Strand Invasion of Supercoiled DNA by Oligonucleotides with a Triplex Guide Sequence

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The use of oligodeoxynucleotides (ODNs) to control gene expression at the DNA level is hindered by sequence recognition strategies. Triplex formation by Hoogsteen hydrogen bonding of the ODN to purines in a homopurine run[1] is most often used, but this critical target sequence requirement limits the applicability of this approach. Ideally, one would like to be able to target any desired site in DNA. Although RecA protein can catalyze binding of ODNs to complementary sequences in DNA,[2] this method faces challenges for implementation in cells. The versatility of Watson−Crick sequence targeting could be realized by displacement loop (D-loop) formation, which, in principle, places no limits on targeting sequence. It does, however, present significant thermodynamic and kinetic barriers.[3] These might be overcome if (a) the rate of D-loop formation could be accelerated and (b) the D-loop could be stabilized.

DNA strand invasion has been observed with homopyrimidine peptide nucleic acids (PNA) at DNA homopurine runs,[4] where tightly bound PNA_2-DNA complexes are formed. Certain PNA oligomers can directly invade DNA to give Watson−Crick duplexes.[5] ODNs targeted to a cruciform in supercoiled DNA form stable D-loops,[6] and D-loop formation can be promoted by a nuclease conjugated to the ODN.[7] Additionally, Kobets et al. have shown that certain areas of chromatin may be available to Watson−Crick recognition and that this modification is eliminated by pretreatment with S1 nuclease.[8] We sought to induce D-loop formation in double stranded DNA using phosphodiester-based oligonucleotides and report here a new method for doing so. This method uses ODNs containing a triplex-forming guide sequence adjacent to the D-loop-forming domain. The guide sequence anchors the D-loop domain in proximity to its complement on the double stranded DNA and allows strand invasion to occur. Additionally, a reactive group in the D-loop domain permanently fixes that structure once formed. This strategy is diagrammed in Figure 1A.

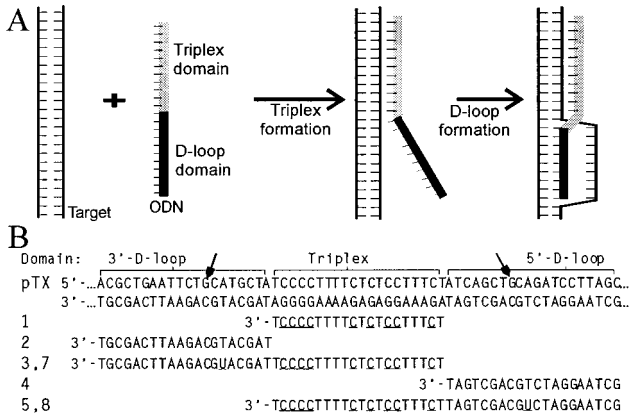

Figure 1. (A) Schematic of strand invasion of double stranded DNA by chimeric ODN. (B) Model system for evaluating triplex-guided D-loop formation. Partial pTX sequence is at the top, with regions for triplex and D-loop formation indicated. *C* = 5-methyl-2′-deoxycytidine; U = 5-(3-aminopropyl- or 3-(bromoacetamido)propyl)-2′-deoxyuridine (in 3 and 5 or 7 and 8, respectively). Arrows denote the guanine bases in the plasmid alkylated by 7 and 8.

ODNs are listed in Figure 1B. The triplex guide sequence present in these ODNs was designed to form a pyrimidine motif triplex[9] with a 20-base homopurine run engineered into plasmid pGFIB1 to give plasmid pTX. The D-loop region of the ODN was targeted to sequences flanking either one side or the other of this homopurine run. ODN **1** contains only the triplex domain and ODNs **2** and **4** contain only the D-loop domains. ODNs **3** and **5** are chimeras with the D-loop domain linked 3′- and 5′-, respectively, to the triplex guide sequence. ODNs **7** and **8** are identical to ODNs **3** and **5** but contain a reactive bromoacetamido group[10] on a dUrd in the D-loop domain. For a target, we prepared linearized (lin), supercoiled (sc), and highly supercoiled (hsc) forms[11] of pTX because negative supercoiling reduces the kinetic and thermodynamic barriers to D-loop formation.[3,6,4c] To control for sequence specificity, we used a highly supercoiled (hsc) preparation of plasmid pGFIB1, which lacks the homopurine run but contains the same flanking D-loop domain sequences as pTX.

