Effect of a Triplex-Binding Ligand on Parallel and Antiparallel DNA Triple Helices Using Short Unmodified and Acridine-Linked Oligonucleotides†

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Received July 20, 1994; Revised Manuscript Received October 14, 1994⊗

ABSTRACT: We have used DNase I footprinting to investigate the effect of a triplex-binding ligand on the formation of intermolecular DNA triple helices at target sites that have been cloned into longer DNA fragments. In the presence of a triplex-binding ligand (*N*-[2-(dimethylamino)ethyl]-2-(2-naphthyl)quinolin-4-ylamine), the concentrations of T_5C_5 and C_5T_5 required to generate DNase I footprints at the target sites $A_6G_6{\cdot}C_6T_6$ and $G_6A_6{\cdot}T_6C_6$, respectively, are reduced by at least 100-fold. Complexes with the acridine-linked oligonucleotides Acr-T_5C_5 and Acr-C_5T_5 are stabilized to a much lesser extent and produce footprints at concentrations similar to those of the unmodified oligonucleotides in the presence of the ligand. The stabilizing effects of acridine modification or the addition of a triplex-binding ligand are not additive. The position and length of the footprints produced by Acr-T_5C_5 and T_5C_5 at the target sequence $A_6G_6{\cdot}C_6T_6$ are unaffected by the ligand. In contrast, footprints at the target site $G_6A_6{\cdot}T_6C_6$ appear 3−4 bases shorter in the presence of the ligand, when viewed from the pyrimidine strand, and 1−2 bases longer on the purine strand. These results are explained by suggesting that the compound binds at T·AT triplets and prevents the transmission of any DNA structural changes into the flanking duplex. The compound has a smaller stabilizing effect on short antiparallel triplexes consisting of G·GC and T·AT triplets. Binding of Acr-G_5T_5 to $A_6G_6{\cdot}C_6T_6$ is enhanced slightly by the compound, which increases the apparent footprinting site, probably by preventing fraying at the 3′-end of the third strand. The compound does not promote the binding of G_5T_5 to $A_6G_6{\cdot}C_6T_6$ or that of Acr-T_5G_5 and T_5G_5 to $G_6A_6{\cdot}T_6C_6$.

Oligonucleotide-directed triple helix formation offers the possibility of achieving sequence specific recognition of DNA and may be useful for designing compounds targeted at individual genes as antiviral or anticancer agents (Moffat, 1991; Chubb & Hogan, 1992). In this approach, a short oligonucleotide binds within the DNA major groove, generating a triple-helical complex in which the third strand is held in place by specific hydrogen bonds to substituents on the duplex base pairs. Since the discovery of poly(A)·2poly(U) in 1957 (Felsenfeld & Rich, 1957), two classes of intermolecular triple helix have been characterized that differ according to the orientation of the third strand.

Structures in which the third strand runs parallel to the duplex purine strand generally are formed by pyrimidine-rich oligonucleotides and are characterized by the formation of T·AT and C^+·GC triplets (Moser & Dervan, 1987; LeDoan *et al.*, 1987; Cooney *et al.*, 1988). The requirement for cytosine protonation means that these structures are only stable at low pH's. Within this motif, other triplets, including G·TA (Griffin & Dervan, 1989) and G·GC (Giovannangeli *et al.*, 1992b), have also been described. In the alternative motif the third strand runs antiparallel to the duplex purine strand and generally is purine-rich. This structure is characterized by G·GC, A·AT and T·AT triplets (Beal & Dervan 1991; Chen, 1991; Pilch *et al.*, 1991). The formation of these triplets is pH independent, although both triplex motifs require the presence of divalent metal ions, especially magnesium.

Although triplex-forming oligonucleotides may be endowed with considerable sequence selectivity, their binding is not strong. One strategy to enhance triplex stability is to attach a DNA-binding agent such as acridine (Sun *et al.*, 1989; Collier *et al.*, 1991), ellipticine (Perrouault *et al.*, 1990), or psoralen (Takasugi *et al.*, 1991; Giovannangeli *et al.*, 1992a) to one end of the triplex-binding oligonucleotide. An alternative approach, with which this paper is concerned, is to design compounds that bind strongly and specifically to triplex, but weakly to duplex, DNA. Several such compounds have been described, including the benzopyroindole derivatives BePI and BgPI (Mergny *et al.*, 1992; Pilch *et al.*, 1993) and coralyne (Lee *et al.*, 1993). BePI has been reported to stabilize triple helices that are rich in T·AT triplets, whereas coralyne exhibits little sequence-binding preference. Intercalation alone cannot account for their effect on triplexes, since most classical intercalators, including ethidium, do not selectively stabilize triplex DNA (Scaria & Shafer, 1991; Mergny *et al.*, 1991).

Wilson *et al.* (1993) designed a series of triplex-stabilizing ligands that are proposed to function by selectively intercalating into triple-helical DNA. The structure of the first of these compounds is shown in Figure 1. These were designed with three factors in mind: (i) the need for a large aromatic surface area for stacking with the three bases in the triplet; (ii) the aromatic system should possess some flexibility since third strand bases are not necessarily coplanar with the duplex base pairs; and (iii) they should be cationic to complement the high negative charge density of the triplex. These

† This work was supported by grants from the Cancer Research Campaign, the Science and Engineering Research Council, and the Medical Research Council. K.R.F. is a Lister Institute Research Fellow.

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⊗ Abstract published in *Advance ACS Abstracts,* November 15, 1994.

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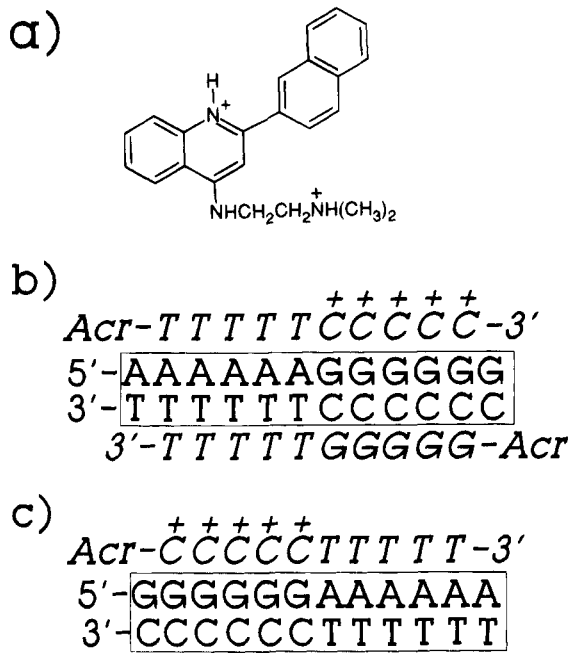

FIGURE 1: (a) Structure of triplex-binding ligand **1** (Wilson *et al.*, 1993). (b and c) Successful triple helices formed at target sites $A_6G_6{\cdot}C_6T_6$ and $G_6A_6{\cdot}T_6C_6$. The duplex is boxed. The third strand oligonucleotides are shown above the duplex form parallel triplexes, consisting of $C^+{\cdot}GC$ and T·AT triplets. In part b, the oligonucleotide shown below the duplex corresponds to an antiparallel triplex consisting of G·GC and T·AT triplets.

compounds have been shown to stabilize poly(dA)·2poly(dT) to a much greater extent than the corresponding duplex (Wilson *et al.*, 1993).

