Footprinting Studies on Ligands which Stabilize DNA Triplexes: Effects on Stringency within a Parallel Triple Helix[†]

Simon P. Chandler,[‡] Lucjan Strekowski,[§] W. David Wilson,[§] and Keith R. Fox*,[‡]

Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K., and Department of Chemistry, Georgia State University, University Plaza, Atlanta, Georgia 30303

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ABSTRACT: We have examined the effect of four triplex-binding ligands on the interaction of the oligodeoxynucleotides T_8NT_8 (N = A, G, C, T) with DNA fragments containing the sequences A_8XA_8·T_8-YT_8 (X = G, C, T; Y = C, G, A) by DNase I footprinting. The ligands form a series of quinoline derivatives with an alkylamine chain in the 4-position and different aryl substituents in the 2-position. By themselves these compounds do not alter DNase I digestion of the DNA duplexes at concentrations up to 100 μM. At a concentration of 10 μM they potentiate triplex formation, lowering the concentration of oligonucleotide required to produce a clear footprint by as much as 100-fold. As well as stabilizing triplexes which consist of well-characterized DNA triplets, they also promote the formation of complexes which contain central triplet mismatches. This reduction in the stringency of triple helix formation may be used to broaden the range of triplex target sequences and enable recognition at sites which contain short regions for which there are no good triplet matches.

The formation of intermolecular DNA triple helices offers the possibility of designing agents with pronounced sequence recognition properties, which may be useful as gene-specific agents (Hélène, 1991a; Moffat, 1991; Chubb & Hogan, 1992). Triple-stranded nucleic acid structures were first detected using synthetic polyribonucleotide and polydeoxyribonucleotide sequences (Felsenfeld *et al.*, 1957; Arnott & Selsing, 1967). More recent studies have examined the formation of intermolecular triple helices and shown that the third strand binds within the major groove of the target duplex nucleic acid (Moser & Dervan, 1987; Le Doan *et al.*, 1987). This binding is sequence specific and is determined by the formation of hydrogen bonds between the third strand bases and substituents on the homopurine strand of the duplex target site (Thuong & Hélène, 1993; Hélène, 1993; Sun & Hélène, 1993). The third strand can lie in either parallel or antiparallel orientation with respect to the homopurine strand, depending upon its sequence composition.

Pyrimidine-rich third strands are oriented parallel to the homopurine target strand and are held in place by the formation of Hoogsteen base pairs; within this motif the best characterized triplets are T·AT and C^+·GC (Moser & Dervan, 1987; Le Doan *et al.*, 1987; Radhakrishnan & Patel, 1994a). As a result of the requirement for protonation of the third strand cytosine, triplexes containing C^+·GC triplets are only stable at low pHs (<6.0). In contrast, purine-rich strands bind in an antiparallel orientation with respect to the target homopurine strand and are stabilized by reverse-Hoogsteen interactions. Specificity is derived from G·GC, A·AT, and T·AT base triplets (Beal & Dervan, 1991, 1992; Durland *et al.*, 1991; Radhakrishnan & Patel, 1993; Radhakrishnan *et al.*, 1993). These triplexes are stable at physiological pHs. The formation of both types of triplex is stabilized by the presence of divalent metal ions.

Intermolecular DNA triplexes have pronounced sequence recognition properties and have been successfully employed to repress transcription of specific genes (Hélène, 1991b; Orson *et al.*, 1991; Postel *et al.*, 1991; Birg *et al.*, 1990; Grigoriev *et al.*, 1992, 1993). However, the stability of these structures is low in comparison with that of double-helical DNA. As a result, several methods have been explored to enhance their stability. One way of achieving this is by tethering a nonspecific DNA binding agent such as acridine or psoralen to either end of the third strand oligonucleotide (Birg *et al.*, 1990; Sun *et al.*, 1989; Collier *et al.*, 1991; Grigoriev *et al.*, 1992; Orson *et al.*, 1994; Washbrook & Fox, 1994). An alternative strategy, with which this paper is concerned, is to develop compounds which specifically bind to triple-helical DNA (better than to duplex structures) and thereby stabilize the formation of intermolecular triple helices. Hélène and co-workers have reported two benzopyridoindole derivatives (BePI and BgPI), which selectively stabilize triple-helical DNA (Mergny *et al.*, 1992; Pilch *et al.*, 1993a,b). Coralyne, an antitumor antibiotic containing four fused aromatic rings, binds to poly(dA)·2poly(dT) and poly(dGA)·2poly(dCT) about 2 orders of magnitude better than to the underlying duplexes (Lee *et al.*, 1993). Although each of these agents appears to bind by an intercalative mechanism, this alone is not sufficient to explain the selectivity for triplex DNA. The classical intercalator ethidium binds to both duplex and triplex structures, binding to poly(dA)·2poly(dT) (Scaria & Shafer, 1991) but not poly(dG)·2poly(dC) (Lee *et al.*, 1979; Morgan *et al.*, 1980; Mergny *et al.*, 1991) and at triplex−duplex boundaries (Sun *et al.*, 1991), but does not selectively stabilize triple-helical DNA. Wilson *et al.* (1993) have designed a different series

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* Corresponding author: Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, U.K. Tel: +703 594374. Fax: +703 594319.

[‡] University of Southampton.

[§] Georgia State University.

