RecA-Catalyzed, Sequence-Specific Alkylation of DNA by Cross-Linking Oligonucleotides. Effects of Length and Nonhomologous Base Substitutions

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ABSTRACT: Oligodeoxyribonucleotides (ODNs) bearing the reactive nitrogen mustard chlorambucil have been used as sequence-directed affinity labeling reagents to investigate the length and homology requirements for RecA-catalyzed alkylation of double-stranded DNA. The cross-linkage reaction, which takes place at the N-7 position of a targeted complementary strand guanine following strand exchange, was highly sequence specific with both a 272 bp DNA fragment and a linearized plasmid. Alkylation required the ODN to be at least 26 nucleotides long and to possess homology to the target in the vicinity of the modification site. The extent of alkylation was improved by using longer ODNs, with a 50-mer giving over 50% reaction. Mismatches inhibited alkylation when they perturbed the structure of the strand exchange product near the targeted guanine. Longer heterology also inhibited alkylation when it prevented strand exchange. Our inability to detect cross-linkage in stable synaptic complexes unable to undergo complete strand exchange is best explained by a model for homologous alignment in which the presynaptic filament approaches from the minor groove of the duplex. Since the N-7 position of guanine is in the major groove, it is inaccessible to the tethered chlorambucil group of the ODN during the search for homology. The reaction specificity of chlorambucil-bearing ODNs suggests that they may have general use as recombinase-mediated DNA targeting agents.

The sequence-specific targeting of an oligodeoxyribonucleotide (ODN)[1] to any arbitrary sequence in dsDNA remains an elusive goal. Such a technology could have obvious uses in molecular biology, diagnostics, and therapeutics. During the past decade considerable progress has been made in understanding how pyrimidine- or purine-rich ODNs recognize homopurine runs in dsDNA and form short triple-stranded complexes (Cheng & Pettitt, 1992; Helene, 1991). Unfortunately, extension of this approach to mixed sequences has been unsuccessful.

An alternative strategy, not limited to homopurine runs, for targeting dsDNA relies on synaptic complex formation catalyzed by a recombinase such as the *Escherichia coli* RecA protein (Cox, 1994; Kowalczykowski & Eggleston, 1994; Radding, 1991; West, 1992). When an ODN is complexed with RecA in the presence of ATPγS, the resultant nucleoprotein or presynaptic filament is able to form a stable synaptic complex with a homologous segment in long dsDNA (Golub et al., 1992; Hsieh et al., 1990, 1992). In the RecA-stabilized three-stranded DNA complex the "incoming" ODN appears to be Watson−Crick hydrogen bonded to the complementary "recipient" strand of the duplex, with the homologous "outgoing" strand residing unpaired in the major groove of the newly formed heteroduplex (Podyminogin et al., 1995). Synapsis is very rapid (Gonda et al., 1985) and efficient (Ferrin & Camerini-Otero, 1991), but mismatched complexes are readily formed, leading to reduced specificity (Hsieh et al., 1992). In non-supercoiled substrates, removal of RecA usually results in spontaneous release of the ODN from both matched and mismatched complexes (Adzuma, 1992; Hsieh et al., 1992, Chiu et al., 1993; Podyminogin et al., 1995).

Chlorambucil is an anticancer nitrogen mustard which primarily alkylates guanine N-7 and to a much lesser extent adenine N-3 in dsDNA (Povirk & Shuker, 1994). We have shown that ODNs which bear a chlorambucil group alkylate dsDNA within a model synaptic complex following strand exchange (Podyminogin et al., 1995). In that complex, the tethered chlorambucil moiety preferentially alkylated nearby guanines in the recipient strand of the original duplex.

An understanding of the efficiency and specificity of the alkylation reaction is important if chlormabucil-bearing ODNs are to be developed as sequence-specific DNA-targeting agents. Here we have determined the minimum ODN length required to support the specific alkylation reaction as well as the effect of mismatches on alkylation efficiency. DNA substates consisted of a linearized plasmid DNA and a PCR amplified fragment from that plasmid. Additionally, we investigated this reaction in a synaptic complex engineered to place the tethered chlorambucil moiety in a region of heterology. This model system allowed us to both investigate the mechanism of sequence searching and more stringently test the specificity of the alkylation reaction. Designed to mimic a pre-strand exchange homology search intermediate, we reasoned that cross-linkability would be determined by whether homologous alignment occurred from the major or minor groove of the duplex. Only a major groove orientation should support alkylation of guanine N-7. Our results indicate that RecA-coated, chlorambucil-bearing ODNs exhibited good sequence specific cross-

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[1] Abbreviations: ATPγS, adenosine 5′-[γ-thio]triphosphate; ds, double-stranded; ODN, oligodeoxyribonucleotide; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; ss, single-stranded.

