formed by $kDNA_{30w}$ in the middle of the neomycin phosphotransferase dsDNA fragment were hybridized to annealing ONs with the following backbones: (◆) DNA (15−70 bases), (■) LNA (9−20 bases), and (▲) 2′-OMe RNA (15−70 bases). (C) Synaptic complexes formed by $kDNA_{45w}$ in the middle of the neomycin phosphotransferase dsDNA fragment were hybridized to the same annealing ONs as in panel B. All of the ONs are listed in Figure 1.

the two synaptic complexes decreased as the length of the annealing ON was extended beyond an optimal value (i.e., 13−20 bases). The relative hybridization efficiencies of annealing ONs with DNA versus 2′-OMe RNA backbones differed for the two synaptic complexes. With the longer complex, the ONs exhibited similar activities, whereas with the shorter complex, the 2′-OMe RNA ONs were signifi-

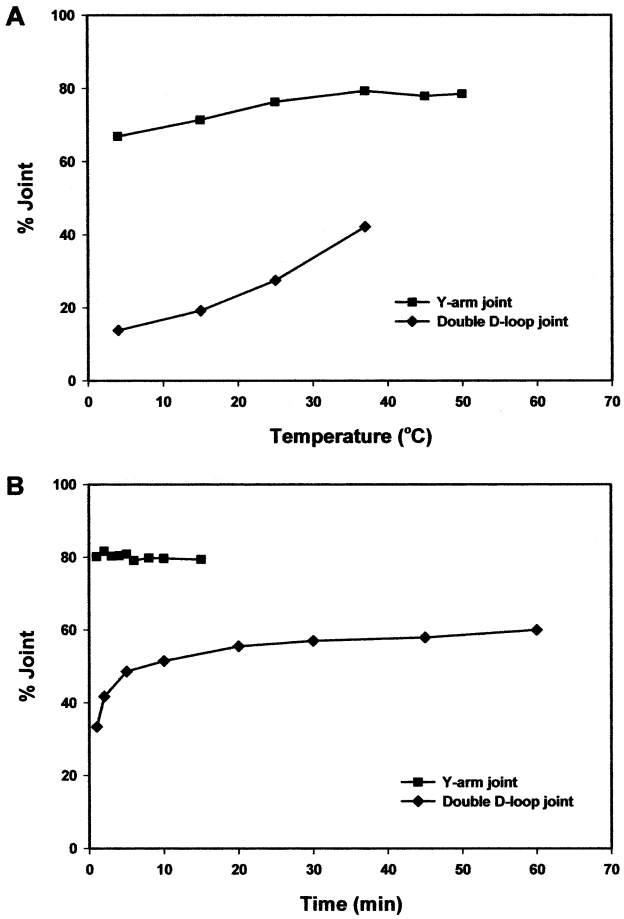

FIGURE 5: Temperature and time dependence of the annealing reaction. Synaptic complexes containing a Y-arm or D-loop joint were formed according to the standard protocol (Y-arm joint, globin target with $gDNA_{30c}$; and D-loop joint, neomycin phosphotransferase target with $kDNA_{30w}$). Complement-stabilized Y-arm (■) or complement-stabilized D-loop (◆) joints were formed by adding $gDNA_{30w}$ or $kLNA_{15c}$ to the respective synaptic complexes at time zero. The annealing reactions were conducted (A) at different temperatures for 5 min or (B) at 37 °C for different times.

cantly more active. Recognizing that the outgoing strand in a RecA-associated D-loop is constrained within a nucleoprotein filament, we find it is likely that hybridization of annealing ONs to the outgoing strand is hindered by its proximity to the RecA filament and by the absence of a free end. Neither of these possibilities, however, accounts for the difference in hybridization efficiency between the two types of annealing ONs. Similarly, binding of RecA protein to an annealing ODN would be expected to inhibit hybridization to both synaptic complexes and not just one.