In this paper, we examine the effect of one of these ligands (shown in Figure 1a) on the formation of both unmodified and acridine-linked triplexes at the target sites $A_6G_6{\cdot}C_6T_6$ and $G_6A_6{\cdot}T_6C_6$, generating the structures shown in Figure 1b, c. It has previously been shown that these duplexes can be selectively bound by Acr-T_5C_5 and Acr-C_5T_5, respectively, forming parallel triplexes containing T·AT and $C^+{\cdot}GC$ triplets (Stonehouse & Fox, 1994; Fox, 1994), which are stable at low pH's. The unmodified oligonucleotides bind less well and only produce clear footprints at low temperatures. Attempts to form similar antiparallel triplexes containing G·GC and T·AT triplets at these sites have been less successful (Fox, 1994). The unmodified oligonucleotides G_5T_5 and T_5G_5 show no interactions, even at low temperatures. Although Acr-G_5T_5 binds to $A_6G_6{\cdot}C_6T_6$, no binding could be detected between Acr-T_5G_5 and $G_6A_6{\cdot}T_6C_6$. This was explained by the suggestion that the antiparallel T·AT triplet is weaker than G·GC and that separation of the acridine and the guanines does not provide a nucleation center strong enough to generate a stable complex (Fox, 1994).

In this paper, we examine the effect of a triplex-binding ligand on the formation of these acridine-linked and unmodified triplexes. In each case, the target sites have been cloned into longer DNA fragments, and the relative binding strength has been assessed by determining the concentration of the third strand required to generate a clear DNase I footprint.

MATERIALS AND METHODS

Chemicals and Enzymes. The acridine-linked oligonucleotides Acr-G_5T_5, Acr-T_5G_5, Acr-T_5C_5, and Acr-C_5T_5 were gifts from Dr. M. J. McLean (Cambridge Research Biochemicals). In these modified oligonucleotides, the 6-chloro-2-methoxyacridin-9-amine is linked to the 5'-end of the oligonucleotide by a pentamethylene chain. They were dissolved in water at a concentration of 360 μM and stored at −20 °C. Unmodified oligonucleotides G_5T_5, T_5G_5, T_5C_5, and C_5T_5 were synthesized on an Applied Biosystems 380B DNA synthesizer and stored at a stock concentration of 1 mM at −20 °C. Triplex-binding ligand **1** (Figure 1a) was prepared as previously described (Wilson *et al.*, 1993) and stored at −20 °C as a 20 mM solution in dimethyl sulfoxide. DNase I was purchased from Sigma and stored at −20 °C as a 7200 units/mL solution in 0.15 M NaCl containing 1 mM $MgCl_2$. Restriction enzymes and reverse transcriptase were purchased from Promega.

DNA Targets. The preparation of plasmids containing the inserts $G_6A_6{\cdot}T_6C_6$ (pGA1) and $A_6G_6{\cdot}C_6T_6$ (pAG1) has been described previously (Stonehouse & Fox, 1994; Fox, 1994). These were cloned into the *Bam*HI site of the pUC18 polylinker. For pGA1 the insert is oriented so that the pyrimidine strand is visualized by labeling the 3'-end of the *Hin*dIII site, whereas pAG1 is inserted in the opposite orientation so that the purine strand is visualized by labeling the 3'-end of the *Hin*dIII site.

DNA fragments containing these inserts were obtained by cutting the plasmids with *Hin*dIII, labeling at the 3'-end using [α-^{32}P]dATP and reverse transcriptase, and cutting again with *Eco*RI. In some instances the other strand was labeled by reversing the order of addition of *Hin*dIII and *Eco*RI. The labeled fragments of interest were separated from the rest of the plasmid DNA on 8% nondenaturing polyacrylamide gels.

DNase I Footprinting. Radiolabeled DNA (1.5 μL), dissolved in 10 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA (at approximately 10 nM strand concentration), was mixed with 1.5 μL of oligonucleotide at a concentration between 150 and 0.15 μM and either 1.5 μL of **1** (30 μM, so as to give a final concentration of 10 μM) or 1.5 μL of buffer. In order to minimize differences in the oligonucleotide concentrations between the ligand-treated and ligand-free samples, these were always prepared in parallel, using the same dilution from the stock oligonucleotide. In this way, although there may be variations in the exact oligonucleotide concentration between experiments, the difference in the concentration within each experiment is kept to a minimum. The oligonucleotides G_5T_5, T_5G_5, Acr-G_5T_5, and Acr-T_5G_5 were diluted in 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$; T_5C_5, C_5T_5, Acr-T_5C_5, and Acr-C_5T_5 were diluted in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. Samples were allowed to equilibrate at room temperature for at least 30 min, before digestion with 2 μL of DNase I as previously described (Stonehouse & Fox, 1994; Fox, 1994). The reactions were terminated after 1 min by the addition of 4.5 μL of stop solution containing 80% formamide, 10 mM EDTA, and 0.1% (w/v) bromophenol blue. Samples were heated to 100 °C for 3 min prior to electrophoresis.

Gel Electrophoresis. The products of DNase I digestion were separated on 10% (*Hin*dIII-labeled) or 13% (*Eco*RI-labeled) polyacrylamide gels containing 8 M urea. These were run for about 2 h at 1500 V. Gels were then fixed in 10% (v/v) acetic acid, transferred onto Whatman 3MM paper, dried under vacuum at 80 °C for 1 h, and subjected to autoradiography at −70 °C with an intensifying screen.