Complex formation was determined by agarose gel electrophoresis after incubation[12] of ODN with plasmid for 42 h at pH 6.0. This pH allows the triplex domain of the ODNs to bind its target homopurine run on pTX. When the gel was run at pH 6.0

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(1) (a) Plum, G. E.; Pilch, D. S.; Singleton, S. F.; Breslauer, K. J. *Annu. Rev. Biophys. Biomol. Struct.* **1995**, *24*, 319−350. (b) Sun, J. S.; Garestier, T.; Helene, C. *Curr. Opin. Struct. Biol.* **1996**, *6*, 327−333.
(2) (a) Hsieh, P.; Camerini-Otero, C. S.; Camerini-Otero, R. D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 6492−6496. (b) Adzuma, K. *Genes Dev.* **1992**, *6*, 1679−1694. (c) Podyminogin, M. A.; Meyer, R. B.; Gamper, H. B. *Biochemistry* **1996**, *35*, 7267−7274. (d) Golub, E. I.; Glazer, P. M.; Ward, D. C.; Radding, C. M. *Mutation Res.* **1996**, *351*, 117−124.
(3) Holloman, W. K.; Wiegand, R.; Hoessli, C.; Radding, C. M. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 2394−2398.
(4) (a) Nielsen, P. E.; Egholm, M.; Berg, R. H.; Buchardt, O. *Science* **1991**, *254*, 1497−1500. (b) Peffer, N. J.; Hanvey, J. C.; Bisi, J. E.; Thomson, S. A.; Hassman, C. F.; Noble, S. A.; Babiss, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10648−10652. (c) Cherny, D. Y.; Belotserkovskii, B. P.; Frank-Kamenetskii, M. D.; Egholm, M.; Buchardt, O.; Berg, R. H.; Nielsen, P. E. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1667−1670.
(5) (a) Wittung, P.; Nielsen, P.; Norden, B. *Biochemistry* **1997,** *36*, 7973−7979. (b) Nielsen, P. E.; Christensen, L. *J. Am. Chem. Soc.* **1996**, *118*, 2287−2288.
(6) Iyer, M.; Norton, J. C.; Corey, D. R. *J. Biol. Chem.* **1995**, *270*, 14712−14717.
(7) Corey, D. R.; Munoz-Medellin, D.; Huang, A. *Bioconjug. Chem.* **1995**, *6*, 93−100.
(8) Kobets, N. D.; Borissov, R. G.; Chernolovskaya, E. L.; Gorn, V. V.; Vlassov, V. V. *Antisense Res Dev* **1994,** *4*, 259−262.
(9) Maher, L. J.; Dervan, P. B.; Wold, B. J. *Biochemistry* **1990**, *29*, 8820−8826.
(10) (a) Meyer, R. B.; Tabone, J. C.; Hurst, G. D.; Smith, T. M.; Gamper, H. B. *J. Am. Chem. Soc.* **1989**, *111*, 8517−8519. (b) Tabone, J. C.; Stamm, M. R.; Gamper, H. B.; Meyer, R. B. *Biochemistry* **1994**, *33*, 375−383.
(11) Supercoiled monomeric DNA was isolated from purified plasmid preparations by gel electrophoresis in 0.8% low melting agarose followed by treatment with exonuclease III and extraction with benzoylated naphthoylated DEAE-cellulose, see: Gamper, H. G.; Lehman, N.; Piette, J.; Hearst, J. E. *DNA* **1985**, *4*, 157−164. Plasmids extracted from *E. coli* usually have a mean superhelical density (s) of approximately −0.06, see: Courey, A. J.; Wang, J. C. *Cell* **1983**, *33*, 817−829. Highly supercoiled target plasmid pTX or control plasmid pGFIB1 were prepared from form I DNA and had a $s = -0.163$, see: Singleton, C. K.; Wells, R. D. *Anal. Biochem.* **1982**, *122*, 253−257. Restriction of pTX with Cla I generated linear DNA ($s = 0$).
(12) All incubations were for 42 h at room temperature in 25 mM sodium cacodylate, pH 6.0, and 10 mM $MgCl_2$. After electrophoresis, labeled products were detected by autoradiography.

