# Sequence-Specific Covalent Modification of DNA by Cross-Linking Oligonucleotides. Catalysis by RecA and Implication for the Mechanism of Synaptic Joint Formation

Mikhail A. Podyminogin, Rich B. Meyer, and Howard B. Gamper*

*MicroProbe Corporation, 1725 220th Street South East, Bothell, Washington 98021*

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ABSTRACT: Oligodeoxynucleotides (ODNs) were conjugated to chlorambucil and used as affinity labeling reagents to study joint molecule formation by the *Escherichia coli* recombinase recA. Chlorambucil is a bifunctional nitrogen mustard which alkylates the N-7 position of guanine in the major groove of double-stranded DNA (dsDNA). Incoming ODNs at least 30 nucleotides long cross-linked to a long homologous duplex DNA in the presence of recA and ATP$\gamma$S. Efficient cross-linkage to the complementary recipient strand of the joint occurred preferentially at guanines positioned 5′ relative to the appended chlorambucil group. The pattern of recipient strand alkylation was identical to that observed within a protein-free duplex and indicated that strand exchange had occurred prior to alkylation. Modification of the outgoing homologous strand of the joint was less efficient and spanned a 15−20 nucleotide long region offset to the 3′ side of the tethered chlorambucil. Alkylation of both recipient and outgoing strands in the same joint molecule occurred with low frequency. By contrast, no affinity alkylation of the displaced strand was observed within a synthetic D-loop. These reaction patterns suggest that the incoming ODN approaches from the minor groove of the duplex to yield a poststrand exchange joint in which the major groove of the newly formed heteroduplex harbors the outgoing strand in an unpaired state. No evidence was obtained for the involvement of a triple-stranded DNA intermediate in recombination.

With the assistance of other enzymes, recA catalyzes the process of genetic recombination in *Escherichia coli* (Cox, 1994; Kowalczykowski, 1991; Kowalczykowski & Eggleston, 1994; Radding, 1991; Roca & Cox, 1990; West, 1992). When the purified enzyme is incubated with single-stranded DNA in the presence of ATP or ATP$\gamma$S, a right-handed, helical presynaptic filament is cooperatively formed. The filament has a helical pitch of 95 Å and an axial rise of 5.1 Å per nucleotide; it contains 6.2 recA monomers and 18.6 nucleotides per helical turn. Two DNA binding sites in the recA monomer permit the assimilation of double-stranded DNA into the presynaptic filament where it is unwound and searched for homology. Matched complexes readily undergo strand exchange in a reaction which does not require exogenous energy. However, extension of the initial joint through both homologous and heterologous regions and resolution of the products of strand exchange require hydrolysis of ATP. The entire process can be carried to completion when a single-stranded circular phage DNA is incubated with its linearized replicative form in the presence of recA and ATP (Cox & Lehman, 1981). In this reaction the incoming single-stranded DNA hybridizes to the complementary recipient strand of the duplex thus displacing the outgoing or homologous strand.

The mechanism by which the presynaptic filament searches for homology has not yet been established. Since recA primarily interacts with the phosphodiester backbone of the incoming single-stranded DNA, it is believed that the bases are involved in sequence scanning (DiCapua & Muller, 1987; Dombroski et al., 1983; Leahy & Radding, 1986). Two models have been proposed for the scanning of base pairs by the presynaptic filament, either one of which could involve a triple-stranded intermediate (Stasiak, 1992). In type 1 models, initial interactions take place within the major groove of the duplex, thus preserving the original Watson−Crick base pairs. Upon homologous alignment, the bases of the incoming strand interact through hydrogen bonding and electrostatic interactions with base pair determinants in the major groove to form a triple strand (Chiu et al., 1993; Hsieh et al., 1990; Howard-Flanders et al., 1984; Rao et al., 1993; Rao & Radding, 1994; Shchyolkina et al., 1994; Umlauf et al., 1990; Zhurkin et al., 1994). Strand exchange occurs by relative rotation of the bases in each triad. In type 2 models, the filament transiently opens the base pairs of the duplex to permit their interaction with the bases of the incoming single strand. Recognition of homology and strand exchange occur simultaneously. In either type of model, the outgoing strand might remain transiently hydrogen bonded to the newly formed heteroduplex in a triple-stranded complex [for example, see Chiu et al. (1993)]. Alternatively, strand exchange might directly release the outgoing strand in an unpaired state. In the presence of ATP$\gamma$S, the products of strand exchange are not resolved and the outgoing strand remains intertwined with the heteroduplex in a recA filament (Burnett et al., 1994; Menetski et al., 1990; Rosselli & Stasiak, 1990). For the purposes of this study, a triple strand or triplex will denote three hydrogen bonded DNA strands while a three-stranded DNA will refer to a nonpaired but plectonemically coiled complex between a duplex and a homologous single strand.

By limiting the extent of shared homology between the molecules or by utilizing a topological block, putative triple-

* Corresponding author. Telephone: 206-485-8566. Fax: 206-486-8336. E-mail: howardg@micro.microprobe.com.

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stranded recombination intermediates can sometimes be trapped and studied. Proximal, distal, and medial joints describe synaptic complexes in which the duplex substrate extends past the 3′ end, the 5′ end, or both ends of the incoming single strand. Under certain conditions distal joints appear to survive deproteinization as stable triple strands (Burnett et al., 1994; Chiu et al., 1993; Hsieh et al., 1990; Rao et al., 1991, 1993). Chemical and enzymatic characterization of these complexes suggests that they are the products of strand exchange wherein the recipient strand is Watson−Crick base paired to the incoming strand and the outgoing strand is hydrogen bonded to sites in the major groove. By contrast, medial joints are much less stable and with the exception of supercoiled complexes do not survive deproteinization. Evidence for triple strand formation in recA-associated medial joints is mixed. Although a psoralen photofixation study provided suggestive evidence for the existence of a triple-stranded complex (Umlauf et al., 1990), a chemical footprinting study was consistent with a joint in which strand exchange had already occurred to yield an unpaired outgoing strand (Adzuma, 1992). We note that there is no direct evidence for a prestrand exchange triplex of the sort proposed by the type 1 models.

In this study we used chlorambucil-containing ODNs as affinity labeling reagents to investigate the structure of synaptic intermediates formed with long double-stranded DNA in the presence of recA and ATPγS. Chlorambucil is a bifunctional nitrogen mustard which preferentially alkylates the N-7 position of guanine in nucleic acid (Doskocil & Sormova, 1965; Price et al., 1968). By tethering this reagent to the incoming ODN, the alkylation profile of the synaptic complex was expected to resolve whether the joint is triple-stranded or three-stranded. The results indicate that chlorambucil-containing incoming ODNs could cross-link to the recipient strand, the outgoing strand, or to both strands in a medial joint provided that the ODN was at least 30 bases long. Alkylation of the recipient strand was highly localized whereas alkylation of the outgoing strand spanned a 15−20 base length. These results have led us to construct a type 2 model for recombination in which the incoming strand invades the duplex from its minor groove concomitantly displacing the outgoing strand into the major groove of the newly formed heteroduplex. Although the outgoing strand remains closely associated with the heteroduplex, we have obtained no evidence for the existence of a poststrand exchange triplex.

## MATERIALS AND METHODS

*Proteins and Chemicals.* Sources of proteins were as follows: recA and T4 polynucleotide kinase (U. S. Biochemical); restriction enzymes (New England Biolabs); proteinase K (Boehringer Mannheim). The recA ran at the expected position in an SDS−polyacrylamide gel and was estimated to be 98% pure. Incubations with different DNA substrates showed it to be free of detectable endonuclease and exonuclease activities. In a standard ATPase assay it hydrolyzed 0.25 μmol of ATP $min^{-1}$ $mg^{-1}$. Concentration of the recA was determined using an absorption of 0.516 at 1 mg/mL. Sources of chemicals were as follows: ATPγS (Boehringer Mannheim); [γ-$^{32}$P]ATP (New England Nuclear); DNA synthesis reagents (Glen Research). The 5′-*O*-(4,4′-dimethoxytrityl)-5-[3-(trifluoroacetamido)propyl]-2′-deoxyuridine-3′-*O*-(2-cyanoethyl)-*N*,*N*-diisopropylphosphoroamidite was synthesized as previously described (Meyer et al., 1989).