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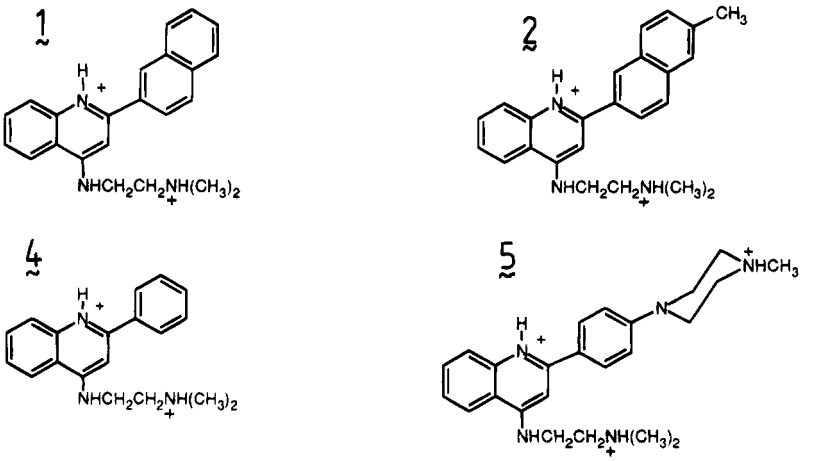

FIGURE 1: Structures of the four triplex-binding ligands used in this work. For consistency the numbers are the same as used in previous work (Wilson *et al.*, 1993).

of triplex-specific ligands which are also proposed to bind by intercalation (Wilson *et al.*, 1993). The structures of these compounds are shown in Figure 1. These were designed to fulfil three criteria: (i) The compounds should have an aromatic area which can stack with the three bases in the triplex; (ii) the aromatic systems should be unfused with torsional flexibility to accommodate the propeller twist of the triplets, in which the three bases are not isoplanar; and (iii) the compounds should be cations to complement the high negative charge of the triple helix. Thermal denaturation curves showed that these compounds stabilize poly(dA)·2poly(dT) much more than the parent duplex. Modeling studies indicate very good stacking of the two planar naphthyl ring systems with the bases at the triplex intercalation site and suggest that this ring system is too large to stack optimally at a duplex intercalation site (Wilson *et al.*, 1993).

In this paper we use DNase I footprinting to examine the effect of these compounds on the stability and stringency of triple helix formation using complexes which contain predominantly T·AT triplets. We have previously studied the formation of triple helices between the oligonucleotides T_8XT_8 (X = A, C, G, T) and DNA fragments containing the target sequences $A_8XA_8{\cdot}T_8YT_8$ (where X = T, C, G and Y = A, G, C) (Chandler & Fox, 1993). Although relatively large concentrations of the third strands were required to generate clear footprints (10 μM at 4 °C), stable triplexes were found between T_8CT_8 and $A_8GA_8{\cdot}T_8CT_8$, T_{17} and $A_8CA_8{\cdot}T_8GT_8$, and T_8GT_8 and $A_8TA_8{\cdot}T_8AT_8$. These generated structures containing central C·GC, T·CG, and G·TA triplets, respectively, species which have been suggested by other studies (Yoon *et al.*, 1992; Griffin & Dervan, 1989). Successful complex formation was characterized by protection at the 5′-end of the target site and enhanced DNase I cleavage at the 3′-end of the duplex purine strand, at the triplex−duplex junction. Several oligonucleotides, which failed to produce clear DNase I footprints, showed a weaker interaction with these target sites as judged by the 3′-enhancements. In this paper we examine the effect of four triple helix binding ligands on the stability of these triple helices. These studies have included strong, weak, and nonproductive triplex combinations as judged by our previous studies (Chandler & Fox, 1993). In this way we show that these ligands can stabilize structures which contain triplex mismatches and suggest that, although this lowers the stringency of triplex formation, it may provide a means for targeting sequences for which there is not a perfect triplet match.

MATERIAL AND METHODS

Chemicals and Enzymes. Deoxyoligonucleotides were purchased from Genosys Biotechnologies Inc., dissolved in water to a concentration of 1 mM, and used without further purification. These stock solutions were stored at −20 °C.

Bovine pancreatic DNase I was purchased from Sigma and stored at −20 °C at a concentration of 7200 units/mL. AMV reverse transcriptase was purchased from Promega; restriction enzymes were purchased from Pharmacia or New England Biolabs. Compounds **1**, **2**, **4**, and **5** were prepared as previously described (Wilson *et al.*, 1993) and stored as 20 mM stock solutions in dimethyl sulfoxide at −20 °C. These were diluted to working concentrations in aqueous solutions immediately before use.

DNA Fragments. Plasmids containing the inserts $A_8GA_8{\cdot}T_8CT_8$, $A_8CA_8{\cdot}T_8GT_8$, and $A_8TA_8{\cdot}T_8AT_8$ cloned into the *Sma*I site of pUC18 were prepared as previously described (Chandler & Fox, 1993). Labeled 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 AMV reverse transcriptase, and cutting again with *Eco*RI. The radiolabeled fragments were separated from the rest of the plasmid on non-denaturing 8% polyacrylamide gels. In some instances the fragments were labeled at the other end by reversing the order of addition of *Eco*RI and *Hin*dIII. The inserts were oriented so that labeling the 3′-end of the *Hin*dIII site labeled the purine-rich strand of $A_8GA_8{\cdot}T_8CT_8$ but the pyrimidine-rich strand of $A_8CA_8{\cdot}T_8GT_8$ and $A_8TA_8{\cdot}T_8AT_8$. The isolated DNA fragments were dissolved in 10 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA at approximately 10 nM strand concentration.