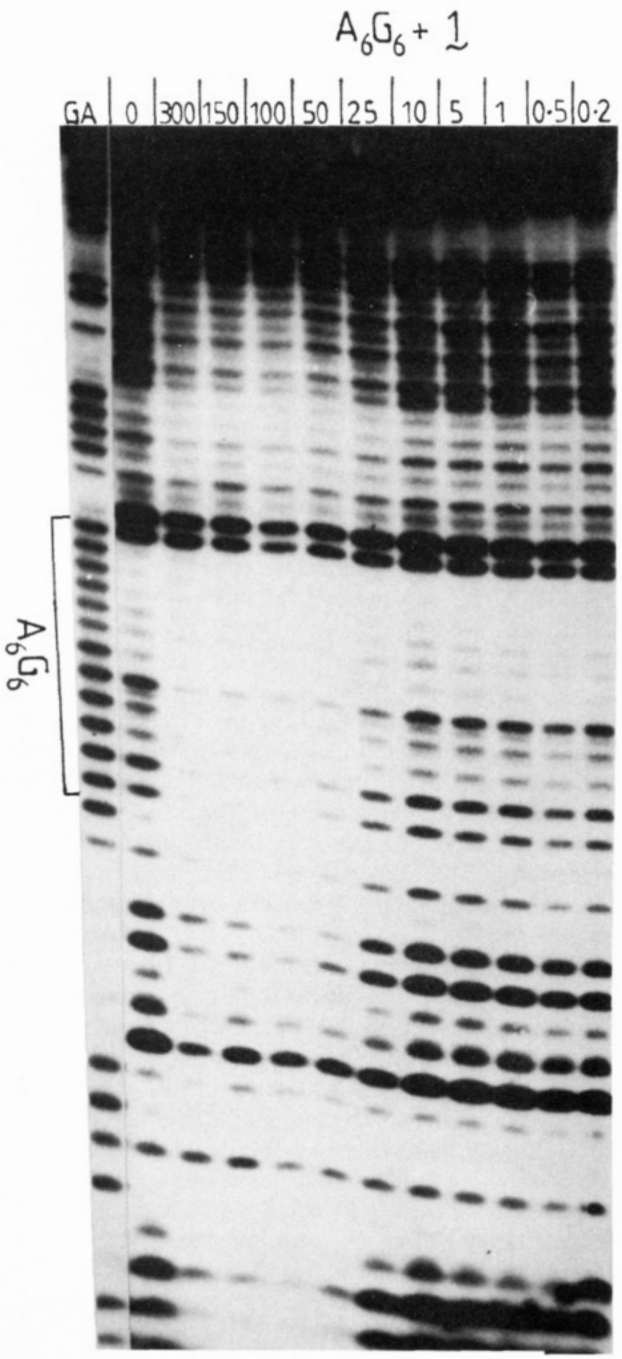

FIGURE 2: Effect of triplex-binding ligand **1** on DNase I digestion of a fragment containing the insert A_6G_6•T_6C_6. The DNA was labeled at the 3′-end of the *Hin*dIII site, revealing the purine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The concentration of **1** (micromolar) is shown at the top of each gel lane. The square brackets show the position and length of the target sequence A_6G_6. The lane labeled GA is a Maxam−Gilbert formic acid−piperidine marker specific for guanine and adenine.

Bands in the digests were assigned by comparison with Maxam−Gilbert dimethyl sulfate−piperidine markers specific for guanine.

RESULTS

Interaction with Duplex DNA. Although compound **1** was designed to bind selectively to triple-helical DNA, it does show some interaction with duplex DNA, raising the T_m of poly(dA)•poly(dT) by 5.5 °C at a drug/phosphate ratio of 0.2 (Wilson *et al.*, 1993). We therefore examined the effect

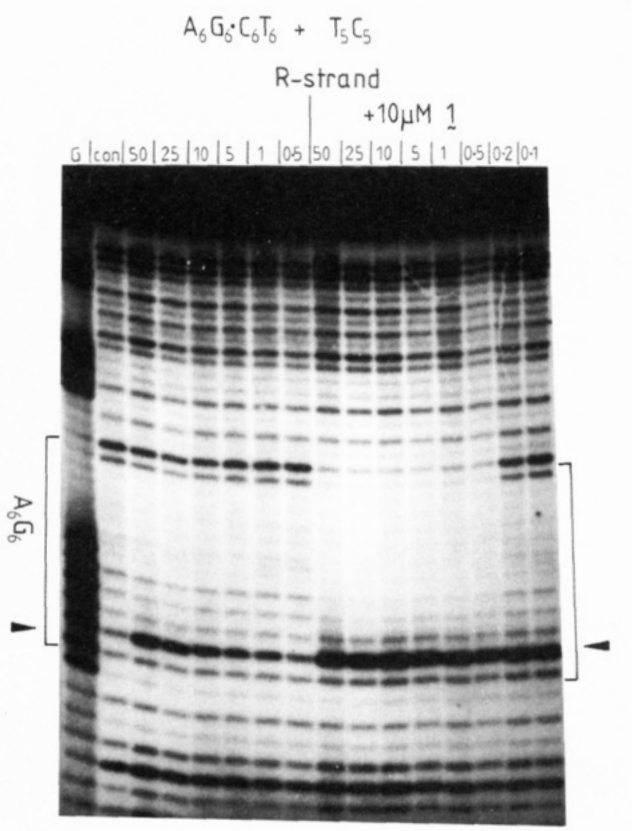

FIGURE 3: Effect of triplex-binding ligand **1** on the interaction of T_5C_5 with a DNA fragment containing the insert A_6G_6•C_6T_6. The DNA was labeled at the 3′-end of the *Hin*dIII site, revealing the purine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence A_6G_6. The lane labeled G is a Maxam−Gilbert dimethyl sulfate−piperidine marker specific for guanine. The arrows show the position of enhanced cleavage at the triplex−duplex boundary.

of this ligand on DNase I digestion of the duplex target sites, before studying its effect on intermolecular triple-helix formation. The results for the interaction with a fragment containing the target site A_6G_6•C_6T_6 at pH 5.5 are presented in Figure 2. It can be seen that concentrations of 25 μM and below do not affect the cleavage pattern. Higher concentrations (50 μM and above) cause a general reduction in cleavage intensity throughout the fragment, which is consistent with nonspecific binding. It is worth noting that the doublet at the upper (5′) edge of the insert (corresponding to the cleavage of CpA and ApA) is less affected than many other bands in the fragment. This will become significant since this doublet is eliminated upon interaction with the third strand oligonucleotide (see the following). A similar interaction was observed on raising the pH to 7.5. Since **1** affects the DNase I digestion pattern at concentrations above 25 μM, we have been careful to perform triplex footprinting experiments at lower concentrations (10 μM) at which the compound alone has no effect.

Parallel (YYR) Triplexes. (1) A_6G_6•C_6T_6 with T_5C_5. Figure 3 shows DNase I footprinting patterns for the interaction of the unmodified decanucleotide T_5C_5 with a DNA fragment containing the insert A_6G_6, measured at pH 5.5 in the presence of 5 mM $MgCl_2$ and in the presence and absence

of 10 μM **1**. We chose to work with 10 μM **1**, since this alone does not affect the digestion pattern of the duplex, and preliminary experiments revealed that it was sufficient to stabilize the footprinting pattern obtained with the third strand; reducing the concentration of **1** to 3 μM did not produce clear footprints in the presence of T_5C_5. It can be seen that, as previously reported (Stonehouse & Fox, 1994), the oligonucleotide alone does not generate a DNase I footprint, although there is some enhanced cleavage at the 3′ (lower) end of the target site (indicated by the arrows), suggesting that there is some weak interaction. However, in the presence of 10 μM **1**, the oligonucleotide produces a clear footprint that is evident at concentrations as low as 0.5 μM. It is especially noteworthy that the doublet at the upper (5′) edge of the target site, which was not affected by the ligand alone, is eliminated in the presence of both **1** and T_5C_5. The enhanced cleavage at the 3′-end of the target site persists at concentrations below 0.1 μM. Therefore, it appears that the triplex-binding ligand has reduced the concentration of oligonucleotide required to generate a footprint by at least 2 orders of magnitude (i.e., from >50 to 0.5 μM), consistent with its ability to bind to triplex DNA selectively. The lack of an effect of 10 μM **1** alone on the DNase I digestion patterns is confirmed by the cleavage pattern at the lowest oligonucleotide concentrations, which resembles that in the control.

Since this compound stabilizes the interaction with the short unmodified oligonucleotide, we examined the binding of the corresponding acridine-linked oligonucleotide 5′-Acr-T_5C_5. The results are presented in Figure 4. It can be seen that, as previously reported (Stonehouse & Fox, 1994), the acridine-linked oligonucleotide generates a clear DNase I footprint at the target site. This is evident with 5 μM, but not with 1 μM, oligonucleotide. This footprint is accompanied by enhanced enzyme cleavage at the 3′-end of the target site (indicated by the arrows), which persists to lower oligonucleotide concentrations. When this experiment is repeated in the presence of 10 μM **1**, although the position and length of the footprint are unaltered, it shifts to lower concentrations and is still visible with 1 μM, but not with 0.5 μM, oligonucleotide. The enhancement also persists at lower concentrations. It appears that the triplex-binding ligand has stabilized the interaction with the acridine-linked oligonucleotide, but to a lesser extent than with the unmodified oligonucleotide (5-fold versus 100-fold). These results have been confirmed by similar experiments on the DNA fragment labeled on the pyrimidine strand (labeled at the 3′-end of the *Eco*RI site).