*Double-Stranded DNAs.* Short double-stranded DNA (dsDNA) fragments 197 and 272 bp long were synthesized using the polymerase chain reaction (PCR) from the *Eco*RI linearized pGEM-4Z plasmid DNA (Promega). One of the PCR primers was chemically phosphorylated to permit selective 5′-$^{32}$P-end-labeling (by T4 polynucleotide kinase) of only one strand of the dsDNA product. pGEM-4Z DNA was amplified as described in Maniatis et al. (1982). All 5′-$^{32}$P-end-labeled dsDNA substrates were purified by nondenaturing PAGE or agarose gel electrophoresis prior to use to remove any nuclease or ssDNA contamination.

*Oligodeoxynucleotides.* ODNs were synthesized by standard phosphoroamidite chemistry on an Applied Biosystems 394 DNA/RNA Synthesizer and purified by reverse-phase high-performance liquid chromatography. The integrity of all ODNs was checked by denaturing polyacrylamide gel electrophoresis (PAGE) followed, if necessary, by preparative PAGE purification. Chlorambucil was conjugated to the ODNs through a 5-(3-aminopropyl) uracil base or a 5′-aminohexyl phosphate end group by postsynthetic acylation using the 2,3,5,6-tetrafluorophenyl (TFP) ester of chlorambucil (Kutyavin et al., 1993). Chlorambucil-containing ODNs were stably stored in aqueous solution at −70 °C.

*Standard Reaction Conditions.* Synaptic complexes were formed by mixing 100 nM (in molecules) incoming ODN, 2 μM RecA protein, and 10−100 nM (in molecules) dsDNA on ice and then increasing the temperature to 37 °C. The reactions were conducted in a 50 μL final volume and contained 10 mM Tris-acetate buffer (pH 7.5), 50 mM sodium acetate, 12 mM magnesium acetate, 1 mM DTT, 1 mM ATPγS, and 5% glycerol. Alkylation of the dsDNA target by the tethered chlorambucil took place over 6 h at 37 °C. At this temperature the first of two 2-chloroethylamino groups present in chlorambucil rearranges with a half-life of 45 min to a highly reactive aziridinium cation (Kutyavin et al., 1993). The second 2-chloroethylamino group undergoes the same rearrangement at a 2−3-fold slower rate. After 6 h of incubation both reactions were nearly complete.

*Characterization of Alkylation Products.* Sites of alkylation by chlorambucil-containing ODNs were detected by denaturing PAGE of deproteinized DNA samples after treatment with hot piperidine. Following the formation and crosslinkage of synaptic complexes, reaction mixtures were diluted 2-fold with water and incubated for 30 min at 37 °C with 0.5% SDS and 200 μg/mL proteinase K. Samples were extracted once with phenol−chloroform, three times with ether and precipitated by ethyl alcohol. To introduce nicks at the positions of alkylated guanines, the pelleted DNA was treated with 10% piperidine for 30 min at 95 °C and precipitated with alcohol. The DNA was recovered by centrifugation, dried, and dissolved in 80% formamide containing 0.1% xylene cyanol and bromophenol blue. Samples were analyzed by 4−12% denaturing PAGE. The sites of DNA cleavage were identified by comparison with sequencing ladders (Maxam & Gilbert, 1977) or with restriction digests. Alkali stable adducts in which the incoming ODN was linked to the labeled target strand were visualized as bands which ran slower than the unmodified target strand. Percent of alkylation was determined by

| ODN | Sequence | Length | % cleavage |
|---|---|---|---|
| 1 | 5′-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG | 50 | 56 |
| 1a | 5′**Chlb**-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAG | 50 | ~10 |
| 1b | 5′-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAG | 50 | - |
| 2 | 5′-AAAAAGCGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG | 40 | 43 |
| 3 | 5′-TAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG | 30 | 38 |
| 4 | 5′-GGTCC**U**CCGATCGTTGTCAG | 20 | 1 |
| 5 | 5′-ATGATCCCCC | 10 | - |

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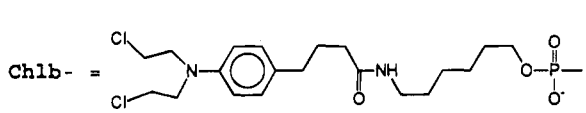
5′...**TACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAG**AAGTAAGTTGGCCGCAGTGTTAT...3′

3′...**ATGTACTAGGGGGTACAACACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTC**TTCATTCAACCGGCGTCACAATA...5′



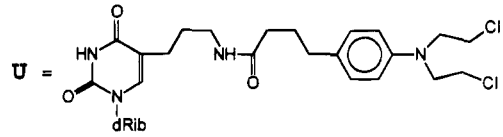




FIGURE 1: Structure of oligonucleotides and their reactive derivatives used for synaptic complex formation. The double-stranded DNA target sequence is shown below and was derived by PCR amplification from pGEM-4Z. The 272 bp PCR fragment contained the entire sequence shown here and ODNs **1**−**4** formed medial joints with this substrate. Only the boldfaced sequence was contained within the 197 bp PCR fragment. ODNs **1**−**4** formed distal joints with this substrate. The DNA strand homologous to the incoming ODN is referred to as the outgoing strand, while the DNA strand complementary to the incoming ODN is referred to as the recipient strand. The efficiency of alkylation-induced cleavage of the recipient strand in a medial joint is shown for each incoming ODN.

phosphorimage analysis (Bio-Rad) of the cleavage product to full length DNA ratio.

## RESULTS

*Experimental Strategy.* Chlorambucil is a bifunctional nitrogen mustard used clinically in cancer chemotherapy. Like other related compounds it reacts primarily with the N-7 position of guanine bases in DNA and RNA to give mono- and di-adducts (Doskocil & Sormova, 1965). Alkylation is preceded by an intramolecular rearrangement to give a highly electrophilic aziridinium cation. The first of two such rearrangements occurs with a half-life of approximately 45 min at 37 °C. We have previously shown that homopyrimidine and homopurine ODNs terminally conjugated to chlorambucil can nonenzymatically bind and cross-link to "complementary" homopurine runs in double-stranded DNA (Kutyavin et al., 1993; J. Lampe, R. Rhinehart, I. Kutyavin, R. Meyer, and H. Gamper, unpublished data). In this study we evaluated the ability of mixed sequence ODNs containing a chlorambucil residue to cross-link to homologous duplexes in the presence of recA and ATPγS. The ODNs ranged in length from 20−50 nucleotides long, and each contained a single chlorambucil residue conjugated to the terminus of the backbone or more usually to an internally situated 5-(3-aminopropyl)uracil base. Conjugation of chlorambucil to the C-5 position of uracil was not expected to impair synaptic joint formation since it is known that T4 phage DNA can participate in recombination mediated by a recA homolog, T4 UvsX, even though the C-5 position of pyrimidines is heavily glycosylated (Yonesaki & Minagawa, 1988). RecA-stabilized medial and distal joints were formed by incubating chlorambucil-containing ODNs with two different PCR-amplified fragments of pGEM-4Z (see Figure 1). Reaction mixtures were incubated in the presence of recA for 6 h to allow consumption of the alkylation reagent. After deproteinization and treatment with hot piperidine, the sites of cross-linkage were visualized as nicks on the end-labeled recipient or outgoing strand by sequencing gel analysis. The disparity in length between the duplex DNA and the incoming ODN substrates guaranteed that synaptic complexes would contain all three strands in a recA-stabilized association.