DNase I Footprinting. The radiolabeled DNA fragments (1.5 μL) containing the target sites were mixed with 1.5 μL of oligonucleotide, dissolved in 10 mM Tris-HCl, pH 7.5, containing 10 mM NaCl and 5 mM $MgCl_2$, and 1.5 μL of the triplex-stabilizing ligands (**1**, **2**, **4**, and **5**) dissolved in the same buffer. In our initial experiments we used the triplex-binding ligands at a concentration of 10 μM, since this has previously been shown to be sufficient to stabilize the binding of T_5C_5 to the target sequence $A_6G_6{\cdot}C_6T_6$ (Cassidy *et al.*, 1994). All the concentrations in the text refer

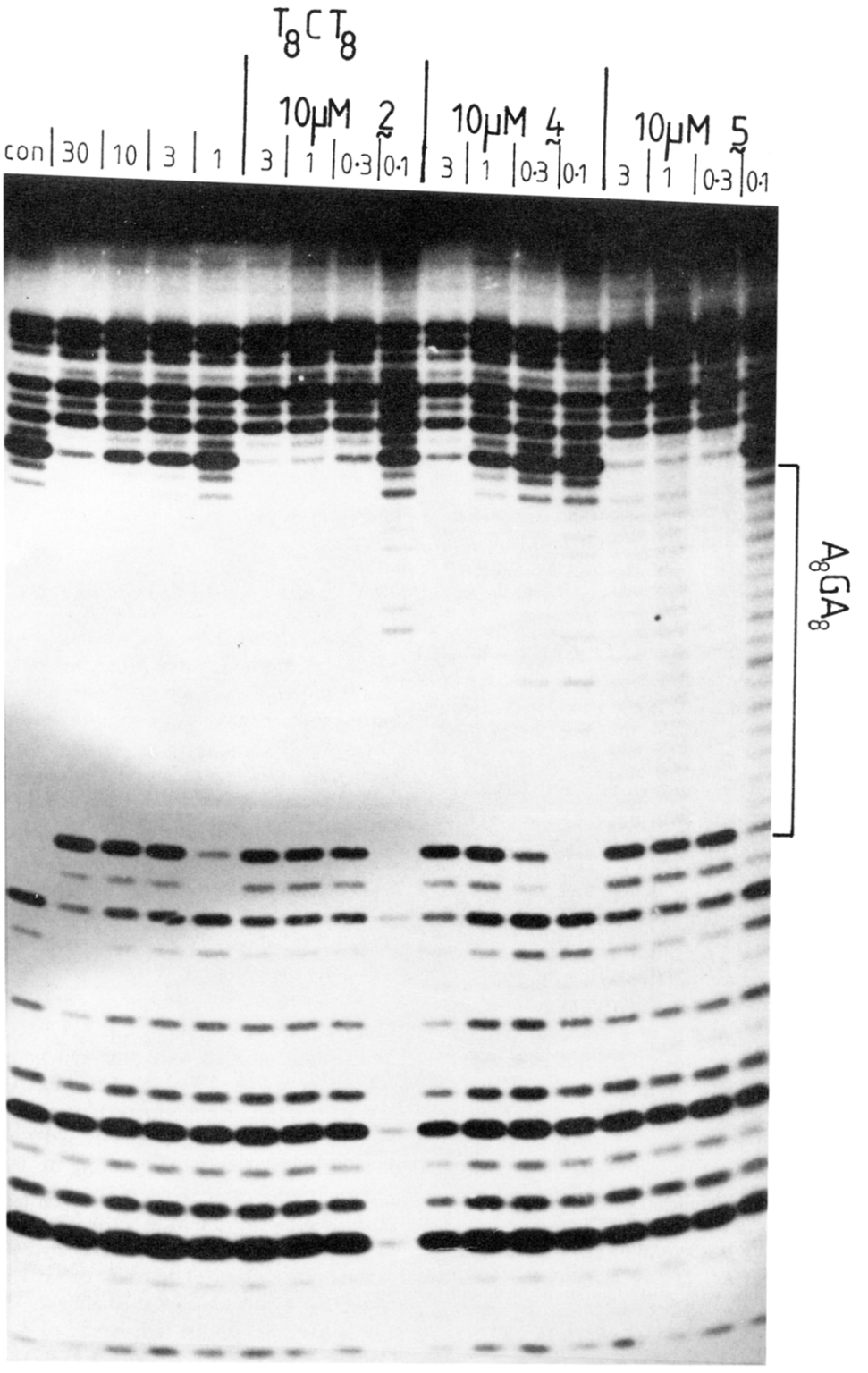

FIGURE 2: DNase I digestion of a fragment containing the insert A_8GA_8•T_8CT_8 with varying concentrations of the oligonucleotide T_8CT_8 in the presence and absence of 10 μM compounds **2**, **4**, and **5**. The DNA is labeled at the 3′-end of the *Hin*dIII site, visualizing the purine-rich strand. Oligonucleotide concentrations (micromolar) are shown at the top of each lane; "con" corresponds to digestion of DNA alone in the absence of oligonucleotide or triplex ligand. The first five lanes show DNase I digestion in the absence of any triplex-binding ligand. The brackets indicate the position and length of the insert.

to the final concentration in the complex mixture. The complexes were allowed to equilibrate at room temperature overnight. The samples were then digested with 2 μL of DNase I (0.01 units/mL dissolved in 2 mM $MgCl_2$, 2 mM $MnCl_2$, and 20 mM NaCl). The digestion was stopped after 1 min by the addition of 4.5 μL of DNase I stop solution (80% formamide containing 10 mM EDTA). Products of the digestion were separated on 10% (for *Hin*dIII labeled) or 13% (for *Eco*RI labeled) polyacrylamide gels, containing 8 M urea and run at 1500 V for 2 h. The gels were then fixed in 10% acetic acid before drying at 80°C and subjecting to autoradiography (overnight) at −70 °C using an intensifying screen. Bands were assigned by comparison with Maxam−Gilbert sequencing lanes specific for guanine or purines. When comparing the oligonucleotide concentrations required to generate DNase I footprints on each fragment in

the presence and absence of added ligand, care was taken to ensure that each sample had been digested to a similar extent, as assessed by the intensity of bands outside the footprinting site.