Since these triplexes contain five adjacent C^+·GC triplets, they are stable only at low pH's, which is necessary for protonation of the third strand cytosine residues. No footprints are produced at pH 6.5 or 7.5 for either unmodified or acridine-linked oligonucleotides, even in the presence of 10 μM **1** (data not shown).

(2) G_6A_6·T_6C_6 with C_5T_5. We have extended these studies on parallel triplexes by examining the effect of this ligand on triplex formation at a target with an identical base composition, but in which the order of the T·AT and C^+·GC triplets has been reversed. The results are presented in Figure 5 for the unmodified oligonucleotide C_5T_5. It can be seen that, as previously reported (Fox, 1994), this oligonucleotide produces a weak footprint at the target site G_6A_6·T_6C_6, which is evident only at concentrations of 50 μM and above. This

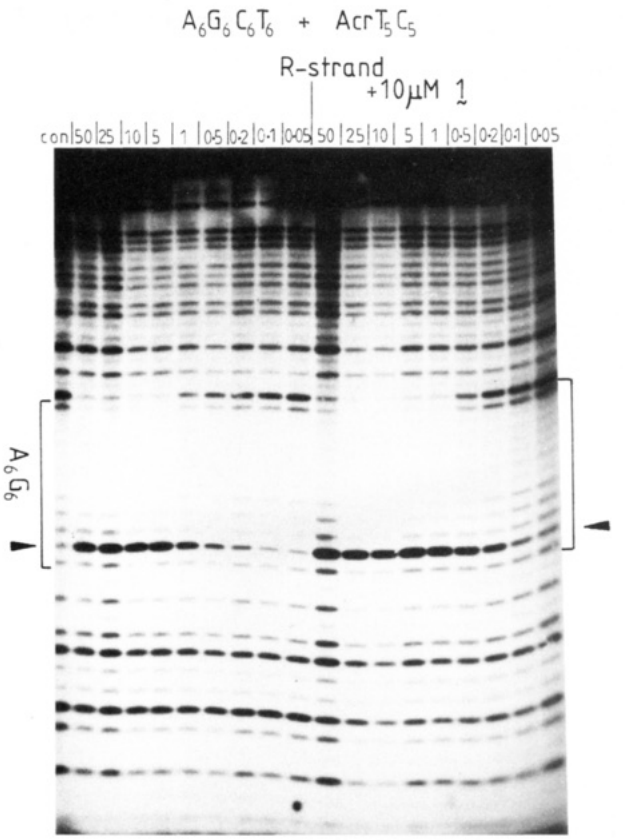

FIGURE 4: Effect of triplex-binding ligand **1** on the interaction of 5′-Acr-T_5C_5 with a DNA fragment containing the insert A_6G_6·C_6T_6. The DNA was labeled at the 3′-end of the *Hin*dIII site, revealing the purine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence A_6G_6. The arrows show the position of enhanced cleavage at the triplex−duplex boundary.

footprint extends above the target site by about three bases. In this instance there is no enhancement at the 3′ (lower) end of the target site, since this phenomenon is observed only with the labeled purine strand (see Figure 6). In the presence of 10 μM **1**, a clear footprint is evident, which persists to much lower concentrations (0.5 μM). However, in this instance the footprint appears to be smaller in the presence of the triplex-binding ligand. The upper (5′) end of the footprint is now coincident with the edge of the target site and is accompanied by the pronounced enhancement of cleavage at this border (immediately above the square brackets). In contrast, cleavage at the 3′ (lower) end of the footprint is not affected by the ligand.

In order to explore this unusual effect further, we have performed similar experiments in which the DNA fragment is labeled on the purine strand of the target sequence (at the 3′-end of the *Eco*RI site). The results are presented in Figure 6. In the absence of the ligand, a footprint is evident at the highest oligonucleotide concentrations. This is accompanied by enhanced cleavage at the 3′ (lower) end of the target sequence at the triplex−duplex junction (indicated by the arrow), which persists to lower concentrations than the footprint. In the presence of **1**, lower concentrations of the oligonucleotide generate a footprint, which is still evident

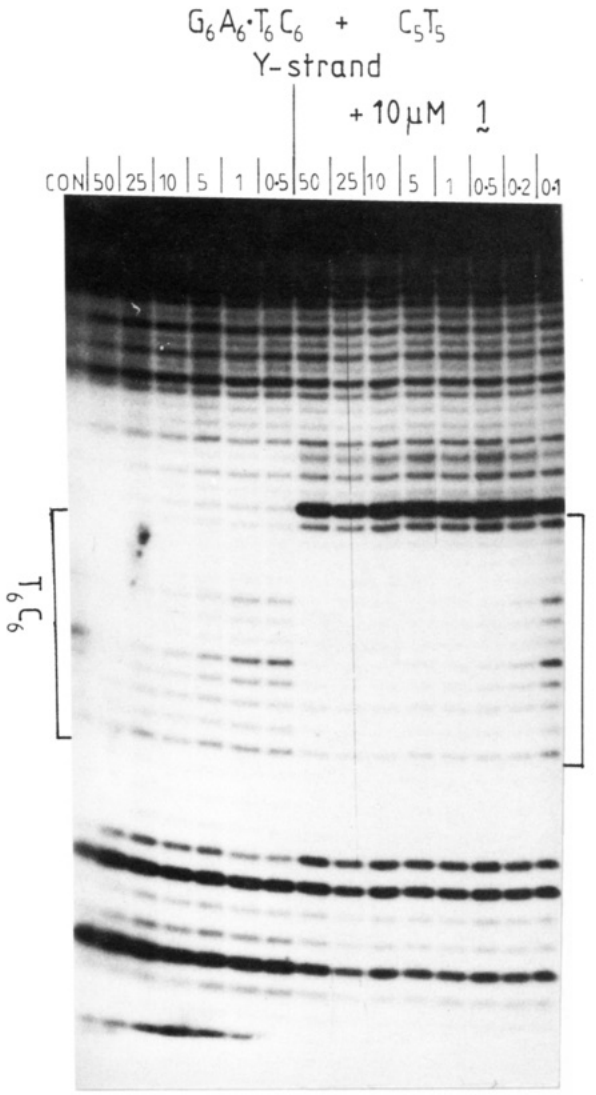

FIGURE 5: Effect of triplex-binding ligand **1** on the interaction of C_5T_5 with a DNA fragment containing the insert $G_6A_6{\cdot}T_6C_6$. The DNA was labeled at the 3′-end of the *Hin*dIII site, revealing the pyrimidine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence T_6C_6.