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linkage and that the homology search occurred from the minor groove of the duplex.

MATERIALS AND METHODS

Proteins and Chemicals. Restriction endonucleases *Ase*I, *Ava*II, *Eco*RI, and *Sca*I were purchased from New England Biolabs. Proteinase K was from Boehringer Mannheim. RecA and T4 polynucleotide kinase were obtained from U.S. Biochemical. 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 that the RecA was free of detectable endonuclease and exonuclease activities. Chemicals (and their sources) 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. A 272 bp long dsDNA fragment derived from the *Eco*RI-linearized pGEM-4Z plasmid DNA (Promega) was prepared by PCR. One of the PCR primers was chemically phosphorylated to permit selective 5′-^{32}P end-labeling (by T4 polynucleotide kinase) of the recipient strand of the dsDNA product. pGEM-4Z DNA was prepared as described in Maniatis et al. (1982). The *Sca*I-linearized pGEM-4Z plasmid (2746 bp in length) was used as a long dsDNA substrate for the RecA reaction after 5′-^{32}P end-labeling of both strands using T4 polynucleotide kinase. Prior to use all end-labeled dsDNA substrates were purified by non-denaturing polyacrylamide or agarose gel electrophoresis 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. All ODNs were analyzed for purity by denaturing PAGE followed by silver staining. When necessary ODNs were further purified by preparative PAGE. Chlorambucil was conjugated to the ODNs through a 5-(3-aminopropyl)uracil base by postsynthetic acylation using the 2,3,5,6-tetrafluorophenyl ester of chlorambucil (Kutyavin et al., 1993). Chlorambucil-containing ODNs were kept in aqueous solution at −70 °C until use.

Standard Reaction Conditions. Reaction mixtures contained 100 nM (in molecules) incoming ODN, 10 nM (in molecules) dsDNA, and 2 μM RecA protein in 50 μL of 10 mM Tris-acetate buffer (pH 7.5), 50 mM sodium acetate, 12 mM magnesium acetate, 1 mM dithiothreitol, 1 mM ATPγS, and 5% glycerol. Following mixing of the reaction components at 4 °C, the complete reactions were incubated 6 h at 37 °C. At the end of this period, alkylation of the recipient strand by the tethered chlorambucil moiety had proceeded through 8 half-lives and was essentially complete.

Characterization of Alkylation Products. Reaction mixtures were diluted 2-fold with water, incubated for 30 min at 37 °C with 0.5% SDS and 200 μg of proteinase K/mL, extracted once with phenol−chloroform and three times with ether, and precipitated by ethyl alcohol. The recovered DNA was treated with 10% piperidine for 30 min at 95 °C to carry out depurination strand scission at sites of alkylated guanines. After alcohol precipitation the DNA pellet was washed with 70% ethanol, dried, and dissolved in 80% formamide containing 0.1% xylene cyanol and bromophenol blue. Aliquots were electrophoresed in a 4%−8% denaturing polyacrylamide gel. Sites of DNA cleavage were identified using Maxam and Gilbert (1977) sequencing ladders or restriction digests as standards. The extent of alkylation was determined using a Bio-Rad GS-250 phosphorimager.

RESULTS

Model System. Experiments were carried out with linearized pGEM-4Z DNA or a 272 bp DNA fragment derived from this plasmid by PCR amplification. Figure 1 shows the structures of "incoming" chlorambucil-bearing ODNs tested against the common sequence present in both double-stranded substrates. The ODNs are aligned above the targeted sequence to illustrate regions of homology and heterology. The region of homology was far removed from the ends of each of the two target duplexes, thus providing a general case for DNA targeting. The chlorambucil moiety was linked to a deoxyuridine which occupied the same position in every ODN. In the presence of RecA and ATPγS, some of these ODNs formed stable synaptic complexes with the duplex substrate, whereupon the tethered nitrogen mustard alkylated the complementary or "recipient" strand of the duplex. Although the formation of synaptic joints is known to be rapid, alkylation proceeded with a half-life of approximately 45 min (Kutyavin et al., 1993). Hydrolysis of chlorambucil and alkylation of RecA (data not shown) were competing reactions which reduced the overall level of DNA cross-linkage. To ensure complete reaction of the chlorambucil moiety, the highly stable synaptic complexes (Golub et al., 1992) were incubated 6 h prior to analysis. The primary guanine N-7 adduct did not undergo detectable depurination even when incubated overnight at 37 °C (data not shown).