*RecA-Stabilized Distal and Medial Joints.* RecA has been shown to associate with ODNs and generate presynaptic filaments that possess the same properties as longer filaments (Hsieh et al., 1990, 1992). In the presence of recA short joints can survive for several hours (Golub et al., 1992) and have been successfully used to investigate the mechanism of strand exchange (Adzuma, 1992; Chiu et al., 1993). RecA can also associate with certain modified ODNs (Leahy & Radding, 1986; Cheng et al., 1988). This appears to hold for chlorambucil-containing ODNs as well. When incubated with recA in the presence of ATPγS, these ODNs alkylated the recombinase thus providing indirect evidence for presynaptic filament formation (data not shown). The ability of the ODNs to sequence specifically alkylate double-stranded DNA only in the presence of recA provides functional evidence for their association with recA in active filaments.

When nonreactive versions of these same ODNs were used to form recA-associated distal or medial joints with the 197 or 272 bp DNA fragments, sequence-specific complex formation could be detected using a restriction inhibition assay (Hsieh et al., 1992). However, following deproteinization no joint molecules could be detected by gel shift analysis, regardless of the length of the incoming ODN or the type of joint. These preliminary experiments (data not shown) predicted that alkylation of the duplex DNA target by chlorambucil-containing ODNs would only take place in the continued presence of recA. This was verified by showing that alkylation was reduced to a near background level when recA was removed early in the 6 h incubation. Others have observed that short medial joints formed with non-supercoiled DNA spontaneously dissociate upon the

removal of recA (Adzuma, 1992; Hsieh et al., 1992; Chiu et al., 1993). However, the dissociation of the deproteinized distal joints formed with the 197 bp fragment was unexpected in light of the work by Hsieh et al. (1990), who were able to characterize protein-free distal joints with as little as 26 bp of homology. Our inability to reproduce these results may be due to differences in protocol and buffer conditions.

In studies such as these it is important to verify that the joint molecules arise from authentic homologous recombination (Kmiec & Holloman, 1994). In the presence of exonuclease, the duplex fragment can be resectioned and joint molecules formed by hybridization. This was not the case here because end-labeled recipient and outgoing strands showed no indication of exonuclease or endonuclease mediated degradation even after 6 h of incubation with an unmodified 50-mer (ODN **1b**) in the presence of recA. Furthermore, our inability to isolate a stable joint molecule from the same reaction mixture following deproteinization is further evidence against resectioning, since a Watson–Crick base paired hybrid should have survived deproteinization and nondenaturing gel electrophoresis.

*Alkylation of the Recipient Strand.* Two chlorambucil-containing 50-mers (ODNs **1** and **1a**) were used to form recA-stabilized joints at the distal end of a homologous 197 bp DNA fragment. Alkylation of the recipient strand by these ODNs is shown in Figure 2. When chlorambucil was conjugated to the 5′ end of the incoming ODN (ODN **1a**), synaptic complex formation was accompanied by alkylation of the recipient strand in the flanking duplex at G-51 and G-52 with a total frequency of approximately 10%. (By convention the bases are numbered from the 5′ end of the subject strand.) An unidentified site in the same strand was also alkylated with 10% efficiency to give a noncleavable product (data not shown). By contrast, a 5′-chlorambucil-tailed homopyrimidine ODN that was Hoogstein paired to a "complementary" homopurine run in a double-stranded DNA fragment exclusively alkylated guanines in the flanking duplex with 80% efficiency (Kutyavin et al., 1993). The reduced reaction efficiency observed with the synaptic complex could reflect competing alkylation of recA, masking of the flanking duplex by the recombinase, or fraying of the 5′ end of the ODN from the complex. When chlorambucil was conjugated to U-36 of the incoming ODN (ODN **1**), three adjacent guanines (G-13, G-14, and G-16) in the recipient strand were alkylated with a total efficiency of about 50%. Only a nominal amount of noncleavable adduct was formed. The ability of chlorambucil to react with internal guanines is consistent with previous studies which showed that the N-7 position of purines in synaptic complexes is not involved in hydrogen bonding (Adzuma, 1992; Jain et al., 1992; Rao et al., 1993). In all the studies described here, alkylation took place over a 6 h period only in the presence of recA. The literature suggests that short synaptic joints are very stable under these conditions (Chiu et al., 1993; Golub et al., 1992).

In light of the various models proposed for synaptic intermediates, it is instructive to ask which can accommodate the alkylation reaction described above for ODN **1**. Two hydrogen bonding schemes have been described for the putative triple-stranded intermediate formed during the initial homologous pairing event. In both models (Rao et al., 1993; Zhurkin et al., 1994), the N-7 of guanine in the recipient strand of the joint does not participate in hydrogen bonding

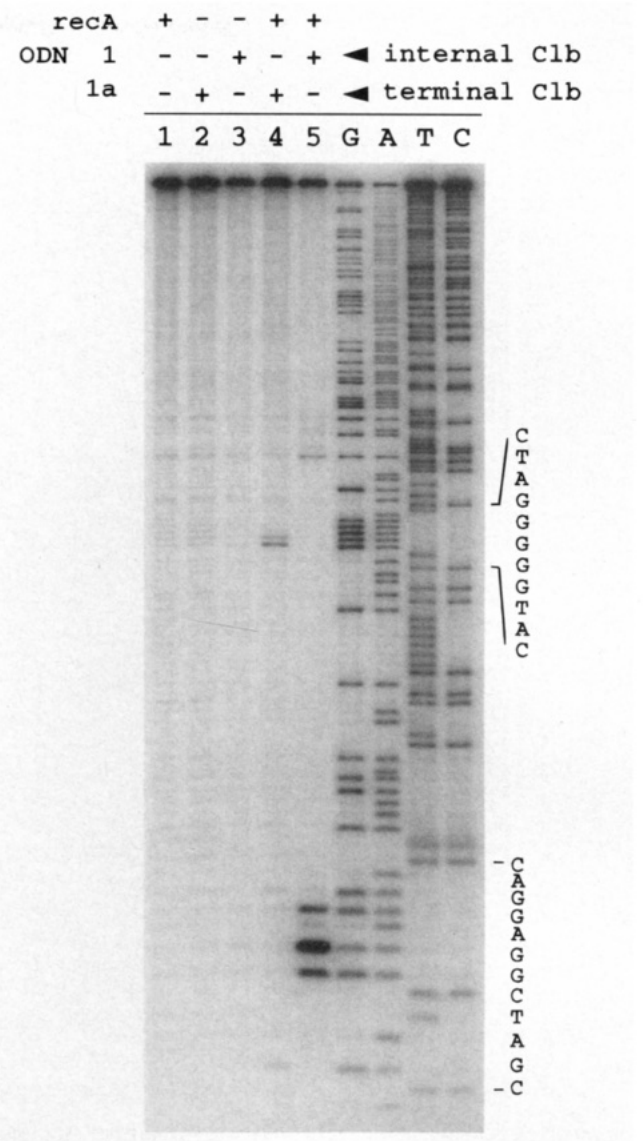



FIGURE 2: Alkylation-induced cleavage pattern of the recipient strand from a recA-stabilized distal joint formed by two incoming chlorambucil-containing 50-mers. Distal joints were formed between the 197 bp DNA fragment and ODNs **1** or **1a**. Samples were deproteinized and treated with hot piperidine prior to analysis of the labeled recipient strand by denaturing PAGE. Lane 1, control lacking ODN; lanes 2 and 3, controls lacking recA; lanes 4 and 5, complete reactions with ODNs **1** or **1a**, respectively. In this and subsequent figures, lanes G, A, T, and C are the Maxam and Gilbert G, A+G, T+C, and C sequencing reactions, respectively.

and is available for reaction. Once strand exchange has occurred, the incoming strand is Watson–Crick hydrogen bonded to the recipient strand. Two structural models have been described for the strand exchange product. One proposes a triple-stranded DNA (Chiu et al., 1993) and the other a three-stranded DNA (Adzuma, 1992). In the triple-stranded model, the bases of the outgoing strand hydrogen bond to the major groove side of base pairs in the newly formed heteroduplex. Although the N-7 position of recipient strand guanines is not hydrogen bonded, it is unavailable for reaction with chlorambucil since the reactants reside in different grooves of the triplex. In the three-stranded model, the outgoing strand is single-stranded and should not interfere with alkylation of the recipient strand. According to this analysis, cross-linkage of the synaptic joint could take place in a prestrand exchange triplex or in a poststrand exchange three-stranded DNA complex. The exact reaction pattern