Time and Temperature Dependence of the Annealing Reaction. Our results indicate that synaptic complexes containing Y-arm and D-loop joints differ significantly in the accessibility of the displaced strand to annealing ONs. To further substantiate this conclusion, we compared the time and temperature dependence of hybridization between the annealing ON and displaced strand for the two types of synaptic complexes. Figure 5A shows the amount of double Y-arm or double D-loop joint formed when hybridizations were conducted at different temperatures with RecA-stabilized Y-arm or D-loop joints. If the synaptic complex was located in the middle of the dsDNA target, the extent of hybridization decreased by 67% when the temperature was lowered from 37 to 4 °C. If, however, the synaptic complex was at the end of the dsDNA, the extent of hybridization decreased by only 16% over the same temperature range. The kinetics of hybridization were also different for the two synaptic complexes (Figure 5B). Formation of the complement-stabilized Y-arm joint was complete in much less than 1 min, whereas formation of the complement-stabilized D-loop joint was still proceeding after 60 min. Interference by secondary structure was ruled out since the same hybridization reaction was extremely rapid when the synaptic complex was replaced with the 70-mer outgoing strand (data not shown). In lieu of other reasonable explanations, we again conclude that the outgoing strand of the internal synaptic complex is rendered less accessible due to the proximity to the RecA filament.

The Displaced Strand of an Internal Synaptic Complex Is Not Uniformly Accessible to Annealing ODNs. Within an internal synaptic complex, the RecA filament stabilizes an otherwise unstable joint by preventing branch migration. Interaction of RecA with the two three-way junctions that bracket the joint might be one way to block such reactions (*30*). If so, accessibility of the displaced strand to annealing ONs might be decreased in the vicinity of the junctions. To test whether this is so, we prepared two synaptic complexes with the 70 bp DNA target, one 30 and the other 45 bp in length. Each complex was separately hybridized to a series of short annealing ONs that spanned the length of the displaced strand. The ONs were synthesized with a LNA backbone and were either 15 or 10 bases in length. The results, summarized in Figure 6, demonstrate that accessibility to the outgoing strand was not equal over its length within the synaptic complex. The central portion had the greatest accessibility, while the junction regions had the least. In both complexes, the 5′-half of the displaced strand was somewhat more accessible to annealing ODNs than the 3′-half. These results are consistent with the hypothesis that RecA protein may preferentially interact with the displaced strand at or near the three-way junctions that bracket an internal synaptic complex.

Reversal of Joint Molecule Formation by an Annealing ON with a DNA Backbone. In Figure 4B, we show that annealing ONs with a LNA or 2′-OMe RNA backbone were more effective than ONs with a DNA backbone in hybridizing to the displaced strand of a short synaptic complex. In light of these results, we investigated whether an annealing ON with a DNA backbone could interfere with or reverse formation of a double D-loop joint by an annealing ON with a 2′-OMe RNA backbone. Annealing ONs, either 15 or 30 bases long and bearing either a DNA or 2′-OMe RNA backbone, were added separately or in combination to an internal synaptic complex that was 30 bases in length. Figure 7 shows that the outgoing strand of this complex hybridized to 2′-OMe RNA ONs that were 15 or 30 nucleotides in length (R_{15} or R_{30}) or to a DNA ON that was 15 nucleotides in length (D_{15}). The outgoing strand, however, did not hybridize to a DNA ON that was 30 nucleotides in length (D_{30}). These control experiments confirmed the results depicted in Figure 4B.

We next determined how the yield of the double D-loop joint was altered when two annealing ONs were added simultaneously or sequentially to the synaptic complex. The R_{15}/R_{30} combinations were revealing because the gel mobility