The pattern and extent of alkylation were determined by treating the labeled DNA with hot piperidine prior to electrophoretic analysis. This treatment converted the alkylated guanines of the recipient strand into nicks. The efficiency with which each ODN listed in Figure 1 alkylated the duplex fragment varied widely (ranging from 0% to 56%) as the molar ratio of reactive ODN to target duplex was held constant at 10:1. These differences reflect the dependency of extent of synaptic complex formation and subsequent cross-linkage efficiency on length of the incoming ODN and degree of homology between the two DNA substrates.

Regardless of the ODN, the alkylation profile of the recipient strand was similar and a typical pattern is shown in Figure 2 for ODN **1**. Cross-linkage clearly required the presence of RecA and ATPγS. The guanine immediately 5′ to the T-A base pair flanking the *Ava*II recognition sequence in the substrate duplex was the primary alkylation site, with secondary alkylation limited to the nearest 3′ guanine and to the penultimate 5′ guanine. On the basis of this pattern, we have proposed that the incoming ODN in a RecA-stabilized synaptic complex is Watson−Crick base paired to the complementary recipient strand of the target duplex, thus displacing the now unpaired outgoing strand (Podyminogin et al., 1995). This would effectively substitute the chlorambucil-conjugated deoxyuridine of the ODN for the thymidine of the outgoing strand in the aforementioned base pair.

ODN	Sequence	Length
1	5′-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG	50
2	5′-AAAAAGCGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG	40
3	5′-TAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG	30
4	5′-AGCTCCTTCGGTCC**U**CCGATCGTTGTCA	28
5	5′-GCTCCTTCGGTCC**U**CCGATCGTTGTC	26
6	5′-CTCCTTCGGTCC**U**CCGATCGTTGT	24
7	5′-TCCTTCGGTCC**U**CCGATCGTTG	22
8	5′-GGTCC**U**CCGATCGTTGTCAG	20
9	5′-CCACCACATCGCCGCATAACCGATCCCTTCGGTCC**U**CCGATCGTTGTCAG	50
10	5′-ATGTTGTGCAAAAAAGCGGTTAGCTTTCCTAACTT**U**TTACCTACCACTGA	50
11	5′-CCACCACATCGCCGCATAACTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAG	50
12	5′-ATGTTGTGCAAAAAAGCGGTTAGCTCCTTCAACTT**U**TTACCTACCACTGA	50
13	5′-AAAAGCGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAGAAGTAAGTTG	49
14	5′-AAAAGCGGTTAGCTCCTTCGACCC**U**CCACTCGTTGTCAGAAGTAAGTTG	49
15	5′-AAAAGCGGTTAGCTCCTTCGACTC**U**CTACTCGTTGTCAGAAGTAAGTTG	49
16	5′-AAAAGCGGTTAGCTCCTTCGACTT**U**TTACTCGTTGTCAGAAGTAAGTTG	49

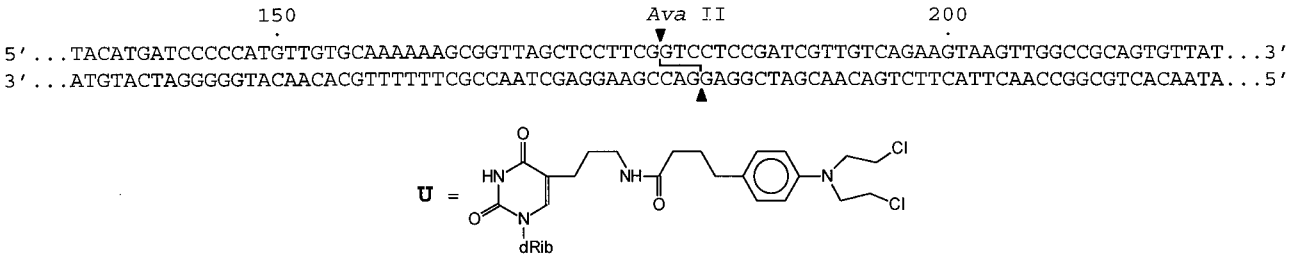

FIGURE 1: Structures of chlorambucil-bearing oligodeoxyribonucleotides used for synaptic complex formation. The sequences of the ODNs are aligned above the homologous region of the 272 bp fragment PCR-amplified from pGEM-4Z. Underlined bases in the ODNs are heterologous with respect to this substrate. We have used the terminology defined by Adzuma (1992) to describe the three strands of a synaptic complex. According this convention, the ODNs are incoming strands and the corresponding complementary and homologous strands of the starting duplex are the recipient and outgoing strands.