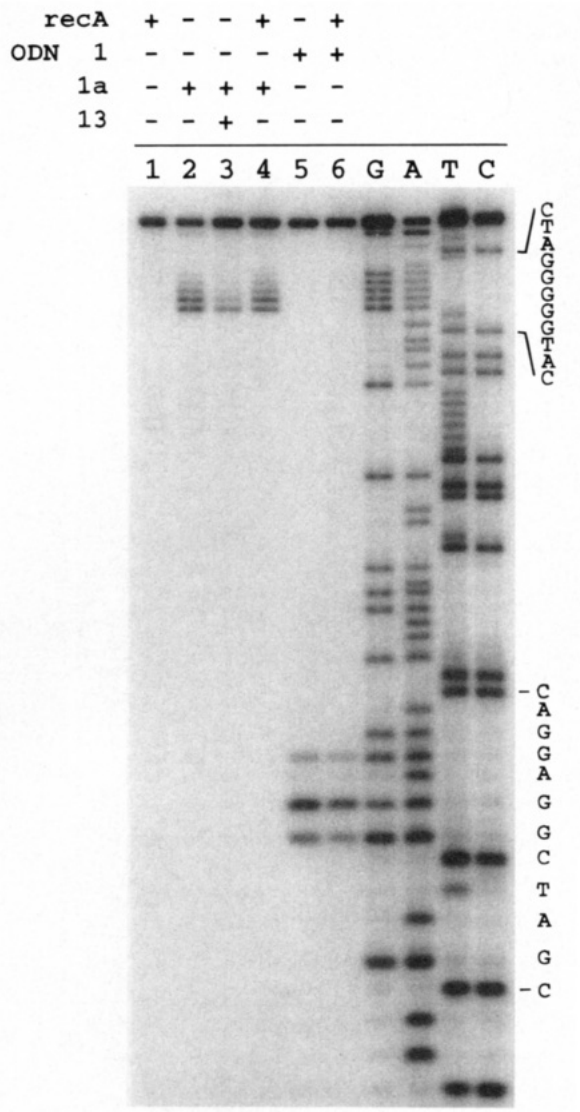



FIGURE 3: Alkylation-induced cleavage pattern of a 60-mer hybridized to ODNs **1** and **1a**. The 60-mer was identical in sequence to a portion of the recipient strand in the 197 bp DNA fragment and overhanged the 5′ ends of ODNs **1** and **1a** by 10 bases. Hybrids (0.1 $\mu$M) were incubated in the standard reaction buffer plus/minus recA (2 $\mu$M) for 6 h at 37 °C. After removal of protein and treatment with hot piperidine the labeled 60-mer from each sample was analyzed for cleavage by denaturing PAGE. Lane 1, control lacking reactive ODN; lanes 2 and 5, hybrids formed with ODNs **1a** or **1**, respectively; lane 3, hybrid formed with ODNs **1a** and **5** (a 10-mer complementary to the overhanging portion of the 60-mer); lanes 4 and 6, hybrids formed with ODNs **1a** or **1** in the presence of recA.

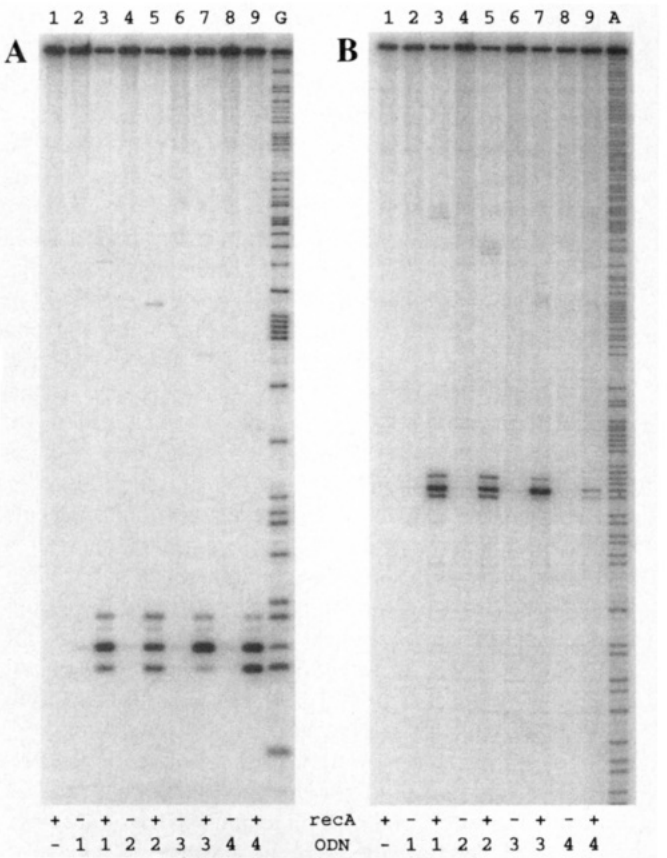



FIGURE 4: Alkylation-induced cleavage pattern of the recipient strand from recA-stabilized (A) distal and (B) medial joints formed by chlorambucil-containing incoming ODNs of variable length. Reactive ODNs **1**−**4** (a length series of 50-, 40-, 30-, and 20-mers) were incubated with the 197 and 272 bp DNA fragments in the presence of recA to form distal and medial joints, respectively. After workup the labeled recipient strand was analyzed for cleavage by denaturing PAGE. In this and subsequent figures the numbers at the bottom of the gel refer to the reactive ODN present in the reaction mixture (see Figure 1). Control incubations lacking recA were routinely carried out and are indicated by the minus sign.

of chlorambucil with adjacent guanines in the recipient strand of the synaptic complex should be different for the two alternative structures.

We reasoned that a comparison of the alkylation pattern of the tethered chlorambucil in an authentic duplex versus the pattern in a homologous synaptic complex should be identical if the joint was a poststrand exchange three-stranded complex and different if it was a pre-strand exchange triplex. The results of such a comparison are found in Figures 2−4. When ODN **1** was incubated with a complementary 60-mer in the presence or absence of recA, the alkylation pattern of the 60-mer (Figure 3, lanes 5 and 6) was identical to that observed with the recipient strand in both distal and medial joints (Figure 2, lane 5 and Figure 4A,B, lane 3). These results support the notion that the chlorambucil reaction takes place in a poststrand exchange three-stranded DNA joint.

A comparison of the alkylation patterns produced by ODN **1a** in a control duplex versus a recA-stabilized distal joint was also carried out. When a hybrid was formed between ODN **1a** and the complementary 60-mer, several guanines in the flanking single-stranded tail were alkylated (Figure 3, lane 2). This alkylation was somewhat inhibited by the presence of recA (Figure 3, lane 4) but was still more far reaching than observed with the recipient strand of a distal joint (Figure 2, lane 4). The reaction patterns, however, became identical when the overhang was double-stranded (Figure 3, lane 3). These results indicate that the DNA immediately proximal to the synaptic joint is double-stranded and possibly shielded from reaction by recA.

*Length Dependence of Alkylation.* Using alkylation and subsequent cleavage of the recipient strand as an indicator of recA-stabilized joint molecule formation, a length series of chlorambucil-containing ODNs were tested for their ability to form distal and medial joints. Figure 4 shows that 50-, 40-, and 30-mers (ODNs **1**−**3**) were competent in forming both types of joints while a 20-mer (ODN **4**) was only able to form significant amounts of a distal joint. All complexes exhibited similar reaction patterns which were limited to alkylation of the three nearest guanines. Distal and medial joints of the same length produced identical reaction patterns.