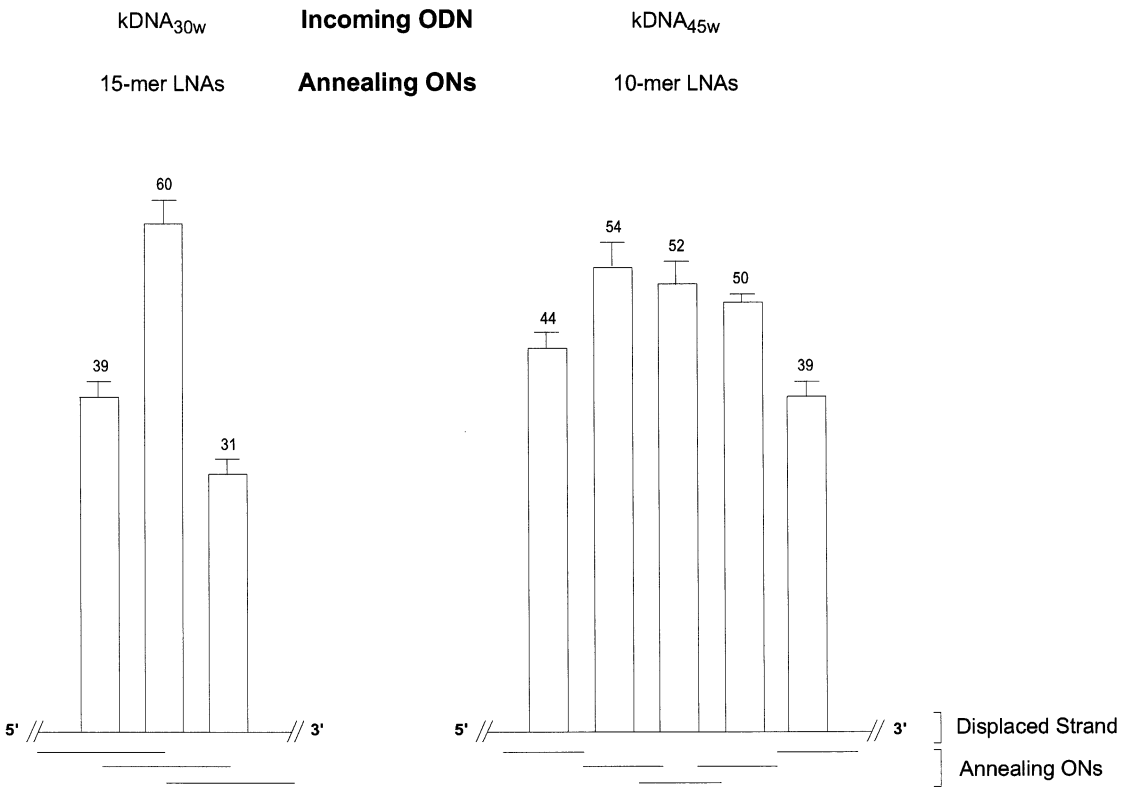

FIGURE 6: Hybridization of overlapping ONs to the outgoing strand of synaptic complexes. Synaptic complexes were formed in the middle of the neomycin phosphotransferase dsDNA fragment using $kDNA_{30w}$ or $kDNA_{45w}$ as incoming ODNs. The outgoing strand of each complex was separately hybridized to a series of overlapping LNA ONs, 15-mers in the case of the 30-base complex and 10-mers in the case of the 45-base complex. After deproteinization, the amount of double D-loop joint was determined by a gel mobility shift assay. Annealing ONs used in these experiments are aligned by complementarity below that portion of the outgoing strand that is displaced in the synaptic complex. The percent yield of the double D-loop joint is averaged from two or more determinations.