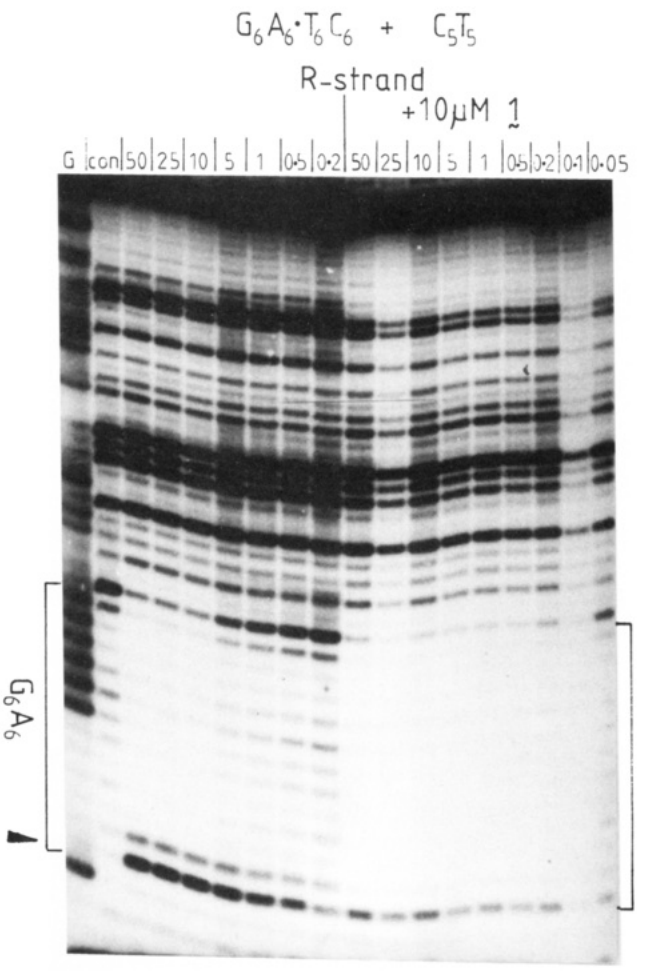

FIGURE 6: Effect of triplex-binding ligand **1** on the interaction of C_5T_5 with a DNA fragment containing the insert $G_6A_6{\cdot}T_6C_6$. The DNA was labeled at the 3′-end of the *Eco*RI site, revealing the purine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence G_6A_6. The lane labeled G is a Maxam−Gilbert dimethyl sulfate−piperidine marker specific for guanine. The arrow shows the position of enhanced cleavage at the triplex−duplex boundary.

at 0.5 μM; bands within the target sequence begin to appear at 0.2 μM oligonucleotide. The upper (5′) end of this footprint is similar to that in the absence of the ligand. In contrast, the footprint extends a few bases further in the 3′ (lower) direction; the enhancement is much weaker and is now barely evident. It appears that, in the presence of **1**, the footprint becomes larger when viewed from the purine strand, but smaller on the pyrimidine strand. Although the two strands appear to behave differently, it is worth noting that in each case the changes occur at the AT end of the target site, possibly suggesting that the triplex-binding ligand exerts its effect at the T·AT triplets. These effects will be considered further in the Discussion.

Figures 7 and 8 present DNase I footprints for the interaction of the acridine-linked oligonucleotide (5′-Acr-C_5T_5) with the same target site. In Figure 7, the DNA is labeled on the pyrimidine strand (3′-end of the *Hin*dIII site; compare with Figure 5). By looking first at the labeled pyrimidine strand (Figure 7), it can be seen that, in the absence of the triplex ligand, Acr-C_5T_5 produces a clear footprint at concentrations as low as 1 μM. As noted for the unmodified oligonucleotide, this footprint extends above the target site by about three bases. It should be remembered that the intercalating acridine moiety is located at the 3′-end of the C_6 tract, at the bottom edge of the insert. When this is repeated in the presence of **1**, a footprint is evident at slightly lower ligand concentrations (0.2 μM). However, there are dramatic changes at the upper (5′) end of the footprint, which are similar to those observed with the unmodified oligonucleotide in the presence of **1**. The footprint is now shorter by about four bases, terminating at the 5′ (upper) edge of the target, and is accompanied by a pronounced enhancement at the triplex−duplex junction. There are no changes at the 3′ (lower) end of the footprint. Figure 8 presents cleavage patterns for this target site labeled on the purine strand (3′-end of the *Eco*RI site; compare with

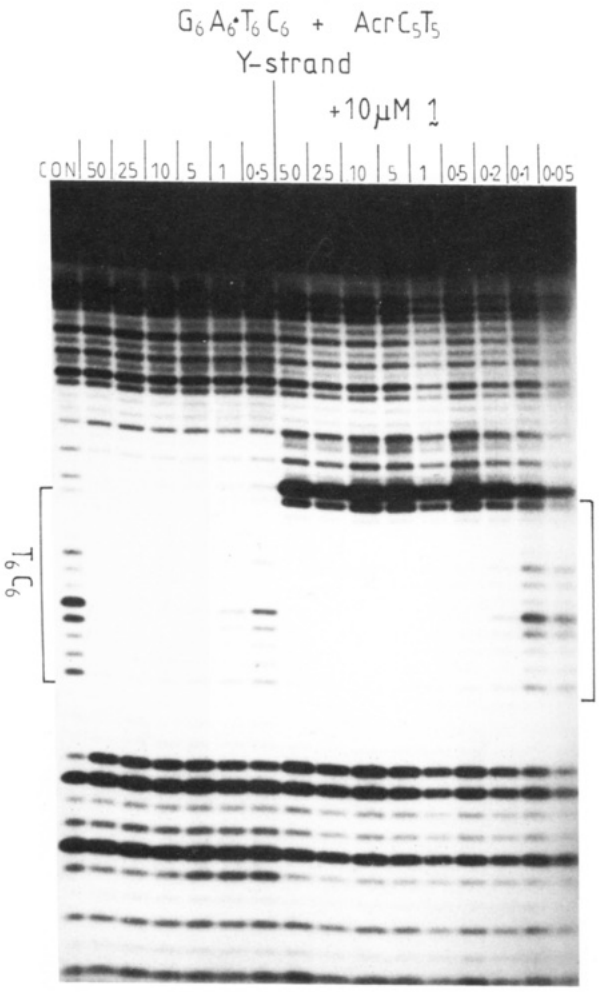

FIGURE 7: Effect of triplex-binding ligand **1** on the interaction of 5′-Acr-C_5T_5 with a DNA fragment containing the insert G_6A_6•T_6C_6. The DNA was labeled at the 3′-end of the *Hin*dIII site, revealing the pyrimidine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence T_6C_6.