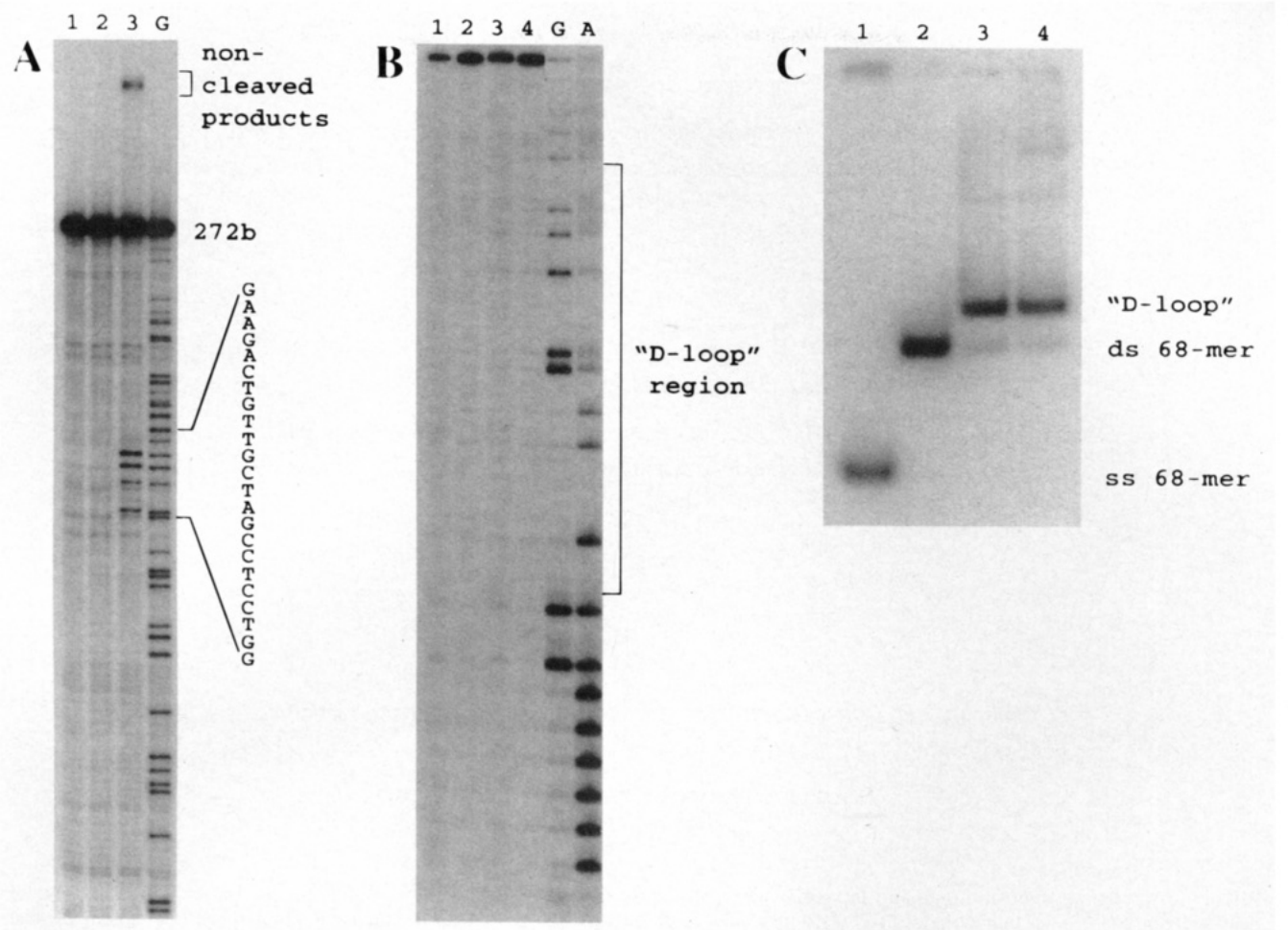



FIGURE 5: (A) Alkylation-induced cleavage pattern of the outgoing strand from a recA-stabilized medial joint formed by ODN **3**. Medial joint molecules were formed with the 272 bp DNA fragment. After alkylation and workup, the labeled outgoing strand was analyzed for cleavage on a denaturing PAGE gel. Lane 1, control lacking ODN **3**; lane 2, control lacking recA; lane 3, complete reaction. (B) Alkylation-induced cleavage pattern of the "outgoing" single strand from a synthetic D-loop formed with ODN **3**. A stable D-loop was formed by hybridizing ODN **3** (100 nM) to a 68-mer replicate of the recipient strand (bases 57−124; 50 nM) as defined in Figure 1. The resultant 30 bp duplex had 19 base long single-stranded arms which were hybridized to a second labeled 68-mer (10 nM). A central 34 base long region of heterology in this ODN (in which only the G's were complementary to the unlabeled 68-mer) prevented it from displacing ODN **3** thus forming a stable D-loop (depicted in Figure 6). The D-loop was incubated in the presence or absence of recA (2 $\mu$M) under standard reaction conditions. After 6 h at 37 °C samples were deproteinized, treated with hot piperidine, and analyzed by denaturing PAGE (6% gel). Lane 1, labeled 68-mer; lane 2, labeled 68-mer bulged hybrid incubated with recA in the absence of ODN **3**; lanes 3 and 4, labeled D-loop incubated in the absence and presence of recA, respectively. (C) Gel shift analysis of synthetic D-loop formation. ODNs were mixed together at the same molar ratios as in (B) and incubated 1 h at room temperature. A nonreactive version of ODN **3** was employed for this analysis. Lane 1, labeled 68-mer; lane 2, labeled 68-mer bulged hybrid; lane 3, D-loop formed by addition of 30-mer to 68-mer bulged hybrid; lane 4, D-loop formed by addition of labeled 68-mer to the 30-mer 68-mer hybrid.

With both types of joints an additional weak alkylation site on the recipient strand could be detected downstream from the proximal end of the joint. The origin of this reaction will be described elsewhere.

The instability of medial joints relative to distal joints is not surprising given that medial joints have two junctions instead of one from which branch migration can take place. Figure 1 lists the efficiencies with which ODNs **1**−**4** alkylated the recipient strand in conjunction with medial joint formation. Yield of recA-stabilized medial joints improved with each incremental increase in ODN length. However, a 20-mer failed to alkylate the recipient strand of the duplex even though a 15-mer was reported to inhibit restriction at a unique site by synaptic joint formation (Hsieh et al., 1992). This difference is reconcilable if the latter joint represents an earlier intermediate in the recombination pathway which is not a substrate for alkylation by chlorambucil.

*Alkylation of the Outgoing Strand.* Lane 3 in Figure 5A shows the cleavage pattern of the outgoing target strand following reaction with ODN **3** in a medial joint and treatment with piperidine. These data are displayed in schematic form in Figure 6A along with data obtained for the recipient strand. In every case cleavage could be attributed to alkylation of the N-7 position of a guanine. However, whereas the alkylation pattern of the recipient strand was highly restricted and relatively efficient, every guanine in a 19 base stretch of the outgoing strand was alkylated at a low frequency. Similar results have been obtained for three other medial joints and one distal joint formed by chlorambucil-containing 30-mers, one example of which is shown in Figure 6C. In all of these joints the envelope of accessible bases in the outgoing strand was 3′-shifted relative to the tethered chlorambucil of the incoming ODN, with maximal reactivity usually observed for G's sited 2−13 nucleotides downstream from the chlorambucil attachment site. The 3′ shift is expected for a tethered reagent which reacts within the major groove of the duplex and has been previously observed for the nicking of DNA by