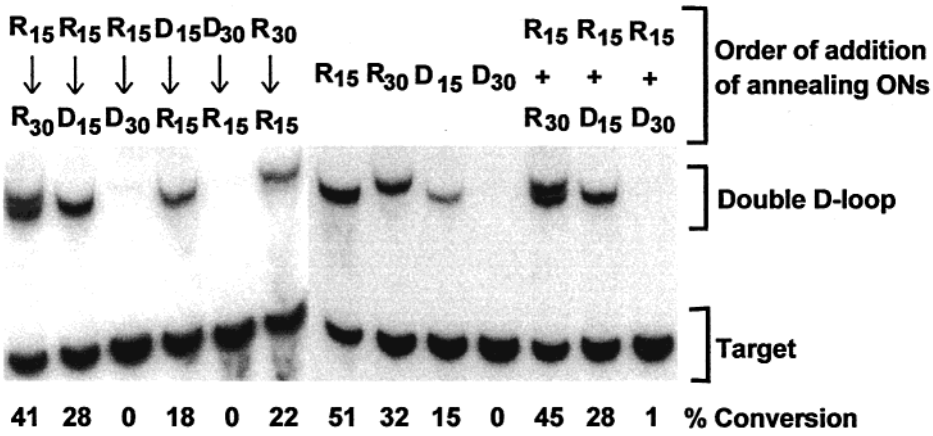

FIGURE 7: Inverse strand exchange with the recipient strand of a synaptic complex. An internal synaptic complex containing $kDNA_{30w}$ as the incoming strand was incubated with the following annealing ONs alone or in combination: $kDNA_{15c}$ (D_{15}), $kDNA_{30c}$ (D_{30}), $kRNA_{15c}$ (R_{15}), and $kRNA_{30c}$ (R_{30}). Reaction mixtures with two annealing ONs contained 6.4 pmol of each. The ONs were added simultaneously (+) or sequentially (→) to the synaptic complex. When the ONs were added sequentially, the first ON added (above arrow) was present for 20 min and the second ON added (below arrow) was present for 10 min prior to stopping the reaction with SDS. Otherwise, all other annealing reactions were carried out for 10 min.

shift assay could resolve the two double D-loop joints. When R_{30} was added first to the synaptic complex, relatively little double D-loop joint was formed by R_{15}, indicating that the annealing reaction was nearly complete after the 10 min incubation with the first ON. When the order of addition was reversed, nearly equal amounts of the two joints were obtained, suggesting that R_{30} had replaced R_{15} in some of the double D-loop joints by strand displacement. The R_{15}/D_{15} combinations were less informative since the two double D-loop joints could not be resolved. The low joint yield when D_{15} was added after R_{15} suggests that some of the double D-loop joint formed by R_{15} was dissociated in the presence of D_{15}.

All three R_{15}/D_{30} combinations were inactive in forming a complement-stabilized D-loop joint regardless of whether the annealing ONs were added simultaneously or sequentially. By assigning dual reactivities to annealing ODNs, we can rationalize their effects. When such an ODN is short relative to the synaptic complex, it can hybridize to the displaced strand to give a double D-loop joint. However, as