Length Dependence of Alkylation. In Figure 3 the extent of specific alkylation of the recipient strand of the 272 bp substrate is plotted as a function of the length of the incoming reactive ODN. The 50-mer alkylated 56% of the substrate, while the 30-mer alkylated 38%, at the same concentration. Below this length, the reaction efficiency decreased precipitously, with the 26-mer being the shortest ODN exhibiting appreciable cross-linking activity. The minimum ODN length needed to sustain alkylation cannot be explained by inability of the incoming ODN to form a nucleoprotein filament or by the failure of that filament to form a sequence-specific synaptic complex. RecA can stably associate with ODNs as short as 9-mers in the presence of ATPγS (Leahy & Radding, 1986), and presynaptic filaments as short as 20-mers can form stable synaptic complexes (Hsieh et al., 1992). Given that the chlorambucil reaction only takes place within a synaptic complex after strand exchange has occurred (Podyminogin et al., 1995), we believe that the extent of cross-linkage provides a gauge of the length dependence for strand exchange. Interestingly, Hsieh et al. (1992) found that a 26-mer was the shortest ODN able to form a D-loop in superhelical DNA following removal of RecA.

Specificity of Alkylation. Early-stage synaptic joint formation is not a highly sequence-specific process. RecA can catalyze the degenerate association of ODNs with both single- and double-stranded DNA (Golub et al., 1993; Hsieh et al., 1992; Rao & Radding, 1993, 1994) and can unwind up to 200−250 bp of heterologous double-stranded DNA during strand exchange (Jwang & Radding, 1992; Morel et al., 1994). Since we are interested in the use of reactive ODNs as RecA-catalyzed DNA-modifying agents, the nucleotide-targeting specificity of the chlorambucil reaction was investigated.

An initial concern was the possibility that chlorambucil-bearing ODNs might react in a random fashion with double-stranded DNA during the RecA-catalyzed homology-search process. The majority of recent studies assume that presynaptic filaments search for homology by scanning the major groove of dsDNA. Since the N-7 position of guanine is located in this groove, it seemed plausible that homology-search complexes could position the chlorambucil close enough to these sites to permit alkylation. To determine if this were so, we synthesized four chlorambucil-bearing 50-mers (ODNs **9**−**12**) each of which contained two sequence domains, one homologous and the other heterologous to the targeted duplex. These are shown in Figure 1, with the heterologous regions underlined. Two pairs of ODNs were generated: **9** and **11**, with the homologous domain at the 3′ end, and **10** and **12**, with the homologous domain at the 5′ end. ODNs **9** and **10** had a 25-base homology region, and ODNs **11** and **12** had a 30-base homology region. Generally, heterology was introduced by changing the correct bases in the ODN to the alternative pyrimidine or purine bases. All four ODNs contained the chlorambucil-conjugated deoxyuridine at the same relative position to the target strand, placing it in both homologous and heterologous domains (see Figure 1 for the alignments).

Alkylation of the recipient strand in synaptic complexes formed by each of the four incoming ODNs is shown in Figure 4. Negligible alkylation was observed when the

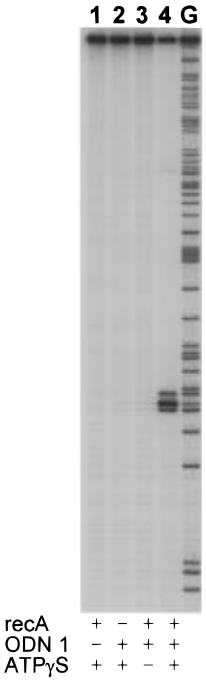

FIGURE 2: Alkylation-induced cleavage pattern of the recipient strand in a synaptic complex formed between ODN **1** and the 272 bp DNA fragment. Complete and control reaction mixtures were formed as indicated. Prior to analysis by denaturing PAGE, reaction aliquots were deproteinized and treated with hot piperidine. In this and subsequent figures, lanes G and A are the Maxam and Gilbert G and A + G sequencing reactions.

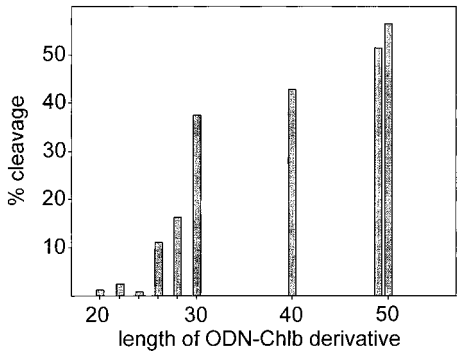

FIGURE 3: Efficiency of alkylation of the recipient strand in RecA-stabilized medial joints formed by chlorambucil-bearing incoming ODNs of variable length. Percent of alkylation of the 272 bp DNA fragment at recipient strand positions G-88, G-89, and G-91 was determined by phosphorimage analysis of dried gels.

chlorambucil residue was present in the heterologous domain of incoming ODNs **10** and **12** or in the 25-base homologous domain of ODN **9**. By contrast, placement of chlorambucil in the 30-base homologous domain of ODN **11** resulted in alkylation of the recipient strand. This is in excellent agreement with the minimum length requirements described earlier, and we infer that strand exchange and subsequent cross-linkage require at least 26 continuous homology matches regardless of the overall length of the incoming ODN.