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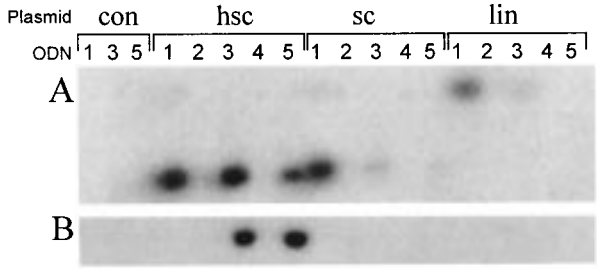

Figure 2. Complexes formed by chimeric ODNs and pTX. The ^{32}P end-labeled ODNs (3.6 nM) were incubated 42 h at room temperature with 36 nM control (con) hsc pGFIB1[11] or with either hsc, sc, or lin[11] pTX forms. Nondenaturing electrophoresis was through 0.8% agarose gels in either (panel A) 90 mM Tris acetate, pH 6.0, and 1 mM $Mg(OAc)_2$ (14 h at 20 V) for detection of triple-stranded complexes or (panel B) 90 mM Tris-borate, pH 8.0 and 2 mM EDTA (1.5 h at 100 V) for detection of D-loop complexes. The plasmid DNA forms were identified by staining a separate gel with ethidium bromide (data not shown).

(Figure 2A), existence of the triplex was confirmed: ODNs **1**, **3**, and **5** formed stable complexes with hsc pTX but did not bind to hsc pGFIB1. ODNs **2** and **4** (D-loop domains only) did not show complexes with any plasmid when assayed at this pH. At pH 8.0 (Figure 2B), stable D-loop complexes would be detected under conditions where the triplex did not exist. In this gel, chimeric ODNs **3** and **5** were bound to hsc pTX, indicating the strand invasion complexes, but not to hsc pGFIB1. No other complexes were observed. Although the guide sequence (ODN **1**) also formed a stable triplex with supercoiled and linearized pTX at pH 6, ODNs **3** and **5** did not (Figure 2A). This could indicate that the D-loop domains of these ODNs destabilized the triplex.

Since these D-loops may be transient in the absence of superhelical strain, we attached a reactive bromoacetamidopropyl group to the D-loop domain in ODNs **7** and **8** to trap this potentially short-lived intermediate with a covalent cross-link. We have previously shown that this side chain, on a deoxyuridine in a 5′-UGC context, efficiently alkylates the guanine residue in the complementary 5′-GCA sequence.[10a] The site of alkylation is readily demonstrated by heat/piperidine-induced strand scission.[10a] Each of the two D-loop domains contained the requisite 5′-GCA sequences. ODNs **7** and **8** were incubated with pTX and analyzed by restriction with Pvu II and denaturing PAGE after heat/piperidine-induced strand scission (Figure 3A). The cleavage patterns indicated that both ODNs alkylated only the targeted guanine residues of pTX and did so with exceptional selectivity. Cleavage at the ODN **7** target site generated a 140 base fragment. The cleavage product of the ODN **8** target site (a fragment 175 bases in length) was obscured by a Pvu II restriction fragment of nearly the same length. As an additional indication of D-loop formation, however, the D-loop domain of ODN **8** covered a Pvu II site (5′-CAGCTG) and inhibited restriction of the displaced strand at that point. This generated the fragment 304 bases in length seen in Figure 3A. Both ODNs reacted most strongly with hsc pTX, although there is an indication of reaction with sc pTX.