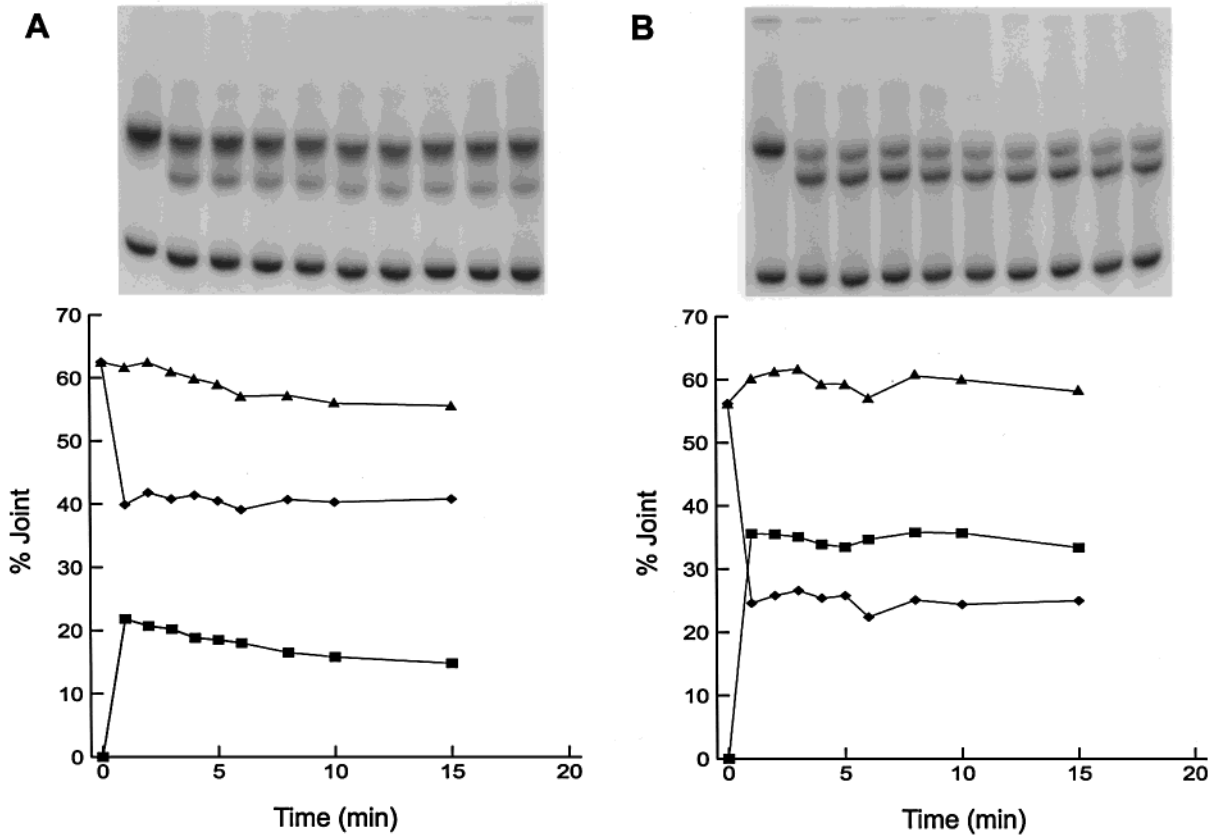

FIGURE 8: Inverse strand exchange with the incoming strand of a synaptic complex. Two RecA-stabilized double D-loop joints were formed according to the standard protocol using $kDNA_{30w}$ as the incoming ODN and either (A) $kLNA_{15c}$ or (B) $kPNA_{18c}$ as the annealing ON. At time zero, 19 pmol of $kDNA_{20w}$ or $kDNA_{25w}$ was added to the joints in gel A or B, respectively. Incubations were continued for 15 min at 37 °C with aliquots removed at the indicated times for subsequent analysis by gel mobility shift analysis. In the autoradiograms, the product of inverse strand exchange runs as a band with intermediate mobility in lanes 2−10: (◆) double D-loop joint with $kDNA_{30w}$, (■) double D-loop joint with (A) $kDNA_{20w}$ or (B) $kDNA_{25w}$, and (▲) total double D-loop joint.

the length of the ODN is increased, this reaction becomes less likely and the ODN preferentially exchanges position with the recipient strand of the heteroduplex. This inverse strand exchange reaction, which is catalyzed by the RecA filament of the synaptic complex, dissociates the heteroduplex by hybridizing annealing and incoming ODNs to one another. As a consequence, a D-loop joint is reversed or a double D-loop joint is converted back to a D-loop joint. In either case, after deproteinization no stable joint remains to be detected. In contrast to D_{30}, R_{30} does not participate in inverse strand exchange and so supports double D-loop formation. The unique ability of annealing ONs with a DNA backbone to exchange with the recipient strand of a synaptic complex explains why these ONs are less effective than those with an RNA-like backbone in generating complement-stabilized D-loop joints.

The Incoming ODN of a Synaptic Complex Can Participate in Inverse Strand Exchange. We next asked whether the incoming strand of a short synaptic complex could undergo inverse strand exchange. For this purpose, we formed complement-stabilized D-loop joints according to the standard protocol using an incoming DNA 30-mer and an annealing LNA 15-mer or PNA 18-mer. Prior to adding SDS, we treated each synaptic complex with a shorter version of the original incoming ODN (i.e., a complementary DNA 20- or 25-mer). Each of these ODNs converted some of the RecA-stabilized double D-loop joint into a new product, with the 25-mer being more effective than the 20-mer (Figure 8). The reactions were complete in less than 1 min, implying that only a fraction of the synaptic complex was susceptible to modification. While the new joint formed by the 25-mer was stable, that containing the 20-mer slowly dissociated. We propose that each of the complementary ODNs has exchanged positions with the incoming ODN of the heteroduplex in a reaction catalyzed by the RecA filament of the synaptic complex. Strand exchange is accompanied by partial collapse of the joint, thereby maintaining the total number of base pairs (see Figure 9C). Increased mobility of the resulting joints is consistent with replacement of the incoming ODN in the heteroduplex with a smaller ODN (see Figure 8). ONs with a 2′-OMe RNA backbone as well as RecA-stabilized double Y-arm joints were not substrates for the inverse strand exchange reaction (results not shown).