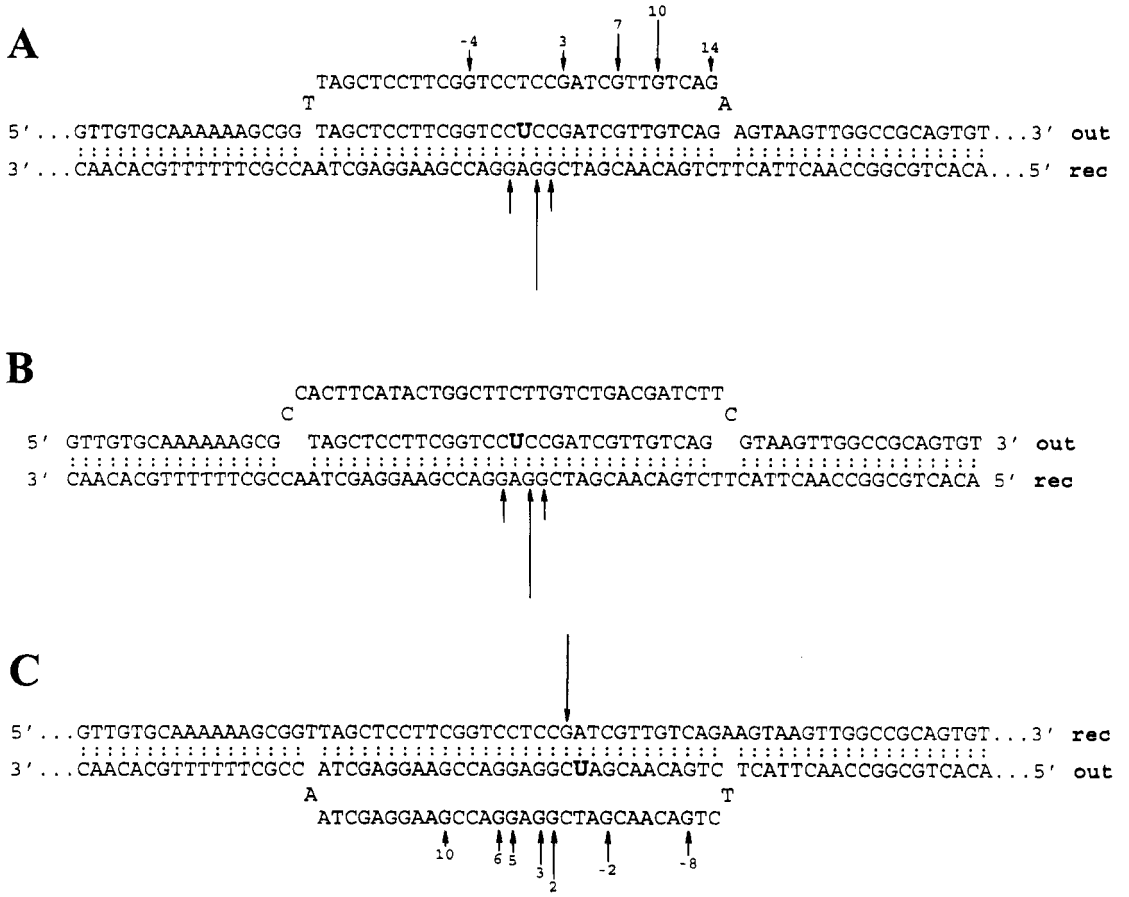



FIGURE 6: Alkylation patterns of outgoing and recipient strands in synaptic complexes and the model D-loop. The 272 bp DNA fragment (A and C) and the 68-mer bulged hybrid (B) were incubated in the presence of recA with the indicated chlorambucil-containing 30-mers (ODN **3** or a reactive complement). After incubation under the standard conditions, the sites of alkylation in the labeled duplex strand were determined as usual. The length of the arrows is proportional to the extent of alkylation-induced cleavage at the indicated bases. Approximately 40% of the recipient strand and 10% of the outgoing strand were cleaved in synaptic joints A and C. No cleavage of the "outgoing" strand was observed in D-loop B. In each structure the incoming 30-mer was hybridized to the recipient strand.

phenathroline-conjugated and triplex-forming ODNs (Francois et al., 1989). Of course, attachment of chlorambucil to the C-5 position of uracil in the incoming strand would orient it within the major groove of the heteroduplex following strand exchange.

Overall alkylation of the outgoing strand in medial joints was usually less than 20% and was equally proportioned between cleavable N-7 guanine adducts and unidentified alkali-stable products (see Figure 5A, lane 3). Elimination of the preferentially alkylated guanine 5′ to the chlorambucil group in the recipient strand of the duplex (by conjugating the cross-linker to a different position in the incoming ODN) did not increase the level of alkylation of the outgoing strand (data not shown). On the basis of reaction profiles of other nitrogen mustards with nucleic acid (Price et al., 1968), the tethered chlorambucil used here could also have reacted with the N-1 of adenine and the N-3 of cytidine, provided the outgoing strand was unpaired at least part of the time. This hypothesis seems plausible in light of the large reaction envelope exhibited by this strand and its low overall level of modification. Stable engagement of the outgoing strand in a hydrogen bonded triplex would probably prevent the formation of alkali-stable adducts and the generation of a broad reaction footprint. In both pre- and poststrand exchange triplexes (Chiu et al., 1993; Rao et al., 1993; Zhurkin et al., 1994) alkylation of the N-7 position of guanines in the outgoing strand by a chlorambucil moiety tethered to the incoming ODN should not occur since the reactants reside in different grooves.

To further explore the conformation of the outgoing strand, the alkylation pattern in an authentic D-loop structure was investigated. The D-loop was formed by hybridization of a 68-mer (the "recipient strand") to both a complementary chlorambucil-containing 30-mer (the "incoming strand") and a partially complementary and labeled 68-mer (the "outgoing strand"). To prevent spontaneous extrusion of the 30-mer by branch migration, the labeled 68-mer was mismatched to its complement over a 34 base long region directly apposed to the 30-mer. All guanines in the looped out region of the 68-mer were preserved as potential sites for alkylation. The D-looped structure (see Figure 6B) was incubated in the presence and absence of recA under standard incubation conditions. Gel mobility shift analysis (Figure 5C) confirmed that the D-loop structure was stable in the reaction buffer. In contrast to the results obtained with synaptic complexes, no cleavage of the outgoing strand in the D-loop was observed regardless of the presence or absence of recA (see Figure 5B, lanes 3 and 4). As usual, however, cleavage was observed at the expected sites in the recipient strand of the D-loop when that strand was labeled (data not shown). In light of these results we suggest that the outgoing strand in oligomeric distal or medial joints resides within or very close to the major groove of the newly formed heteroduplex. While not stably hydrogen bonded to the heteroduplex, the

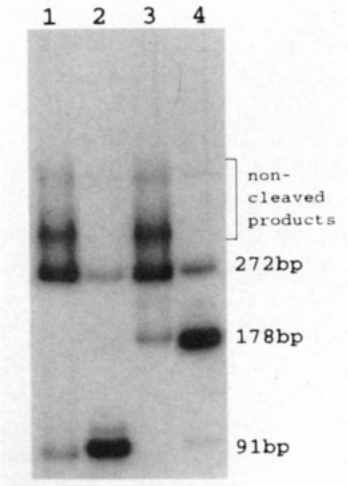



FIGURE 7: Site-specific double strand breakage of duplex DNA after medial joint formation with a chlorambucil-containing 30-mer. The 272 bp DNA fragment was incubated with ODN **3** in the presence of recA under standard reaction conditions. Samples were diluted 2-fold with water, deproteinized, and incubated 48 h at 50 °C to cleave the DNA at sites of alkylated guanine. This nondenaturing procedure preserved the duplex. Double strand breaks were detected by nondenaturing PAGE (8% gel). Lanes 2 and 4, standards formed by *Ava*II restriction of amplicon in which the recipient or outgoing strand had been end-labeled, respectively; lanes 1 and 3, complete reaction mixtures with amplicon in which the recipient or outgoing strand had been end-labeled, respectively. The two restriction fragments approximate the lengths of the cleavage products expected from alkylation of both strands of the parental duplex by a single chlorambucil-containing 30-mer in a medial joint.

three DNA strands in the complex remain plectonemically coiled.