The heterologous single-stranded overhang protruding from the above joints probably resembled a nucleoprotein filament in close association with dsDNA, such as would be the case in a homology-search complex. Because the homologous domain of each of these ODNs formed a stable sequence-specific synaptic complex with the DNA substrate (Hsieh et al., 1992), the protruding overhang cannot move

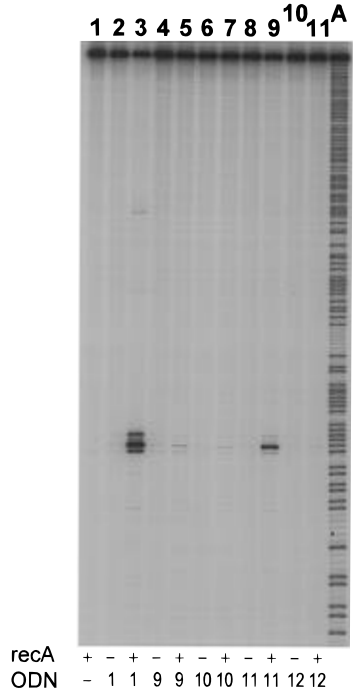

FIGURE 4: Alkylation-induced cleavage of the recipient strand in RecA-stabilized synaptic joints formed by the chimeric chlorambucil-bearing incoming ODNs **9**−**12**. As depicted in Table 1, each incoming 50-mer ODN had homologous and heterologous domains relative to the targeted 272 bp DNA fragment. The homologous domain was 25 nucleotides long in ODNs **9** and **10** and 30 nucleotides long in ODNs **11** and **12**. Chlorambucil was linked to the homologous domain of ODNs **9** and **11** and to the heterologous domain of ODNs **10** and **12**. Joint molecules were formed as usual and after workup the labeled recipient strand was analyzed for cleavage by denaturing PAGE.

and so can be likened to an immobile homology-search complex. In those overhangs which contained a chlorambucil-linked deoxyuridine, the nitrogen mustard might have alkylated the duplex if close contact were made with the major groove. Since this was not the case, presynaptic filaments of the type described here may not interact with the major groove of dsDNA in a homology-search complex.

Recently, Rao and Radding (1993) showed that an ODN identical in sequence but opposite in polarity to one of the two strands in a duplex could form a stable complex in the presence of RecA. This example of sequence selective recognition was interpreted to take place through hydrogen bonding of the ODN to the major groove of the duplex (Rao & Radding, 1994; Rao et al., 1995). We synthesized ODN **3** with reversed polarity and evaluated its ability to alkylate the recipient strand of the 272 bp fragment in the presence of RecA. No reaction was observed (data not shown). The putative sequence selective search complex formed by this ODN with the duplex fragment could not undergo strand exchange. If any alkylation had occurred, it would have taken place in an early-recognition complex. The inability of the tethered chlorambucil to react within such a complex is a further indication of the exceptional sequence specificity of the cross-linkage reaction and of its dependency upon RecA-mediated strand exchange.

Another concern with respect to specificity was cross-linking within stable, partially mismatched synaptic complexes. To address this question two, three, or four bases flanking each side of the chlorambucil-modified deoxyuridine in ODN **13** were changed to create mismatches relative to

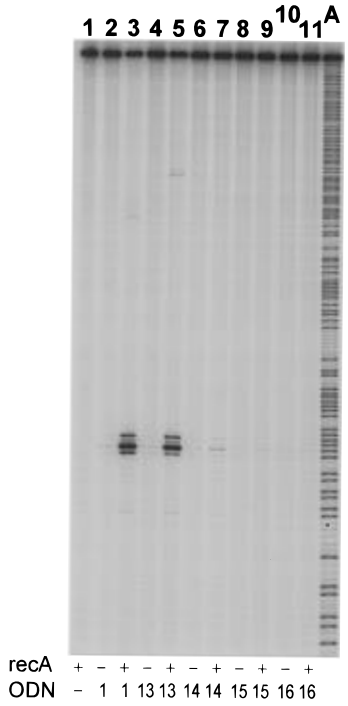

FIGURE 5: Alkylation-induced cleavage of the recipient strand in RecA-stabilized medial joints formed by mismatched chlorambucil-bearing incoming ODNs. Joint molecules were formed between the 272 bp DNA fragment and reactive 49-mers possessing 2, 3, or 4 mismatches (relative to the recipient strand of the fragment) on both sides of the chlorambucil-linked deoxyuridine (ODNs **14**−**16**, respectively). Joints formed by reactive ODNs **1** and **13** were completely matched and served as positive controls for alkylation. After workup of the reaction mixtures, the labeled recipient strand was analyzed for cleavage by denaturing PAGE.