DISCUSSION

Annealing ONs Can Hybridize to the Displaced Strand of a RecA Synaptic Complex. It is well-established that RecA protein catalyzes strand exchange between homologous single-stranded and double-stranded DNA substrates in the presence of ATPγS, a slowly hydrolyzable analogue of ATP. When all three DNA strands are equal in length, the outgoing strand is released upon heteroduplex formation (*37*). Here we have carried out strand exchange with incoming ODNs that are homologous to either the middle or end of a dsDNA target. When these substrates are used, the synaptic complex is flanked on one or both sides by a parental duplex and the

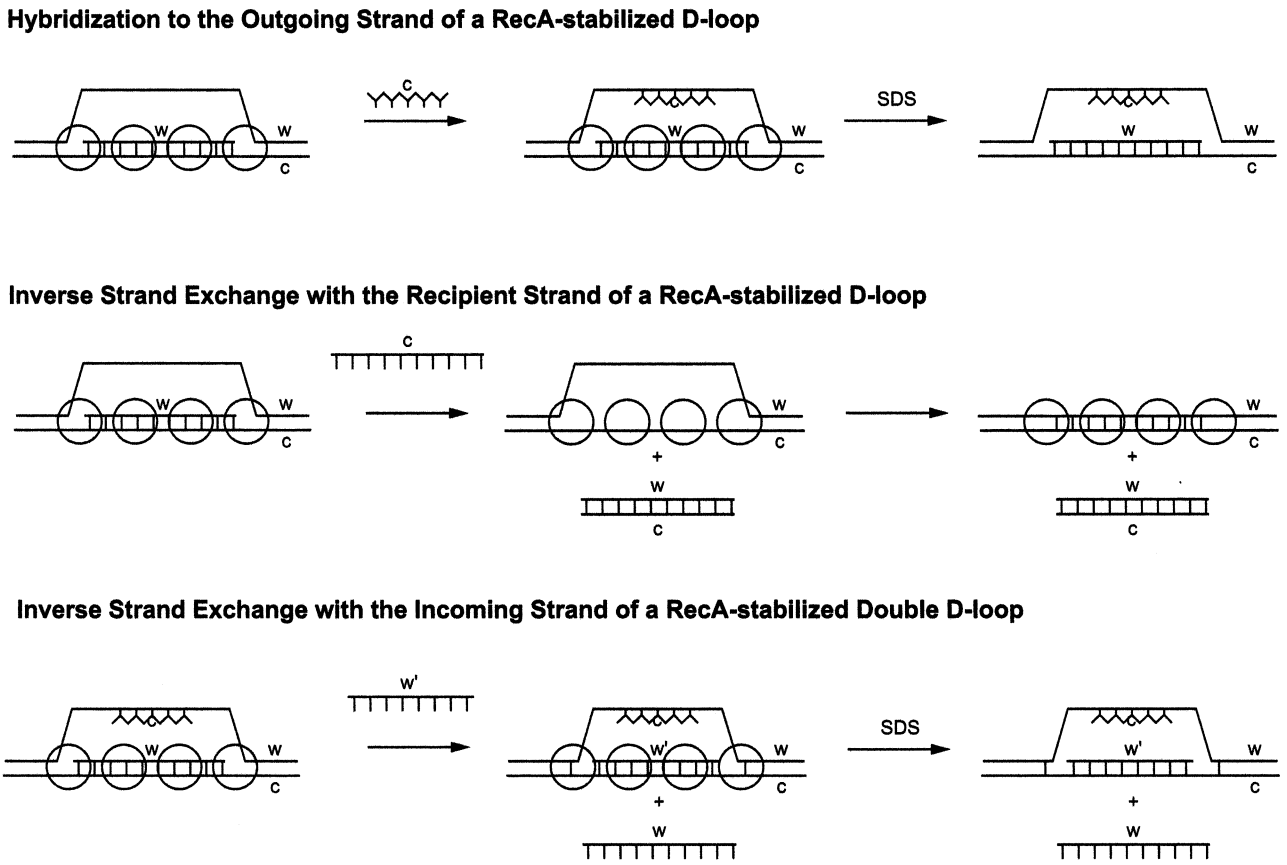

FIGURE 9: Short internal synaptic complexes are dynamic structures that undergo hybridization and inverse strand exchange reactions. ONs with modified backbones can hybridize to the outgoing strand of a synaptic complex but are not substrates for inverse strand exchange. In contrast, ODNs can participate in both types of reactions provided that they are appropriate in length. Hybridization is favored when the ODN is much shorter than the synaptic complex, while inverse strand exchange is favored when the ODN is equal in length to the synaptic complex. Inverse strand exchange should occur with both RecA-stabilized D-loop and double D-loop joints. DNA is represented by a smooth line, RNA, LNA, or 2′-OMe RNA by a wavy line, and RecA protein by a circle. Actual joints would be longer than those depicted here and in the protein-free state would be susceptible to partial collapse by branch migration.

outgoing strand is not released. Using ON probes that are complementary to the displaced portion of the outgoing strand, we have demonstrated that it is single-stranded, a conclusion reached by others using footprinting techniques (*27*−*30*) or fluorescence resonance energy transfer (*31*). Hybridization of the outgoing strand to ONs with modified backbones (Figure 9A) implies that the annealing reaction is not actively mediated by the RecA filament.

While the displaced portion of the outgoing strand is unpaired in both types of synaptic complexes, hybridization proceeds more readily when the joint has a free end. The displaced strand in this type of joint rapidly hybridizes to complementary ONs of any length and effectively competes with homologous single-stranded molecules in hybridization reactions. These results support a Y-arm structure in which the free end of the outgoing strand is distant from the RecA filament and no longer interacts with the protein.