*Bifunctional Cross-Linkage.* Having shown that a chlorambucil-containing ODN could alkylate either strand of the targeted duplex in a synaptic joint and taking into consideration that chlorambucil is a bifunctional reagent, we asked whether both strands could be alkylated by a single tethered chlorambucil in the same joint molecule. Medial joints were formed and incubated as usual. Following deproteinization, however, the reaction mixtures were incubated 48 h at 50 °C to nick both strands at sites of alkylated guanines (Meyer et al., 1989). By substituting this procedure for the standard heat/piperidine treatment, depurination strand scission took place under nondenaturing conditions. The labeled DNA was then analyzed by gel electrophoresis in the absence of urea. This analysis should detect breaks but not nicks. The results presented in Figure 7 show that the targeted 272 bp dsDNA was indeed cleaved into two smaller fragments of the size expected for an interstrand cross-linkage event. The low level of cleavage reflects the infrequency with which the homologous strand was alkylated. Disproportionation of singly nicked duplexes into the two smaller fragments by a transient denaturation/renaturation process was deemed unlikely given that the melting temperatures of the cleaved fragments were estimated to be at least 80−85 °C. Also visible in the autoradiogram is a slower moving product which probably represents the full length duplex stably cross-linked to the incoming 30-mer ODN via alkylation of adenines or cytidines in the outgoing (homologous) strand. These results are again consistent with a model for the synaptic complex in which the outgoing strand is in very close proximity to the newly formed heteroduplex at the time of alkylation.

Table 1: Alkylation Patterns Predicted by Different Models of the Synaptic Complex

| model | alkylation[a] of recipient strand | alkylation[a] of outgoing strand |
|---|---|---|
| prestrand exchange | | |
| triple-stranded DNA | yes | no |
| post-strand exchange | | |
| triple-stranded DNA | no | no |
| three-stranded DNA | yes | possible |
| D-loop | yes | no |
| experimental | yes | yes |

[a] Alkylation of a nearby guanine by a chlorambucil moiety tethered to the incoming strand of a recA-stabilized synaptic complex. Models of a prestrand exchange triplex have been described by Rao et al. (1993) and Zhurkin et al. (1994), a model of a poststrand exchange triplex has been presented by Chiu et al. (1993), and a model of a three-stranded DNA has been described by Adzuma (1992). The alkylation of a D-looped structure in the presence and absence of recA has been described here (see Figures 5 and 6).

## DISCUSSION

*Structure of Cross-Linkable Synaptic Joints.* On the basis of the results presented here, we postulate that oligomeric distal and medial joints formed in the presence of recA and ATPγS are neither triple-stranded nor D-looped. Instead these joints are stable species in which the incoming and recipient strands are Watson−Crick base paired and the outgoing strand, which resides in the major groove of the newly formed heteroduplex, is unpaired. Table 1 summarizes the predicted reactivities of a chlorambucil-tethered incoming ODN with the recipient and outgoing strands in each candidate structure for the synaptic complex. When such ODNs were employed as affinity cross-linking agents in the recA reaction, the alkylation pattern of the recipient strand was highly restricted and mirrored the pattern obtained with an authentic duplex similarly conjugated. In both cases the tethered chlorambucil efficiently alkylated the N-7 position of nearby guanines. No evidence was obtained for a different reaction pattern that would have occurred if the recipient strand had been part of a prestrand exchange triplex [see model in Rao et al. (1993)]. Nor was alkylation blocked as would have been expected if the outgoing strand had remained hydrogen bonded to the heteroduplex in a post-strand exchange triplex [see model in Chiu et al. (1993)]. Our data agree with earlier studies which showed that strand exchange takes place in the presence of ATPγS (Menetski et al., 1990; Rosselli & Stasiak, 1990). Cross-linkage probably occurs in a recA-stabilized three-stranded DNA in which both the heteroduplex and the outgoing strand are in close proximity to each other yet are not hydrogen bonded. The extent of cross-linkage observed with the recipient strand indicates that most if not all of the synaptic complexes have a three-stranded structure.

The alkylation pattern of the outgoing strand also supports a three-stranded DNA structure. Cross-linkage of the chlorambucil-containing ODN to this strand was unrestricted along a nearly 20 nucleotide length in distal and medial joints. Every guanine in this region was alkylated with low efficiency. Adenine and cytidine adducts, which require the bases to be unpaired, also appear to have been formed. The broad reaction envelope as well as the formation of alkali stable adducts argue against the involvement of this strand in a triplex at the time of crosslinkage. The hydrogen bonding patterns of possible poststrand exchange triplexes

would preclude cross-linkage of the incoming ODN to either strand of the original duplex (Chiu et al., 1993; Zhurkin et al., 1994). However, alkylation of both these strands can be accommodated within a three-stranded DNA joint in which the outgoing strand is unpaired.

Despite the single-stranded character of the outgoing strand, distal and medial joints do not appear to have Y-arm or D-loop like structures, respectively. When introduced into a stable D-loop molecule, the tethered chlorambucil behaved quite differently and was unable to react with the looped out region of the "outgoing strand" even in the presence of recA. The detection of bis-adducts formed between a single tethered chlorambucil and both strands of the targeted duplex in medial joints places the outgoing strand in close proximity to the major groove of the heteroduplex. This placement is further substantiated by the 3′ offset of the alkylation envelope in the outgoing strand relative to the tethered chlorambucil.

Two factors prevent release of the outgoing strand from the vicinity of the heteroduplex in a medial joint. First, this strand is held in place at both ends of the junction by its participation in the flanking duplexes. Second, the synaptic complex itself physically constrains both the heteroduplex and the outgoing strand within the larger recA filament, which does not dissociate in the presence of ATP$\gamma$S (Menetski et al., 1990; Rosselli & Stasiak, 1990). Noting that the outgoing strand in distal joints has a free end but nevertheless remains associated with the heteroduplex, we surmise that physical containment within a recA filament is the primary block to strand resolution. If the structural parameters for the recA−dsDNA complex also hold for synaptic joints, then the three-stranded DNA resulting from strand exchange has a maximum diameter of approximately 30 Å or less (Egelman & Yu, 1989) while the surrounding recA filament has a diameter of approximately 110 Å (DiCapua et al., 1982; Egelman & Stasiak, 1986). Although constrained within the recA filament and plectonemically coiled around the heteroduplex, the wide alkylation footprint of the outgoing strand suggests to us that this strand can slip sideways relative to the heteroduplex, otherwise the tethered chlorambucil would not be able to reach the more distant bases in the envelope. The loss of registry associated with sliding makes it less likely that three-stranded and triple-stranded DNA exist in dynamic equilibrium with each other in the joint.

In the absence of a triple-stranded conformation, distal and medial joints should be susceptible to extrusion of the incoming strand by branch migration. The close association of an unpaired outgoing strand should actually facilitate this process. This expectation was met when recA was removed from the oligomeric joints described here. Alkylation did not take place following deproteinization of the synaptic complex. Branch migration and release of the incoming strand would be expected to take place very rapidly if the deproteinized joints possessed a D-loop structure. The step time for three-stranded branch migration at 37 °C has been estimated to be 8000 bp $s^{-1}$ (Radding et al., 1977). Assuming that branch migration occurred as a random walk process, the time required for dissociation of a 50 bp long deproteinized joint would be approximately 15 ms (Feller, 1957). Somehow, an intact recA filament inhibits this process.

The differential stability of recA-associated distal and medial joints also supports a three-stranded DNA structure. Using alkylation of the recipient strand as a measure of synaptic complex formation, the shortest incoming ODNs observed to form distal and medial joints were a 20-mer and a 30-mer, respectively. (Note that the minimum effective ODN lengths required for distal and medial joint formation were not determined.) Since branch migration can proceed from two junctions in a medial joint but from only one junction in a distal joint, the differential stability can be rationalized within the framework of the model presented here.

*Recognition of Homology.* On the basis of the affinity cross-linkage studies described here, we hypothesize that homologous alignment of a presynaptic filament with duplex DNA occurs from the minor groove without any triple-strand formation. As discussed earlier, the alkylation pattern obtained here differs from what models of the putative prestrand exchange triplex would predict (see Table 1). Furthermore, the exquisite sequence specificity of recipient strand alkylation would probably have been lost if the homology search occurred from the major groove. In that case the tethered chlorambucil of a homology-search complex would be expected to randomly alkylate guanines in the recipient and outgoing strands given that the N-7 position is exposed to the major groove and that the intimacy required for base pair scanning would position the tethered chlorambucil close enough to react with guanines in the duplex. Our failure to detect any random alkylation of either strand even when using a 10-fold molar excess of incoming ODN is understandable if the homology search occurs from the minor groove. Also understandable is the inability of recA to catalyze alkylation of the "outgoing strand" in the synthetic D-loop described earlier (see Figure 5B, lane 4). When incubated with this molecule recA might be expected to coat the single-stranded portion of the "outgoing strand" effectively converting it into an "incoming strand" which would now be part of a nonhomologous search complex. If this "incoming strand" associates with the minor groove of the adjacent duplex, no reaction would be expected with a chlorambucil moiety tethered to the major groove of this same duplex.