RESULTS

Effect of Various Compounds on Triplex Formation between T_8CT_8•A_8GA_8 and T_8CT_8. Although T_8CT_8 binds to the target sequence A_8GA_8•T_8CT_8 at pH 7.5, forming one C•GC and 16 T•AT triplets, the interaction is not strong and requires low temperatures or high oligonucleotide concentrations (Chandler & Fox, 1993). DNase I footprinting patterns showing this interaction are presented in Figure 2. As previously noted DNase I cleavage of the target sequence is poor, typical of A_n•T_n tracts, so that interaction with the third strand is most easily assessed by the protection evident at the 5′(upper)-end of the target and the enhanced cleavage generated at the 3′(lower)-end, at the triplex−duplex junction (Chandler & Fox, 1993). Looking at the tracks on the left-hand side of Figure 2 it can be seen that, in the absence of the triplex-binding ligand, bands at the top of the target site are only attenuated at the highest oligonucleotide concentration (30 μM), though the enhancement at the 3′-end persists to lower concentrations. The remainder of Figure 2 shows the effect of 10 μM each of compounds **2**, **4**, and **5** on the concentration of T_8CT_8 required to generate a footprint. Similar data showing the effect of 10 μM **1** on the binding of T_8CT_8 to this target site, under identical conditions, are presented in Figure 4. Not one of the compounds alone has any detectable effect on DNase I digestion of the target sequences at this concentration. In each case it can be seen that these compounds potentiate triplex formation, generating footprints at lower oligonucleotide concentrations. For example, in the presence of **2**, the bands immediately above the target sequence are still absent with 1 μM oligonucleotide and show an attenuated cleavage at 0.3 μM. For compounds **1**, **2**, **4**, and **5** footprints are evident with 0.1, 1.0, 3.0, and 0.3 μM oligonucleotide respectively, suggesting a rank order of potency for stabilizing this triplex of **1** > **5** > **2** > **4**, which is similar to the order deduced from thermal denaturation studies with T_{19}•A_{19}•T_{19} (Wilson *et al.*, 1993).

Figure 3 shows the effect of varying the concentration of the two best triplex-stabilizing ligands (compounds **1** and **5**) on the binding of 10 μM T_8CT_8 to the target site A_8GA_8•T_8CT_8. It can be seen that, under these conditions, **1** facilitates triplex formation at a concentration as low as 1 μM, while **5** requires greater than 3 μM ligand. In each case the enhanced DNase I cleavage at the triplex−duplex boundary persists to lower concentrations and is still evident at 0.1 μM ligand. It is also worth noting that, in contrast to the result shown in Figure 2, the 3′-enhancement is not evident at the highest ligand concentrations (10 μM). This difference may be due to slippage of the third strand, under the forcing conditions of high ligand and oligonucleotide (10 μM each) used in Figure 3, generating structures in which the third strand is not optimally positioned, thereby masking the enhanced cleavage. Since **1** appears to be the most potent ligand, in agreement with previous studies (Wilson *et al.*, 1993), we have used this ligand in most of the studies described below, examining its effect on the stringency of triple helix formation.

Triplex Formation at T_8CT_8•A_8GA_8. We have previously studied the binding of oligonucleotides of the type T_8XT_8