the recipient strand of the duplex. In this series of 49-mers (**13**−**16**) the deoxyuridine was positioned in the middle of the ODN so that the sequences on both sides could still form stable synaptic complexes. Inhibition of *Ava*II restriction was used to verify that the mismatched ODNs formed RecA-stabilized joints (data not shown). Within these joints the chlorambucil residue was no longer able to efficiently alkylate the recipient strand (Figure 5). Reaction was completely abolished by six and eight mismatches (see lanes 9 and 11). In these synaptic complexes one or more of the targeted guanines in the recipient strand were involved in mismatches. When the joint contained four mismatches, reaction was barely detectable (see lane 7). In this joint the chlorambucil-linked deoxyuridine in the incoming ODN and the three reactive guanines in the recipient strand were not mismatched. The dependence upon the correct sequence is again consistent with the alkylation reaction taking place within a Watson−Crick base-paired hybrid.

To further delineate the effect of mismatches on the cross-linkage reaction, the 40-mers listed in Table 1 were evaluated. Relative to the perfectly matched control (ODN **17**), the rest of the ODNs contained one (ODNs **18**−**24**), two (ODNs **25**−**27**), or four (ODN **28**) mismatches. Negligible alkylation was observed when the joint had two or four mismatches near the chlorambucil-linked deoxyuridine or the reactive guanines. However, alkylation was not completely inhibited by the presence of only one mismatch. A single mismatch at or adjacent to the major alkylation site inhibited cross-linkage by 73% or 45%, whereas each of two mismatches 5′ to the chlorambucil linked deoxyuridine inhibited alkylation by approximately 60%. More distant mismatches had little effect on the cross-linkage reaction. We conclude that mismatch-induced perturbations in the vicinity of the cross-linkage site can partially or completely inhibit alkylation within synaptic complexes. In this context the cross-linkage reaction serves as an additional tier of discrimination to improve the specificity of chlorambucil-bearing ODNs.

Alkylation of Plasmid DNA. To determine whether the efficiency and specificity of the RecA-mediated alkylation reaction would be retained with a longer DNA substrate, the chlorambucil-bearing ODNs were incubated with a plasmid from which the 272 bp fragment had been PCR amplified. pGEM-4Z was linearized by digestion with *Sca*I and 5′ end-labeled. The linear DNA was incubated for 6 h in the presence of RecA with a 10-fold molar excess of ODN **1** or **3**. Following a second restriction of the deproteinized DNA, a portion of each sample was treated with hot piperidine. The products were analyzed on a denaturing PAGE gel. Autoradiography (see Figure 6) of the gel demonstrated RecA-dependent sequence-specific cross-linkage of the respective ODNs to the recipient strand (lanes 5 and 7) and subsequent nicking (lanes 11 and 13) of that strand adjacent to the *Ava*II site shown in Table 1 due to ODN-targeted chlorambucil alkylation. Use of 50- and 30-mer ODNs to deliver chlorambucil to the plasmid DNA generated cross-links with efficiencies of 75% and 50%, respectively. Interestingly, these efficiencies are significantly greater than those obtained using the shorter amplified fragment as a target and could reflect a preference for long substrates by RecA. On the other hand, random degradation of the longer DNA fragments during piperidine treatment could artifactually increase the cleavage frequency. These data confirm the suggestion that modified ODNs, with RecA catalysis, might sequence-specifically deliver reactive groups to targeted sites in more complex samples, such as genomic DNA.

DISCUSSION

We had previously shown that RecA-coated, chlorambucil-bearing ODNs can affinity cross-link to both strands of a homologous dsDNA in a putative post-strand exchange synaptic joint (Podyminogin et al., 1995). Here we have determined the length dependence and sequence specificity of the alkylation reaction. RecA-catalyzed cross-linkage required the incoming ODN to be at least 26 bases long and to possess no more than one mismatch to the recipient strand in the vicinity of the alkylation site. The extensive heterology found in an immobile homology-search complex did not support alkylation. As will be discussed, the distinctive cross-linkage patterns of the recipient and outgoing strands provide independent evidence that homologous alignment and strand exchange takes place from the minor groove of the targeted duplex and that alkylation is a post-strand exchange event. The sequence specificity of chlorambucil-bearing ODNs lead us to suggest that they might have potential uses as recombinase-mediated DNA-targeting agents.