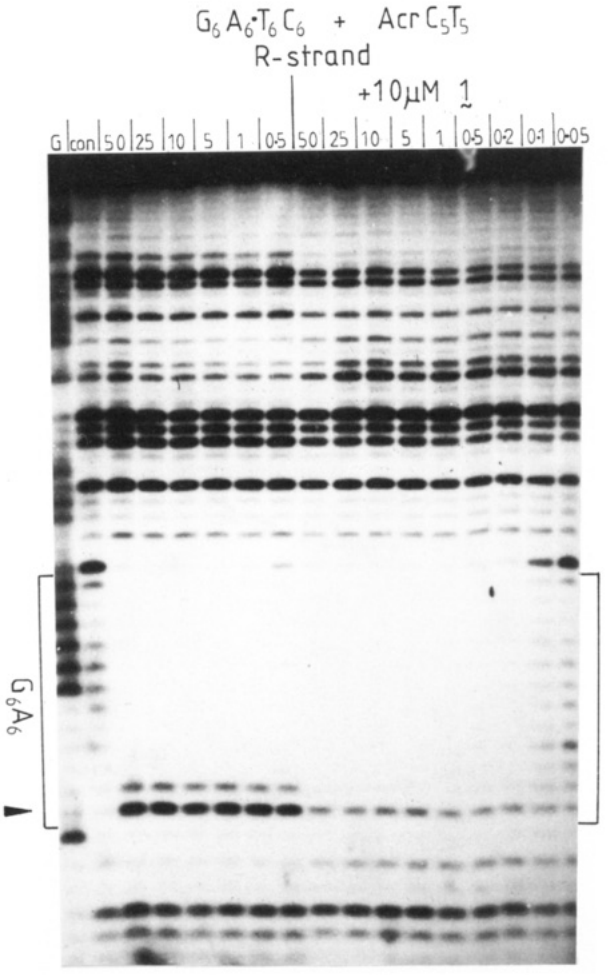

FIGURE 8: Effect of triplex-binding ligand **1** on the interaction of 5′-Acr-C_5T_5 with a DNA fragment containing the insert G_6A_6•T_6C_6. The DNA was labeled at the 3′-end of the *Eco*RI site, revealing the purine strand of the target site. The experiment was performed in 50 mM sodium acetate (pH 5.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence G_6A_6. The lane labeled G is a Maxam−Gilbert dimethyl sulfate−piperidine marker specific for guanine. The arrow shows the position of enhanced cleavage at the triplex−duplex boundary.

Figure 6). In the absence of the ligand, a clear footprint is evident at concentrations of 1 μM and above (bands within the target site begin to appear at 0.5 μM), which is accompanied by enhanced cleavage at the 3′ (lower) end of the target site (indicated by the arrow). In the presence of **1** this footprint persists to lower oligonucleotide concentrations (0.2 μM). It appears that although **1** has stabilized the complex formed with the acridine-linked triplex, the change in oligonucleotide concentration required to produce a footprint is less than that with the unmodified oligonucleotide. As noted for the unmodified oligonucleotide, the footprint in the presence of **1** extends one or two bases farther in the 3′ (lower) direction. The enhanced cleavage at the triplex−duplex boundary is no longer evident. Upon raising the pH to 7.5, no footprints were detected for either the unmodified or the acridine-linked oligonucleotide, even in the presence of 10 μM **1** (not shown).

Antiparallel (RRY) Triplexes. The experiments described earlier demonstrate that **1** reduces the concentration of oligonucleotides required to generate these parallel triplexes at pH 5.5. The results are most easily interpreted by suggesting that the compound has a greater effect on T•AT than on C^+•GC triplets. We were interested to examine whether it could also affect the formation of antiparallel (RRY) triplexes at the same target sites, and therefore we have examined the binding of G_5T_5 and T_5G_5 (both unmodified and acridine-linked) to the target sites A_6G_6•C_6T_6 and G_6A_6•T_6C_6, respectively.

(1) A_6G_6•C_6T_6 with G_5T_5. We have previously demonstrated that Acr-G_5T_5 forms a triple helix with the target sequence A_6G_6•C_6T_6 containing antiparallel G•GC and T•AT triplets (Fox, 1994); no footprint was detected with the unmodified oligonucleotide G_5T_5. Figures 9 and 10 present the effect of **1** on the formation of this triplex with Acr-G_5T_5. In Figure 9, showing the labeled purine strand, the oligonucleotide generates a clear DNase I footprint at concentrations of 5 μM and above. In the presence of **1**, the footprint persists at slightly lower oligonucleotide concentrations (0.5 μM). In addition, it can be seen that

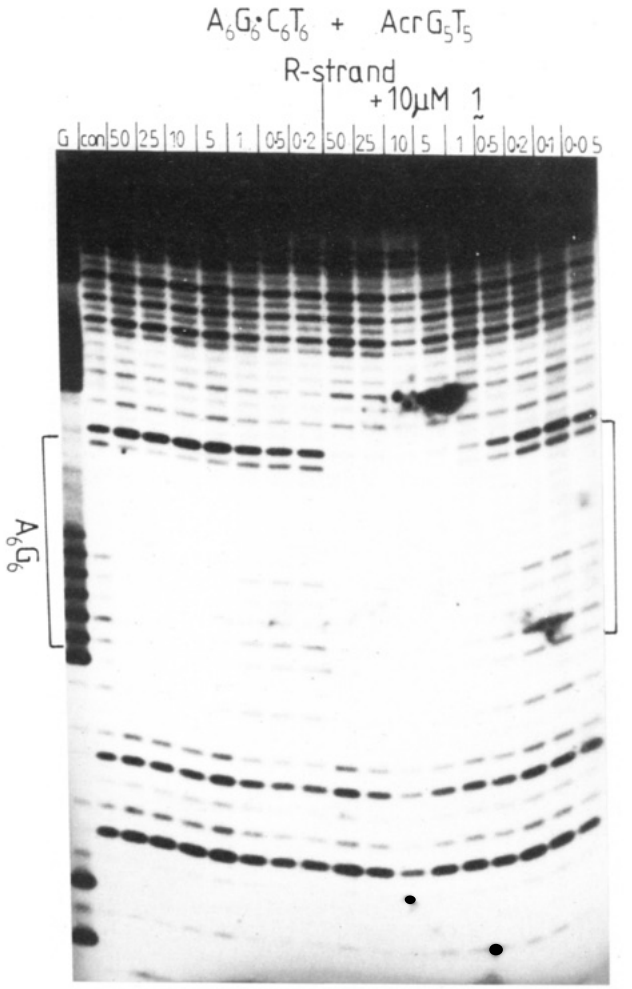

FIGURE 9: Effect of triplex-binding ligand **1** on the interaction of 5′-Acr-G_5T_5 with a DNA fragment containing the insert A_6G_6•C_6T_6. The DNA was labeled at the 3′-end of the *Hin*dIII site, revealing the purine strand of the target site. The experiment was performed in 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence A_6G_6. The lane labeled G is a Maxam−Gilbert dimethyl sulfate−piperidine marker specific for guanine.

the footprint extends by an additional 2−3 bases in the 5′ (upper) direction. This effect is located in the region of the T•AT triplets, at the end distal to the intercalating acridine. Figure 10 shows a similar experiment performed on the labeled pyrimidine strand. Again, it can be seen that slightly lower concentrations of the oligonucleotide are required to generate the footprint in the presence of **1**. The ligand causes the footprint to extend by an additional 1−2 bases in the 3′ (lower) direction. In this instance, the triplex-binding ligand has increased the length of the footprint on both DNA strands, in contrast to the results with G_6A_6•T_6C_6 and either Acr-C_5T_5 or C_5T_5, for which the footprint was increased on the purine strand, but decreased on the pyrimidine strand. These differences will be considered further in the Discussion.

Experiments with the unmodified oligonucleotide G_5T_5 failed to produce any footprints, at concentrations as high as 100 μM, even in the presence of the triplex-binding ligand. It therefore appears that the ligand has different effects on the parallel and antiparallel triplexes.