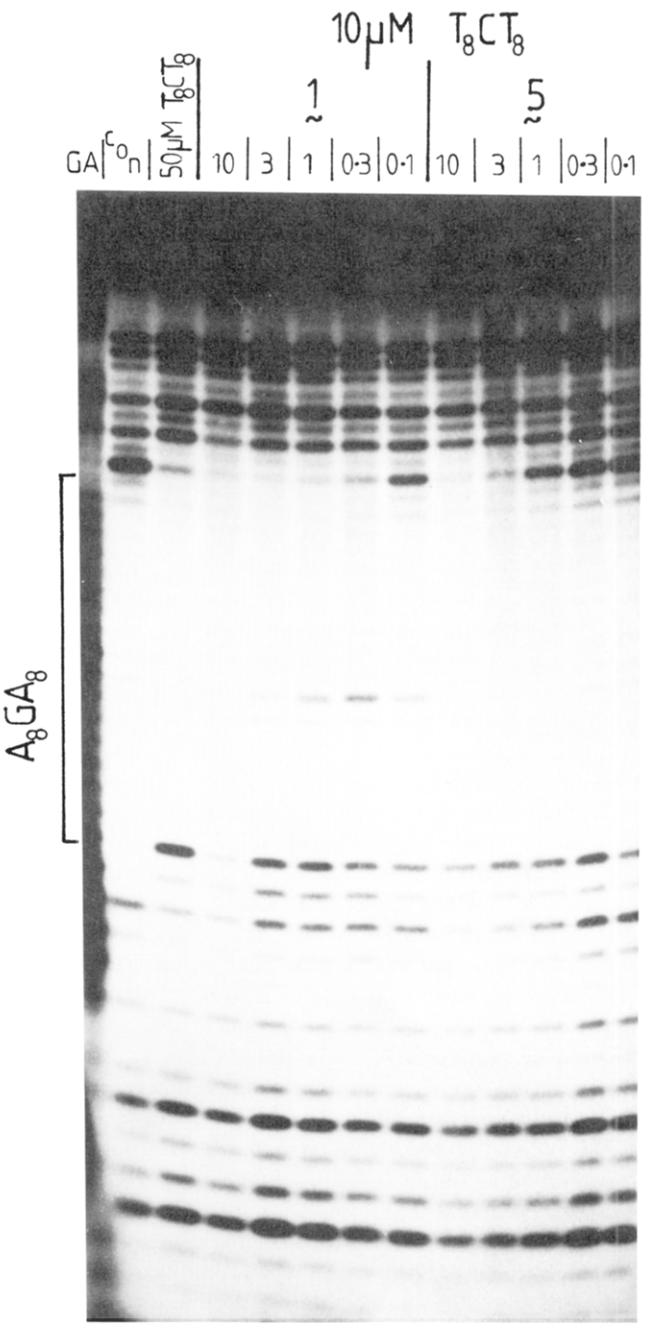

FIGURE 3: DNase I footprinting of a fragment containing the insert A_8GA_8•T_8CT_8 with 10 μM T_8CT_8 in the presence of varying concentrations of compounds **1** and **5**. The concentration of the triplex-binding ligand (micromolar) is shown at the top of each lane. The DNA is labeled at the 3′-end of the *Hin*dIII site, visualizing the purine-containing strand. "con" corresponds to digestion of the DNA alone. The third lane (labeled 50 μM T_8CT_8) corresponds to digestion of the DNA fragment in the presence of 50 μM T_8CT_8, with no added triplex-binding ligand. The track labeled "GA" is a Maxam−Gilbert formic acid−piperidine marker specific for guanine and adenine. The bracket indicates the position and length of the insert.

to a DNA fragment containing the target sequence T_8CT_8•A_8GA_8 (Chandler & Fox, 1993), in an attempt to identify the effect of central triplet mismatches on triple helix stability. As well as a footprint with T_8CT_8, a weaker but significant interaction was seen with T_8GT_8 and T_8TT_8 evidenced only by enhanced DNase I cleavage at the triplex−duplex boundary. No interaction was seen with T_8AT_8. Since these DNA triplexes present a series with a range of affinities, we have examined the effect of some putative triplex-binding