Recognition of Homology. The lack of dectectable cross-linkage when the chlorambucil moiety was tethered to the heterologous portion of the ODN was unexpected in view of the popular belief that sequence recognition by a presynaptic filament occurs from the major groove of the duplex (Stasiak, 1992). Most current models for homology recognition invoke the formation of a DNA triple strand in which the bases of the incoming ODN are hydrogen bonded to base

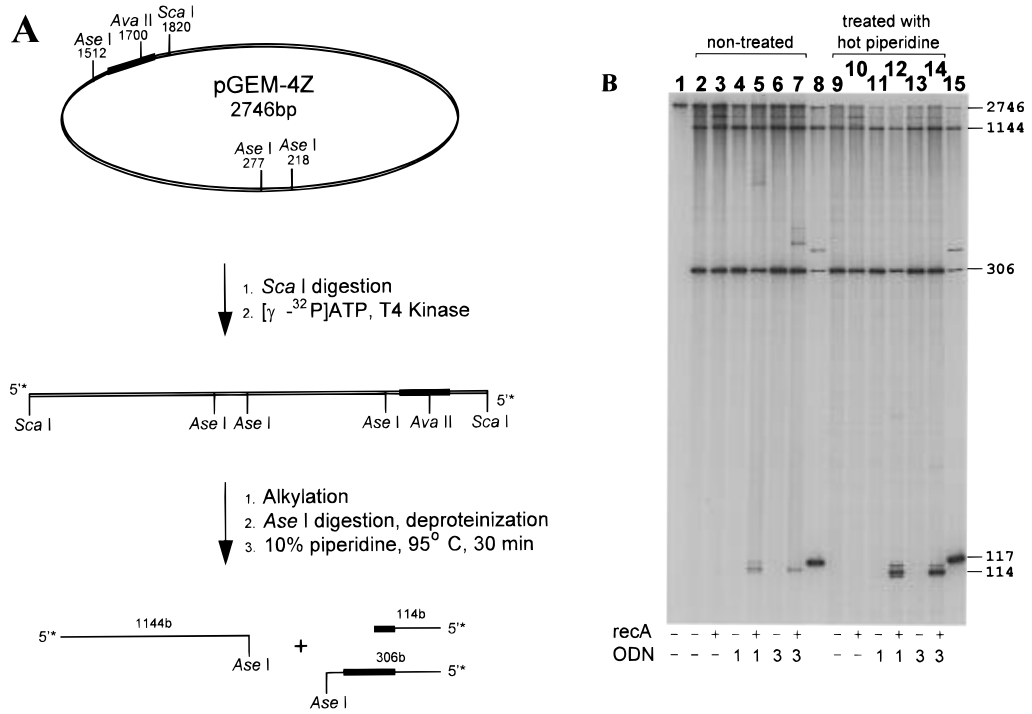

FIGURE 6: (A) Schematic representation of the sequence specific RecA-mediated cross-linkage and cleavage of *Sca*I-linearized pGEM-4Z plasmid DNA by 30- or 50-mer chlorambucil-bearing ODNs. (B) Electrophoretic analysis of the RecA-catalyzed, alkylation-induced nicking of pGEM-4Z DNA by ODNs **1** or **3**. End-labeled plasmid DNA (10 nM) was treated in the presence of RecA (2 μM) with the incoming ODN (0.1 μM) under standard reaction conditions. After deproteinization the plasmid DNA was restricted with *Ase*I and analyzed in a 4% denaturing polyacrylamide gel for cleavage of the recipient strand before (lanes 2−7) and after (lanes 9−14) treatment with hot piperidine. Lane 1, control FIII pGEM-4Z DNA; lanes 8 and 15, FIII pGEM-4Z DNA after sequential digestion with *Ava*II and *Ase*I.

Table 1: Inhibition of Alkylation-Induced Cleavage of the Recipient Strand in RecA-Stabilized Medial Joints Formed by Mismatched Chlorambucil-Bearing Incoming ODNs[a]

ODN	sequence	normalized % of cleavage
17	5'-CGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTCAGAAGTAA	100
18	5'-CGGTTAGCTCCTTCGGTCC**U**TCGATCGTTGTCAGAAGTAA	27
19	5'-CGGTTAGCTCCTTCGGTCC**U**CTGATCGTTGTCAGAAGTAA	55
20	5'-CGGTTAGCTCCTTCGGTCC**U**CCGCTCGTTGTCAGAAGTAA	91
21	5'-CGGTTAGCTCCTTCGGTCC**U**CCGATTGTTGTCAGAAGTAA	94
22	5'-CGGTTAGCTCCTTCGGTCC**U**CCGATCGTTGTTAGAAGTAA	95
23	5'-CGGTTAGCTCCTTCGGTCT**U**CCGATCGTTGTCAGAAGTAA	38
24	5'-CGGTTAGCTCCTTCGGTTC**U**CCGATCGTTGTCAGAAGTAA	43
25	5'-CGGTTAGCTCCTTCGGTCT**U**TCGATCGTTGTCAGAAGTAA	<1
26	5'-CGGTTAGCTCCTTCGGTCC**U**TTGATCGTTGTCAGAAGTAA	<1
27	5'-CGGTTAGCTCCTTCGGTTT**U**CCGATCGTTGTCAGAAGTAA	<1
28	5'-CGGTTAGCTCCTTCGGTTT**U**TTGATCGTTGTCAGAAGTAA	<1