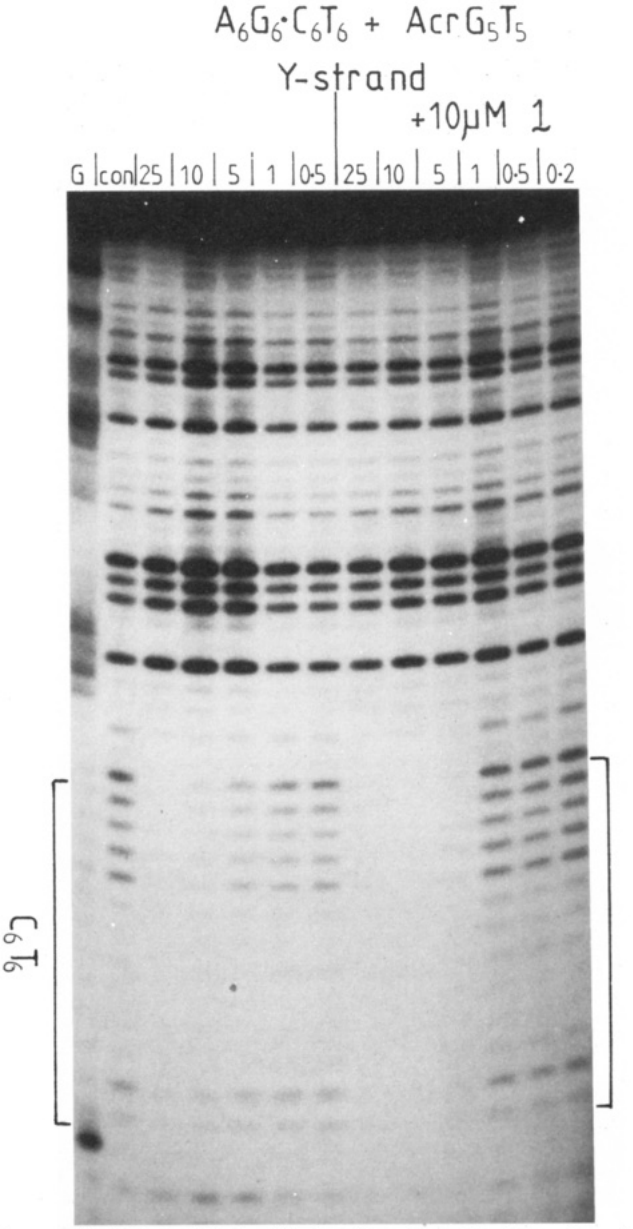

FIGURE 10: Effect of triplex-binding ligand **1** on the interaction of 5′-Acr-G_5T_5 with a DNA fragment containing the insert A_6G_6•C_6T_6. The DNA was labeled at the 3′-end of the *Eco*RI site, revealing the pyrimidine strand of the target site. The experiment was performed in 10 mM Tris-HCl (pH 7.5) containing 5 mM $MgCl_2$. The lane labeled con shows control DNase I cleavage of this DNA in the absence of both **1** and the oligonucleotide. Oligonucleotide concentrations (micromolar) are shown at the top of each gel lane. The right-hand lanes show cleavage in the presence of 10 μM **1**. The square brackets show the position and length of the target sequence C_6T_6. The lane labeled G is a Maxam−Gilbert dimethyl sulfate−piperidine marker specific for guanine.

(2) G_6A_6•T_6C_6 with T_5G_5. In contrast with the results described earlier with A_6G_6•C_6T_6 and Acr-G_5T_5, we previously have been unable to detect the formation of an intermolecular triple helix between Acr-T_5G_5 and the target sequence G_6A_6•T_6C_6 (Fox, 1994). We reasoned that the antiparallel T•AT triplet is weaker than the G•GC, so that when the five G•GC triplets are separated from the intercalating acridine, there is not a sufficiently strong nucleation center to generate a stable complex. We anticipated that **1** might stabilize these weaker triplets and lead to successful complex formation. However, neither Acr-T_5G_5 nor unmodified T_5G_5 generated a footprint, at concentrations as

high as 100 μM, in the presence of 10 μM **1** (data not shown).

DISCUSSION

Triplex Stabilization. The results presented in this paper demonstrate that **1** stabilizes certain intermolecular triplexes, reducing the oligonucleotide concentration required to generate DNase I footprints. Although we have not examined the effect of this compound on the stringency of triplex formation, it clearly is not a nonspecific phenomenon for several reasons. Firstly, oligonucleotides with incorrect orientations do not produce footprints; secondly, **1** only promotes the formation of triplexes containing C^+•GC triplets at low pH, and thirdly, not all potential triplex structures are stabilized by the compound, which has a greater effect on parallel than antiparallel structures. The effect of **1** is greatest on the interaction of unmodified oligonucleotides T_5C_5 and C_5T_5 with the target sequences A_6G_6•C_6T_6 and G_6A_6•T_6C_6, respectively, for which the concentration of third strand required to produce a footprint is reduced by at least 100-fold. These short unmodified oligonucleotides produce little or no protection from DNase I cleavage at concentrations below 50 μM, yet in the presence of **1**, they yield clear footprints at concentrations less than 1 μM. The compound has a much smaller effect on the binding of the corresponding acridine-linked oligonucleotides, reducing the effective concentration by about 5-fold. The observation that the interaction with acridine-linked oligonucleotides is potentiated, albeit by a small amount, is significant since it suggests that these ligands are not simply binding at the 5′-end of the oligonucleotide at the triplex−duplex junction. In this position, compound **1** and the intercalating acridine moiety would compete for binding and so are unlikely to improve triplex formation. It may be significant that, in the presence of **1**, lower concentrations of Acr-C_5T_5 are required to produce a footprint than of Acr-T_5C_5, at their respective target sites. This is consistent with the proposal that **1** selectively stabilizes the parallel T•AT triplet (see below), since in Acr-C_5T_5 the acridine moiety is situated the farthest from the block of T•AT triplets.

It appears that although either the presence of **1** or the modification with a 5′-acridine moiety stabilizes triplex formation, their effects are not additive. Indeed, in the presence of **1**, similar amounts of acridine-linked or unmodified oligonucleotides are required to produce DNase I footprints. This might be because a single acridine intercalation event provides the same stabilization effect as (several) interactions with **1**, or possibly because these compounds exert their effects by different and mutually exclusive mechanisms.

Although **1** has a dramatic effect on the formation of parallel triplexes containing C^+•GC and T•AT triplets, it has a much smaller effect on structures containing antiparallel G•GC and T•AT triplets. It promotes a slight increase in the binding of Acr-G_5T_5 to A_6G_6•C_6T_6, but has no effect on the unmodified oligonucleotide, which does not produce a footprint at concentrations up to 100 μM. Similarly, it does not promote any interaction between T_5G_5 and G_6A_6•T_6C_6, in either the acridine-linked or unmodified form, even though this structure potentially contains the same G•GC and T•AT triplets. We have previously suggested that this latter structure is not stable because the weaker antiparallel T•AT triplets separate the acridine moiety from the G•GC triplets, and so does not generate a strong enough nucleation center (Fox, 1994). Since **1** does not facilitate the formation of this structure, it seems that it does not bind to the antiparallel T•AT triplet as well as to the parallel form. Parallel and antiparallel triplets are not isohelical and they have backbones that lie in different positions within the major groove. The binding of the intercalator between the third strand bases therefore will be different for parallel and antiparallel T•AT triplets. A compound designed to give optimal stacking interactions with a parallel triplex (such as **1**) may be positioned within an antiparallel structure so that the second aromatic ring is in poor base overlap with the third strand thymine. It may be necessary to design a different series of ligands for interaction with antiparallel triplex motifs. Since the binding of Acr-G_5T_5 is slightly improved by **1**, the compound must possess a weak interaction with G•GC and/or T•AT triplets, but the interaction is not sufficient to stabilize a complex with the unmodified oligonucleotide.