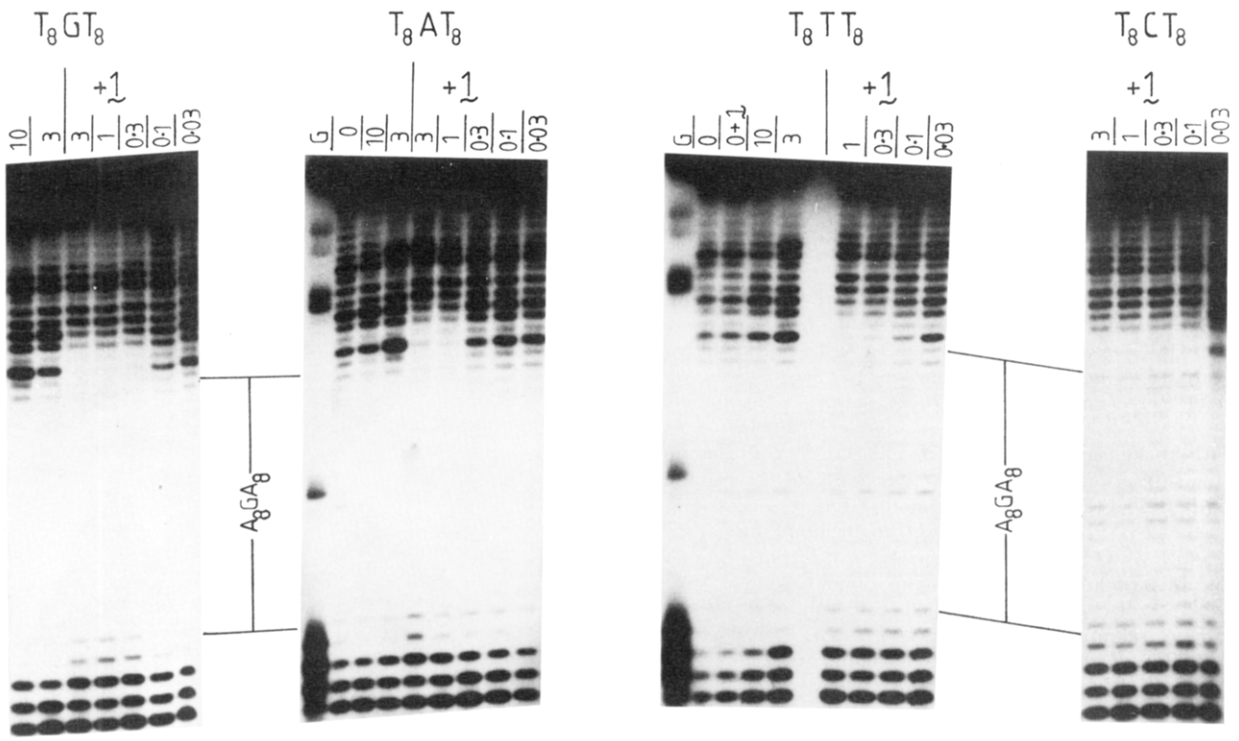

FIGURE 4: DNase I footprinting of a fragment containing the insert $A_8GA_8{\cdot}T_8CT_8$ with varying concentrations of the oligonucleotides T_8GT_8, T_8AT_8, T_8TT_8, and T_8CT_8 in the absence and presence of 10 μM compound **1**. The DNA is labeled at the 3′-end of the *Hin*dIII site visualizing the purine-rich strand. Oligonucleotide concentrations (micromolar) are shown at the top of each lane; "0" corresponds to digestion of DNA alone in the absence of oligonucleotide or triplex ligand. The track labeled "0+**1**" shows DNase I digestion in the presence of 10 μM compound **1**, with no oligonucleotide. The brackets indicate the position and length of the insert. The track labeled "G" is a Maxam−Gilbert dimethyl sulfate−piperidine marker specific for guanine. In the first three panels (T_8GT_8, T_8AT_8, and T_8TT_8) the lanes on the left show digestion in the absence of added triplex-binding ligand; those on the right were performed under identical conditions, but with the addition of 10 μM **1**. Data for T_8CT_8 are only presented in the presence of 10 μM **1**; the effect of this oligonucleotide on this DNA fragment, in the absence of ligand, can be seen in Figure 2.

ligands on their ability to generate DNase I footprints. The effect of 10 μM **1** on the formation of this triple helix is shown in Figure 4. Under these conditions (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, and 5 mM $MgCl_2$, 20 °C) none of the oligonucleotides generates a DNase I footprint at concentrations as high as 10 μM. DNase I digestion in the control (lane 0) is identical to that in the presence of 10 μM oligonucleotide alone. Footprints were observed with T_8CT_8 as previously described (Chandler & Fox, 1993) on raising the oligonucleotide concentration to 30 μM or lowering the temperature to 4 °C. When the incubations are repeated in the presence of 10 μM **1**, clear footprints can be seen with each of the oligonucleotides, evidenced by protection at the 5′(upper)-end of the target site, though the effective concentration range is different for each one. It can be seen that T_8AT_8, which by itself does not produce a footprint with 100 μM oligonucleotide at 4 °C (Chandler & Fox, 1993), generates a footprint at concentrations as low as 1 μM, though this is removed on decreasing the concentration to 0.3 μM. T_8GT_8 and T_8TT_8, which showed evidence for a weak interaction in the absence of ligand (Chandler & Fox, 1993), produce footprints which each persist to 0.3 but not 0.1 μM, while T_8CT_8, which contains the perfect match, yields a clear footprint at 0.1 μM but not 0.03 μM. These results are summarized in Table 1 and suggest that the triplex-binding ligand **1** has increased the stability of these triplex complexes by more than 300-fold (T_8CT_8 footprints at 0.1 μM in the presence of the ligand, in contrast to 30 μM in its absence). Although this increased binding strength has caused a relaxation in the stringency

Table 1: Minimum Oligonucleotide Concentrations Required To Generate DNase I Footprints on Fragments Containing the Target Sites $A_8GA_8{\cdot}T_8CT_8$, $A_8TA_8{\cdot}T_8AT_8$, and $A_8CA_8{\cdot}T_8GT_8$ in the Presence of 10 μM **1**

	lowest concentration of third strand generating a footprint (μM) in the presence of 10 μM **1**			
target site	T_8CT_8	T_8TT_8	T_8GT_8	T_8AT_8
$A_8GA_8{\cdot}T_8CT_8$	0.1	0.3	0.3	1.0
$A_8TA_8{\cdot}T_8AT_8$	0.3	0.1	0.1	1.0
$A_8CA_8{\cdot}T_8GT_8$	0.1	0.03	0.1	3.0

of triplex formation, the order of binding of the various oligonucleotides is the same as seen in the absence of the ligand, i.e., $T_8CT_8 > T_8GT_8 = T_8TT_8 > T_8AT_8$. It should also be emphasized that this is not a general nonspecific effect, stabilizing all three-stranded structures irrespective of the sequence of the third strand, since an unrelated G-rich oligonucleotide, $(GGA)_5$, showed no triplex formation at this target site in the presence of compound **1**.

Since compound **1** appeared to be so effective in stabilizing these triplex structures, enabling the binding of oligonucleotides which contain a single mismatch, we also examined the effect of the other three triplex-binding ligands on the stringency of triplex formation at this target site. The results are presented in Figure 5, which shows the effect of 10 μM compounds on the formation of triplexes between 10 μM T_8XT_8 (X = G, T, A) and the target sequence $A_8GA_8{\cdot}T_8CT_8$. It can be seen that, as shown in Figure 4, **1** stabilizes all three complexes, protecting from DNase I cleavage at the 5′(upper)-end and generating enhanced cleavage at the

FIGURE 5: DNase I footprinting of a fragment containing the insert A_8GA_8·T_8CT_8 with 10 μM of the oligonucleotides T_8GT_8, T_8AT_8, T_8TT_8, and T_8CT_8 in the presence and absence of 10 μM compounds **1**, **2**, **4**, and **5**. The DNA is labeled at the 3′-end of the *Hin*dIII site visualizing the purine-rich strand. Lanes **1**, **2**, **4**, and **5** correspond to the addition of 10 μM of the respective triplex-binding ligand. "con" corresponds to digestion of DNA alone in the absence of oligonucleotide or triplex ligand, while "0" refers to digestion in the presence of the oligonucleotide and in the absence of any added ligand. The brackets indicate the position and length of the insert. The track labeled "GA" is a Maxam−Gilbert formic acid−piperidine marker specific for guanine and adenine.