Residence of the outgoing strand in the major groove of the heteroduplex might be a direct consequence of the incoming strand approaching the duplex from the minor groove. Figure 8 shows that uptake of the incoming strand prior to strand exchange and release of the outgoing strand after strand exchange should take place in opposite grooves of the two duplexes. Hence, if the incoming strand searches for homology from the minor groove of the duplex, then the outgoing strand should be displaced to the major groove of the heteroduplex. By contrast, if homologous alignment occurs in the major groove of the duplex, then the outgoing strand should end up in the minor groove. Disposition of that strand in the major groove would require it to go around one of the two phosphodiester backbones of the heteroduplex. While we cannot exclude free interchange of the outgoing strand between minor and major grooves of the heteroduplex, we view it as unlikely. Given that alkylation of the outgoing strand occurred in the major groove of the heteroduplex, we propose that strand exchange takes place from the minor groove of the duplex.

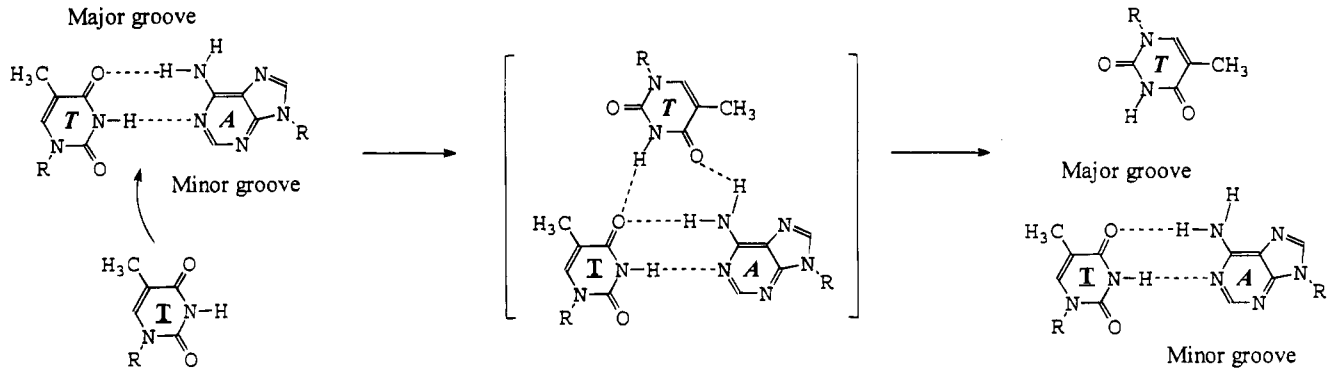



FIGURE 8: Model of strand exchange showing how uptake of the incoming base and expulsion of the outgoing base occur in opposite grooves of the DNA. In this example homologous alignment takes place from the minor groove of the duplex thus releasing the outgoing strand into the major groove of the heteroduplex. A poststrand exchange triplex can be incorporated into the pathway as an optional intermediate. The T-A-T triplet proposed by Chiu et al. (1993) is shown here. The incoming base is underlined, while the outgoing and recipient bases are italicized.

How might recognition be accomplished in a type 2 model? We note that the recA filament is able to bind both ssDNA and dsDNA in a deep groove formed by the filament itself. This groove has two binding sites which accommodate the single-stranded and double-stranded DNAs. Studies have shown that recA interacts with the phosphodiester backbone of ssDNA (Leahy & Radding, 1986) and with the minor groove of dsDNA (DiCapua & Muller, 1987; Dombroski et al., 1983). During the search for homology, the minor groove of the dsDNA presumably threads past recA which acts as a wedge to transiently disrupt base pairs. The unpaired bases of the outgoing strand interact with residues in the recA, leaving the bases of the recipient strand free to interact with the bases of the incoming strand. The unwound and extended conformation of the three strands in the recA filament should facilitate transient unpairing of the moving duplex. Once homologous alignment takes place, lateral movement of the duplex stops and strand exchange takes place.

Our model calls into question the role of triple-stranded complexes in mediating the homologous alignment of strands. Recognition of homology from the minor groove probably proceeds by a type 2 mechanism, since isomorphic triplets cannot be formed using the hydrogen bonding sites accessible in that groove. Although a triple-stranded complex might exist immediately after strand exchange, as previously discussed the alkylation pattern of the outgoing strand is consistent with an unpaired state. The close proximity of this strand to the major groove of the heteroduplex might result in chemical and enzymatic footprints which resemble a triple-stranded complex. Alternatively, a poststrand exchange triple strand might be the preferred configuration of certain distal joints under specific conditions. Recently, naked ODNs of varied sequence have been incubated with recA-coated duplex DNA in a reaction that is formally the reverse of classical recombination (Rao & Radding, 1994). This reaction yielded stable, degenerate complexes which were proposed to represent triple-stranded DNA held intact by the recA filament. In these triplexes the ODN presumably resided in the major groove of the duplex. It is not clear how the formation of these complexes relates to homologous recognition in the forward recA reaction.

*Cross-Linkage Efficiency.* As described above, alkylation of the recipient strand by chlorambucil took place in recA-stabilized joints that contained three-stranded DNA. Cross-linkage efficiency was never greater than 60%. We attribute this to the presence of competing nucleophiles, including water, ATPγS, purines in the incoming and outgoing DNA strands, and imino and sulfhydryl groups in recA itself. Siting of the chlorambucil near the end of a joint enhanced these competing reactions and was therefore avoided (M. A. Podyminogin and H. B. Gamper, unpublished observations). We cannot exclude the possibility that some joints failed to cross-link because they contained a poststrand exchange triplex with the structure proposed by Chiu et al. (1993) or Zhurkin et al. (1994).

Studies are planned to determine how the substitution of ATP for ATPγS modulates the efficiency and specificity of recipient strand cross-linkage by chlorambucil-containing ODNs. The level of cross-linkage observed here would not be possible if recA-stabilized medial and distal joints were unstable species. Given that the chlorambucil reacts with a half-life of 45 min at 37 °C, alkylation of the recipient strand must occur within a stable, long-lasting structure. The stability of synaptic joints formed in the presence of ATPγS has been noted previously (Golub et al., 1992). When ATP is employed, short presynaptic filaments are less stable (Leahy & Radding, 1986) and medial and distal joints undergo turnover (Burnett et al., 1994; Jain et al., 1995; Reddy et al., 1994). These phenomena may reduce the efficiency of recipient strand alkylation. It has also been observed that strand exchange in the presence of ATP can proceed past DNA lesions, mismatches, or short heterologous sequences (Bianchi & Radding, 1983; DasGupta & Radding, 1982; Kim et al., 1992; Livneh & Lehman, 1982). The specificity of the cross-linkage reaction would be decreased in the presence of ATP if alkylation occurred in mismatched heteroduplexes.

*Affinity Modification of Synaptic Complexes.* The wealth of information provided by chlorambucil-tethered incoming ODNs demonstrates the usefulness of affinity labeling reagents in unraveling the structure of synaptic intermediates. In these intermediates two of the three strands are identical. When a reactive molecule is conjugated to the incoming or outgoing strand, it becomes distinguished from its mate. Furthermore, conjugation uniquely positions the reagent within the complex such that otherwise reactive sites are obscured from modification. The specificity of reaction and its modulation by local structure make it a powerful technique. We intend to investigate the use of other affinity

labels appended to the incoming, outgoing or recipient strands of the joint.

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