In contrast, the outgoing strand in an internally situated synaptic complex is less accessible to complementary ONs. It hybridizes poorly or not at all to longer ONs that extend beyond the junctions of the complex, and relative to free ssDNA, it hybridizes at a reduced rate to shorter complements. With no free ends, the displaced portion of the outgoing strand is restrained within the synaptic complex in the proximity to the heteroduplex. Enhanced hybridization to the central portion of the outgoing strand indicates that this strand interacts more strongly with RecA protein near the junctions of the complex. Such interactions may be important in stabilizing the post-strand exchange synaptic complex but nonetheless interfere with hybridization.

The annealing reaction is even more restricted when the internal synaptic complex is extremely short. Here the efficiency of hybridization is highly dependent upon the type of backbone in the annealing ON. Only ONs with an RNA-like backbone that targeted the central portion of the displaced strand were effective hybridization agents. In the case of ONs with a DNA backbone, we have shown that inverse strand exchange reduces the yield of the double D-loop joint. For unknown reasons, PNA and phosphorothioate backbones were also inhibitory.

A RecA-stabilized D-loop joint should present a topological barrier to hybridization between an outgoing strand and a longer single-stranded complement that overhangs both ends of the joint. In this case, plectonemic coiling, which accompanies hybridization, can only occur if one end of the complement rotates about the outgoing strand. Obviously, the longer the complement, the less likely it will be able to "thread the needle". The likelihood of hybridization would be further reduced if the ends of a long annealing ON interact with the flanking dsDNA of the synaptic complex. In our experiments, this may be the case since excess RecA protein probably promotes extension of the nucleoprotein filament past the junctions of the synaptic complex (*44*). Homologous incorporation of an ON into these flanking regions could

interfere with subsequent hybridization of the ON to the outgoing strand. By eliminating homology to flanking dsDNA sequences, we have found that the hybridization of long ONs to the outgoing strand of a synaptic complex can be enhanced (data not shown).

The hybridization reaction described here may provide an alternative route for initiating recombination-dependent DNA replication and yet another example of how RNA transcripts in vivo could participate in homologous recombination (*45*, *46*). Our observation that ONs with a LNA or 2′-OMe RNA backbone can hybridize to the outgoing strand of a short synaptic complex suggests that transcripts in the cell could participate in a similar reaction and that the hybridized RNA could serve as a primer for the initiation of DNA synthesis. This pathway for RNA uptake is distinct from the recently described inverse strand exchange reaction (*19*, *20*). In that reaction, dsDNA in a RecA or Rad51 filament strand exchanges with a homologous single-stranded DNA or RNA.