3′(lower)-end of the insert at the triplex−duplex boundary. Similarly compound **5** stabilizes the interaction with T_8GT_8 and T_8TT_8; with T_8AT_8 no footprint is evident. Although **2** does not produce footprints with any of these oligonucleotides, a clear enhancement is evident at the triplex−duplex boundary with T_8GT_8 but not T_8TT_8 and T_8AT_8. Compound **4** does not facilitate triplex formation with any of these three oligonucleotides. These results confirm the rank order of triplex-stabilizing ability as **1** > **5** > **2** > **4** and the relative affinity of the third strands as T_8CT_8 > T_8GT_8 = T_8TT_8 > T_8AT_8.

In our previous experiments we were unable to detect the formation of parallel (purine-rich) triple helices at this target site using a third strand of the type A_8NA_8. We therefore tested whether these ligands could enhance the stability of this complex, consisting largely of A·AT triplets. These experiments were unsuccessful and did not produce any DNase I footprints. It therefore appears that these triplex-

binding ligands may stabilize various triplexes to different degrees and may be specific for the parallel T·AT triplet.

Triplex Formation on the Target Sequence T_8AT_8·A_8TA_8. We have previously shown that the sequence T_8AT_8·A_8TA_8 can be recognized by the oligonucleotide T_8GT_8, generating a complex which contains a central G·TA triplet (Chandler & Fox, 1993), a triplet which has been observed in different sequence contexts by other groups (Griffin & Dervan, 1989; Radhakrishnan *et al.*, 1992). A weaker interaction with the oligonucleotide T_8TT_8 was also suggested by enhanced DNase I cleavage of the triplex−duplex boundary at the 3'-end of the purine strand. We have performed a series of experiments similar to those shown in Figure 4, examining the effect of **1** on the binding of the four oligonucleotides to a DNA fragment containing the target sequence T_8AT_8·A_8-TA_8 (not shown). Although none of the oligonucleotides alone alter the DNase I cleavage pattern at a concentration of 10 μM, in the presence of the ligand all four oligonucleotides produce DNase I footprints. The results are summarized in Table 1, showing the lowest concentration producing a footprint. In the presence of the ligand, T_8GT_8, which should generate a central G·TA triplet, produces a clear footprint at concentrations as low as 0.1 μM, as does T_8-TT_8. T_8CT_8 and T_8AT_8 also produce footprints, but require oligonucleotide concentrations of 0.3 and 1.0 μM, respectively. It appears that although **1** has reduced the stringency of triplex formation, the rank order of binding of the various oligonucleotides is unchanged. The oligonucleotide containing a central adenine requires the highest concentration to produce a footprint (1 μM), though it should be noted that this is much lower (by at least 100-fold) than in the absence of the ligand, for which no footprints have been observed. Within this context, in the presence of 10 μM **1**, the rank order of triplet stability appears to be G·TA = T·TA > C·TA > A·TA.

Triplex Formation on the Target Sequence T_8GT_8·A_8CA_8. DNase I footprinting studies with the target sequence T_8-GT_8·A_8CA_8 showed a strong interaction with T_8TT_8 and a weaker interaction with T_8CT_8 (Chandler & Fox, 1993). The stronger interaction contains a central T·CG triplet as previously reported (Yoon *et al.*, 1992). We have examined the effect of **1** on the interaction of the four oligonucleotides with the fragment containing the target sequence T_8GT_8·A_8-CA_8 (not shown). None of these oligonucleotides, when added alone, alter the DNase I cleavage pattern at a concentration of 10 μM. However, in the presence of the ligand all four oligonucleotides produce clear DNase I footprints. The results of these experiments are summarized in Table 1, showing the lowest concentration producing a footprint. With T_8TT_8, generating the central T·CG triplet, the footprint is still evident with 0.03 μM oligonucleotide. In contrast T_8AT_8 requires at least 3 μM to alter the DNase I digestion pattern. T_8CT_8 and T_8GT_8 each yield footprints at 0.1 μM. Once again the oligonucleotide containing a central adenine has the lowest affinity, though even this has been enhanced by the ligand. Within this context, in the presence of 10 μM **1**, the rank order of triplet stability appears to be T·CG > C·CG = G·CG > A·CG.

DISCUSSION

The results presented in this paper demonstrate that these ligands can stabilize DNA triplexes which consist largely of T·AT triplets, enabling stable complex formation at much lower oligonucleotide concentrations. Examination of the concentrations required to generate DNase I footprints suggests that the affinity for the third strand has in some instances been increased by more than 300-fold in the presence of 10 μM compound **1**.