[a] Joint molecules were formed between the 272 bp DNA fragment and reactive 40-mers possessing 1, 2, or 4 mismatches relative to the recipient strand of the fragment. Mismatched bases are underlined. **U**, chlorambucil conjugation site (see legeng to Figure 1). In each case the amount of cleavage was normalized relative to that obtained with a completely matched 40-mer (ODN **17**).

pair determinants accessible from the major groove (Rao et al., 1993; Zhurkin et al., 1994). If one of these models is correct, then alkylation of the duplex should have taken place within the immobile homology search complexes formed by ODNs **10** and **12**. The absence of alkylation suggested to us that homologous alignment of strands might involve the minor groove of the duplex. In this case the preferred reaction site for chlorambucil (the N-7 of guanine) would no longer be accessible and cross-linkage would not be observed. Inhibition of joint molecule formation by the minor groove binding agent distamycin is consistent with this alternative model (Kumar & Muniyappa, 1992).

Perhaps the most convincing evidence in support of a presynaptic filament scanning the target duplex from the minor groove are recently published affinity cross-linkage and cleavage studies conducted with RecA-coated ODNs

bearing a chlorambucil group (Podyminogin et al., 1995) or an Fe-EDTA group (Baliga et al., 1995) conjugated to the C-5 position of a deoxyuridine analog. According to our recent model (Podyminogin et al., 1995), these reactions probably took place in a RecA-stabilized, post-strand exchange joint in which the displaced outgoing strand resided in the major groove of a right-handed heteroduplex. From the perspective of the recipient strand, the tethered reagent occupied the major groove of the newly formed heteroduplex and as a result the alkylation or cleavage of this strand was shifted toward the 5′ end. However, from the perspective of the displaced outgoing strand, the same reagent occupied the minor groove of the now hypothetical duplex between outgoing and recipient strands. As a result, modification of the outgoing strand was shifted toward the 3′ end. The generation of these patterns by both tethered reagents is best explained by the initial homology-search taking place in the minor groove.

Double-Stranded DNA Modification by Reactive ODNs. The RecA-dependent alkylation of dsDNA by a chlorambucil-bearing ODN appears to be highly efficient and reasonably specific. In work to be described elsewhere, a semiquantitative ligation-mediated PCR technique has shown that alkylation of a single-copy gene in human genomic DNA occurred with an efficiency equal to that obtained with a simple plasmid. This specificity contrasts with the results of two previous reports (Cheng et al., 1988; Kosaganov et al., 1994) which employed reactive ODNs in conjunction with RecA to cross-link plasmid DNA. Differences in the type of cross-linking agent and the position of conjugation probably account for the reduced specificity relative to this study.

RecA-catalyzed cross-linkage of ODNs to dsDNA might have application in the physical mapping of chromosomes or the screening of recombinant DNA libraries. In the presence of RecA, chlorambucil-containing ODNs which are linked to a reporter group (such as biotin) or a cleaving agent (such as Cu-phenanthroline or Fe-EDTA) could be used to sequence specifically tag or cleave dsDNA. In these applications the chlorambucil-mediated cross-link would impart specificity to the RecA reaction by only permitting highly homologous joints to survive deproteinization.

Within the cell, it is conceivable that cross-linkable ODNs might be able to harness naturally occurring recombinases to alkylate and inactivate disease-causing genes much like antisense ODNs rely upon RNase H to cleave the mRNA hybridized within a DNA·RNA heteroduplex. Increasing evidence points to the existence of eucaryotic analogs of RecA (Ogawa et al., 1993; Shinohara et al, 1993; Story et al., 1993) and to the ability of eucaryotic cells to recombine short regions of homology (Ayares et al., 1986; Moerschell et al., 1988; Rubnitz & Subramani, 1984). While the homologous recombination frequency of transfected DNA with the genome of mammalian cells is very low (typically 10^{-4}−10^{-7} on a per cell basis; Capecchi, 1989), synaptic complexes should form with much greater efficiency (Thomas et al., 1986; Zheng & Wilson, 1990). The presence of a cross-linked ODN downstream from a promoter would be expected to block transcription (Grigoriev et al., 1993; Young et al., 1991). If repair of the cross-link was error prone, then targeted mutagenesis could permanently inactivate deleterious genes or correct genetic defects (Havre & Glazer, 1993).

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