Further evidence of D-loop formation was provided by the S1 nuclease cleavage pattern shown in Figure 3B. Brief (15 min) treatment of the complexes formed between chimeric ODN **3** or **5** and either supercoiled or highly supercoiled pTX gave cleavage only in the homologous strand and only at the sites corresponding to the D-loop domains of the respective ODN. This is indicative of the single stranded character of the homologous strand at these sites. Even though sc pTX did not show a D-loop with ODN 3 or 5 in the gel shift assay (Figure 2B), the S1 nuclease cleavage shows the ODN 5 forms a D-loop with the 5′-domain. This was also seen in the cross-linking pattern of ODN 8 with sc pTX (Figure 3A). Lack of detectable cleavage in the control lanes

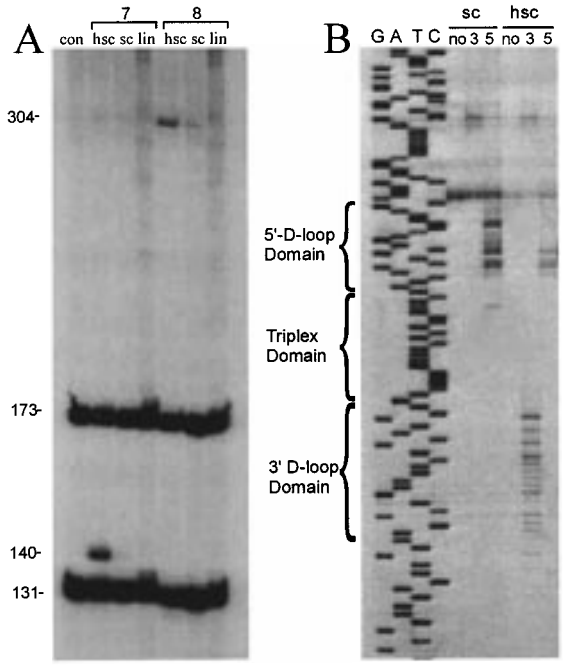

Figure 3. A. Targeted alkylation and inhibition of Pvu II restriction by chimeric ODNs. ODNs **7** and **8** (820 nM) were incubated[12] with 3.85 nM control (con) hsc pGFIB1 or pTX (forms hsc, sc, and lin; molar ratio of ODN to plasmid = 212:1). Reactions were diluted into 10 mM Tris, pH 8.0, containing 1 mM EDTA, processed through a Centricon 30 membrane to remove excess ODN, and restricted with Pvu II to generate three fragments 131, 173, and 3632 bp in length. Fragments were visualized after end-labeling, treatment with hot piperidine for 20 min at 90 °C, lyophilization, and 6% denaturing PAGE. Targeted alkylation of pTX by ODNs **7** and **8** occurs at guanine residues 193 and 228 of the pyrimidine-rich strand, whereas Pvu II restriction occurs 5′ of positions 53, 226, and 357. B. S1 nuclease cleavage of D-loops. ODNs (3 μM) were incubated[12] with 30 nM sc or hsc pTX, followed by 2.5 units of S1 nuclease for 15 min. Products were isolated by addition of EDTA to a final concentration of 50 mM, then extraction with phenol/chloroform and ethanol precipitation. Primer extension (primer: 5′CCCTGGCGC-CGCTTCTTTGAG, 104 nt upstream from the *Eco*RI restriction site used for inserting the triplex-forming region, of the purine-containing strand was with Sequenase (Amersham Life Science Inc., Cleveland, OH) in the presence of [α-^{35}S]ATP. Lanes are labeled with the pTX form and ODN used; no = no ODN.

containing no ODN indicates that this highly supercoiled plasmid is indeed double stranded in the region shown in Figure 3B.

These results show that appropriately designed ODNs can invade supercoiled double stranded DNA at sites apparently undistinguished by unusual primary or secondary structure. These chimeric ODNs form sequence specific complexes composed of adjacent triplex and D-loop regions. Our design allowed triplex formation to orient the Watson−Crick domain of the chimera so that it is poised to hybridize with its complementary strand in the adjacent duplex, perhaps during DNA breathing. D-loop formation was demonstrated by gel mobility, affinity alkylation, and S1 nuclease cleavage. The unrestrained superhelical state of transcriptionally active genes[13] may render them particularly susceptible to targeting in this manner. This approach may expand the recognition repertoire and application breadth of DNA-targeted ODNs, especially if implemented with short triplex domains and enhanced binding affinity in both domains of the chimera.

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(13) (a) Jupe, E. R.; Cartwright, I. L. *EMBO J.* **1993**, *12*, 1067−1075. (b) Ljungman, M.; Hanawalt, P. C. *Nucleic Acids Res.* **1995**, *23*, 1782−1789.