Apparent Site Size. Before one considers the effect of **1** on the footprinting site size, it is worth remembering that these footprints do not arise from steric occlusion of the enzyme by the third strand. DNase I cuts from the DNA minor groove, whereas the third strand oligonucleotide is located in the major groove. Crystal structures of DNase I, bound to short oligonucleotides, reveal that the DNA is bent away from the enzyme toward the major groove (Lahm & Suck, 1991; Weston *et al.*, 1992), suggesting that DNA flexibility may be an important feature of its cleavage mechanism. In addition, because of its relatively large size, DNase I overestimates ligand- and protein-binding-site sizes. Since DNase I cuts by binding across the DNA minor groove, footprints are staggered across the two strands by 2−3 bases in the 3′-direction (Drew & Travers, 1984). Footprints of these intermolecular complexes must arise from triplex-induced changes in DNA structure and/or rigidity. It should also be remembered that several studies have shown that the DNA is distorted at the triplex−duplex, junction (Sun *et al.*, 1991; Collier *et al.*, 1991; Stonehouse & Fox, 1994), possibly involving a bend in the DNA (Chomilier *et al.*, 1992). In addition, there is some uncertainty about the exact structure adopted by the underlying duplex, concerning whether it is more like A- or B-DNA (Arnott & Selsing, 1974; Howard *et al.*, 1992).

The size of the DNase I footprint produced by T_5C_5 or Acr-T_5C_5 at the sequence A_5G_6•C_6T_6 is not affected by the presence of **1**, as expected. In contrast, when Acr-C_5T_5 or C_5T_5 binds to the target sequence G_6A_6•T_6C_6, the apparent site size is altered by the ligand. This is now 3−4 bases shorter when viewed from the labeled pyrimidine strand, but 1−2 bases longer on the purine strand. This effect is not caused by the acridine moiety, since the same result is obtained for both the acridine-linked and the unmodified oligonucleotides. Examination of the radiolabeled pyrimidine strand reveals that, in the presence of the ligand, the upper (5′) edge of these footprints is located at the boundary of the insert, whereas in the absence of the ligand, the footprint extends by an additional 3−4 bases. Since DNase I footprints are usually staggered in the 3′-direction (not the 5′) we would expect the upper edge of the footprint to lie close to the end of the target site. It therefore appears that, in this instance, the unusual pattern is produced by the oligonucleotide in the absence of the ligand, generating a

footprint that is longer than expected in the 5′-direction (upper). This reverts to the predicted size in the presence of **1**. A similar, although different, change is evident on the labeled purine strand. In the absence of the ligand the footprint terminates at the 3′-edge of the insert, whereas one would normally expect it to extend 2−3 bases in the 3′-direction (lower), beyond the actual binding site. This is exaggerated by the presence of enhanced DNase I cleavage at the triplex−duplex boundary, evident only on the purine strand. In the presence of **1** the footprint extends for an additional two bases in the 3′-direction (lower). It therefore appears that, regardless of the origin of the differences in apparent site size, the pattern is unusually large in the absence of the ligand and returns to a more predictable configuration in the presence of the ligand. In the presence of the ligand, the footprint is staggered across the two strands in the 3′-direction by 2−3 bases, whereas the oligonucleotide alone produces an unusual 5′-stagger. One explanation for this phenomenon is that since DNase I footprints of triplexes represent changes in DNA structure and/or flexibility, the oligonucleotide-induced changes continue beyond the actual target site, whereas in the presence of triplex-binding ligand **1**, these are restricted to the actual site of the interaction.

It is not clear why these changes in site size are evident for triplex formation at $G_6A_6{\cdot}T_6C_6$ but not at $A_6G_6{\cdot}C_6T_6$. A simple explanation might be that it depends on the relative positions of the T·AT and C^+·GC triplets. Complexes with T residues at the 3′-end of the third strand (or C's at the 5′-end) show these changes in apparent site size. Since the changes are also apparent with Acr-C_5T_5, it seems reasonable to suppose that the effect arises from the positions of the T residues since these are located the farthest from the intercalating acridine moiety. Since no such effect is observed for T_5C_5 or Acr-T_5C_5 binding to $A_6G_6{\cdot}C_6T_6$, it seems reasonable to suggest that either the ligand does not interact with C^+·GC, or that its effect on this triplet is different from that on T·AT.

Another possible explanation for the changes in apparent footprinting site size concerns the precise orientation of the DNA phosphate groups. Although DNase I is sensitive to the width of the minor groove, some bonds within good cleavage regions show anomalously poor cutting and *vice versa* (Drew & Travers, 1984), an effect that has been suggested to result from the orientation of the scissile phosphodiester bond. Since the triplex-binding ligand **1** possesses a cationic tail, this may have a direct interaction with the phosphate groups.

For the interaction between Acr-G_5T_5 and $A_6G_6{\cdot}C_6T_6$, **1** produces an *increase* in the size of the DNase I footprint when viewed from either the purine or the pyrimidine strand and therefore deserves an explanation different from that proposed for the parallel (YYR) triplexes, described earlier. Since the antiparallel T·AT triplet is thought to be much weaker than G·GC, it is possible that the T's at the 3′-end of the third strand (distal to the acridine) are not held rigidly in place and may be fraying from the triplex. If **1** stabilizes this triplet, then by preventing the terminal fraying, it may stabilize the interaction of the entire oligonucleotide and thereby increase the apparent footprinting site size. However, it should be emphasized that this stabilization must be much weaker than the effect on the parallel T·AT triplet, since the ligand does not assist the interaction with the unmodified oligonucleotide G_5T_5. In addition, it does not promote the binding of Acr-T_5G_5 to $G_6A_6{\cdot}T_6C_6$. It therefore appears that if the antiparallel T·AT triplets are held in place by the strong binding of Acr-G_5, then they can be further stabilized by the ligand; however, this alone will not promote the formation of these triplets.

Enhanced Cleavage. As previously noted, the parallel triple helices generate enhanced DNase I cleavage at the 3′-end of the duplex purine strand (Stonehouse & Fox, 1994; Fox, 1994). This is thought to be due to a local change in DNA structure at the junction. In several instances, in the presence of **1**, these enhancements persist to lower oligonucleotide concentrations than the footprints. This difference most likely arises because enhancements represent an increase in DNase I cleavage against a low background in the control, and so may be evident when only a small proportion of the target sites is occupied. In contrast, the formation of a footprint requires that a large proportion of the sites is occupied significantly to reduce the band intensity. A similar effect has been observed previously with oligonucleotides containing single triplet mismatches, which fail to produce footprints, yet induce some enhanced DNase I cleavage, and has been taken as indicating a weaker interaction (Chandler & Fox, 1993).

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