Homologous ODNs Can Strand Exchange with the Incoming or Recipient Strands of a RecA Synaptic Complex. When hybridized to a short synaptic complex, annealing ONs with a DNA backbone yielded less double D-loop joint than ONs with a 2′-OMe RNA or LNA backbone. We propose that annealing ONs with a DNA backbone are unique in their ability to interact in two ways with a small internal synaptic complex. First, they can hybridize to the outgoing strand of such a complex. This reaction is observed when the annealing ODN is shorter than the incoming ODN, and it generates a RecA-stabilized double D-loop joint. Second, they can participate in an inverse strand exchange reaction with the recipient strand of the heteroduplex (Figure 9B). This reaction predominates when annealing and incoming ODNs have equal lengths. Inverse strand exchange with the recipient strand of a synaptic complex reverses joint molecule formation.

Exchange of strands in a synaptic complex was not limited to the recipient strand of the heteroduplex. The incoming ODN in a RecA-stabilized double D-loop can also participate in an inverse strand exchange reaction with homologous ODNs. When the incoming strand was replaced with a shorter ODN, the exchange reaction could be monitored by a gel mobility shift assay. As depicted in Figure 9C, the number of base pairs is unaltered in the smaller double D-loop joint. However, despite maintenance of base pairing, replacement of the incoming ODN was never complete. This could reflect two populations of the synaptic complex, only one of which is competent for inverse strand exchange.

The inverse strand exchange reactions observed with a medial 30 bp RecA synaptic complex provide direct evidence that such complexes are dynamic structures even in the presence of ATPγS. It is telling that inverse strand exchange required the use of ONs with a DNA backbone. In this regard, Shibata and co-workers (*12*, *47*) have proposed that DNA is uniquely suited for strand exchange. Within the RecA filament, stacking interactions between adjacent base and sugar moieties stabilize an elongated form of DNA that facilitates base pair switching between strands. The 2′-hydroxyl group of RNA interferes with these stacking interactions and renders RNA a poor substrate for recombination (*48*, *49*). Consistent with this model, ONs with a 2′-OMe RNA backbone did not exchange with the incoming strand of a small synaptic complex.

No inverse strand exchange reactions were detected when the synaptic complex was 45 bp long or when it was oriented at the end of a dsDNA. Longer complexes are probably more stable and less likely to exchange with an exogenous ODN, while release of the displaced strand from the RecA filament of a terminal synaptic complex may induce a conformational change that inhibits inverse strand exchange.

Four-Strand Exchange. Four-strand exchange reactions provide an alternative pathway to double D-loop joints. In an earlier study, we showed that complement-stabilized joints are more likely to form when using an incoming DNA−RNA duplex instead of an all-DNA duplex (*23*). On the basis of the results reported here, we propose that synaptic complexes initially formed between two double-stranded substrates contain a heteroduplex flanked by both an outgoing strand (from the target duplex) and an annealing strand (from the incoming duplex). When the annealing strand has a DNA backbone, inverse strand exchange is favored over hybridization, thus inhibiting the formation of a double D-loop joint. However, the reverse applies when the annealing strand of the incoming duplex has an RNA or DNA−RNA backbone since inverse strand exchange should not occur. Chimeric DNA−RNA double hairpin vectors have been successfully used as gene targeting agents in mammalian cells (*50*).

A similar situation appears to apply to short duplexes in which the Watson−Crick strands are not covalently linked to one another. Hence, the $gDNA_{30c}$−$gDNA_{30w}$ and $kDNA_{30w}$−$kDNA_{25c}$ duplexes did not exhibit strand exchange activity, whereas the $kDNA_{30w}$−$kLNA_{15c}$ duplex did (see Figure 2). The fact that double D-loop formation is more efficient when $kDNA_{30w}$ and $kLNA_{15c}$ are added sequentially indicates that synaptic complex formation by $kDNA_{30w}$ is inhibited when that ODN is hybridized to $kLNA_{15c}$. Via addition of free annealing ODN to a preformed synaptic complex, this barrier to joint molecule formation can be avoided. We will describe elsewhere applications of RecA-mediated double D-loop formation.

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