Differences between the Ligands. Previous studies with these compounds examined their effects on the thermal denaturation profiles of dT_{19}·dA_{19}·dT_{19} and showed that, although they each have only a small effect on the duplex melting temperature, their interactions with the triplex vary markedly (Wilson *et al.*, 1993). Compounds **1**, **2**, and **5** stabilize the triplex by about 30 °C, while **4**, which contains a smaller phenyl substituent on the quinoline ring, has a lesser effect (Wilson *et al.*, 1993). Similar differences between the ligands are shown in the present study, for which **4** produces a smaller, though significant, effect. The relative stabilizing effect of these compounds appears to be **1** > **5** > **2** > **4**, compared with **1** > **2** > **5** > **4** in the previous study. This order is shown by their effects both on the concentration of T_8CT_8 required to produce a footprint with the target sequence A_8GA_8·T_8CT_8 (Figures 2 and 4) and on the binding of the other oligonucleotides to this sequence (Figure 5). The minor differences in relative affinities may arise from the different conditions employed in the present work. First, the footprinting studies were performed at 20 °C, whereas simple melting profiles only give information about the relative binding affinities at the transition temperatures. Second, we have used low salt concentrations (10 mM NaCl and 5 mM $MgCl_2$ compared with 200 mM NaCl). Third, although the concentrations of oligonucleotides and stabilizing ligands are still higher than might be achieved in human therapy, the concentration of the components is much lower than that used in previous studies which employed stoichiometric amounts of the triplex and ligand. In contrast, in the present study the concentration of the target site is vanishingly small (approximately nanomolar) and more closely resembles pharmacological situations, where binding is limited by the equilibrium constant rather than the stoichiometric ratio of the reacting species. Fourth, we have used target sequences which contain triplets other than T·AT.

Effect of Ligand Concentration. Figure 3 reveals that 10 μM T_8CT_8 produces clear footprints on the target sequence A_8GA_8·T_8CT_8 in the presence of **1** and **5** at concentrations of 1 and 3 μM, respectively. This confirms the relative stabilizing effect of these compounds as **1** > **5**. Since these experiments were conducted in conditions where the ligands are present in excess of the radiolabeled DNA, these variations must reflect differences in their relative affinities for DNA triplexes. In each case the enhanced DNase I cleavage at the triplex−duplex boundary persists at ligand concentrations as low as 0.1 μM. Does this data give any information on the number of ligand molecules necessary to stabilize each triplex? Most intercalating ligands have affinities for duplex DNA around 10^5−10^6 M^{-1}. If we assume that these ligands have a similar affinity for triplex DNA, then a concentration of 1 μM will achieve between 10 and 50% occupancy of the available sites on a triplex target. The lower estimate might suggest that only a few ligands per target site are required to stabilize the interaction. Indeed it is possible that these might be bound at specific locations within the structure, possibly at the triplex−duplex

boundary, which has been shown to present a good intercalation site (Collier *et al.*, 1991).

Stringency of Triplex Formation. As well as increasing the affinity of oligonucleotides which have a good match for their target sites, forming well-characterized base triplets (e.g., T_8CT_8 binding to $A_8GA_8{\cdot}T_8CT_8$), the compounds facilitate triplex formation with oligonucleotides which did not affect DNase I cleavage patterns, producing neither footprints or enhancements (Chandler & Fox, 1993), and which contain central triplex mismatches. In the presence of 10 μM **1** all the oligonucleotides, at a concentration of 3 μM, yield footprints at every target site. This reduction in stringency, permitting at least one triplet mismatch, is especially striking for the third strand T_8AT_8, which by itself shows no interaction with any of the target sites. We previously noted that a mismatch containing a third strand adenine consistently generates the least stable complex (Chandler & Fox, 1993). However, even in the presence of the triplex-binding ligands, this oligonucleotide is required at higher concentrations than the others to form stable complexes. On the basis of the oligonucleotide concentrations required to generate a footprint it appears that the relative stability of mismatches containing a third strand adenine is A·TA = A·GC > A·CG (Table 1), though this order differs from that derived from thermal denaturation studies on short triplexes (Fossella *et al.*, 1993) which suggested A·GC > A·CG > A·TA. Despite this reduced stringency, the interaction still possesses considerable specificity; single base changes alter the affinity for the target sites, and unrelated oligonucleotides do not affect the DNase I cleavage pattern. Previous footprinting studies (Chandler & Fox, 1993) showed that some oligonucleotides induced enhanced DNase I cleavage at the 3′-end of the purine strand, even though they did not produce clear footprints (for example, T_8GT_8 and T_8TT_8 with $A_8GA_8{\cdot}T_8CT_8$; T_8CT_8 with $A_8CA_8{\cdot}T_8GT_8$, and T_8TT_8 with $A_8TA_8{\cdot}T_8AT_8$). In the presence of the triplex-binding ligands these combinations generate clear DNase I footprints which persist to relatively low oligonucleotide concentrations. This confirms our previous suggestion that enhanced cleavage, in the absence of clear footprints, can indicate a weaker interaction.

Out of all the combinations tested in the presence of the triplex-binding ligands, the most stable (i.e., requiring the lowest oligonucleotide concentration) is between T_8TT_8 and $A_8CA_8{\cdot}T_8GT_8$, generating a complex containing a central T·CG triplet. This triplet has been previously described in several studies (Yoon *et al.*, 1992; Radhakrishnan & Patel, 1994b), but it is generally thought to be less stable than T·AT and $C^+{\cdot}$GC triplets. At first sight it therefore appears surprising that this complex requires a lower oligonucleotide concentration than that between T_8CT_8 and $A_8GA_8{\cdot}T_8CT_8$, which should contain a central C·GC triplet. However, it should be noted that all the present experiments have been performed at pH 7.5, whereas lower pHs are generally required to achieve protonation of the third stand cytosine. These results more likely reflect the stability of the unprotonated C·GC triplet rather than $C^+{\cdot}$GC.

Since these compounds are known to stabilize the T·AT triplet, it might be argued that they are promoting the formation of triplets at each half of the target sites, in the $T_8{\cdot}A_8$ tracts. This would leave the remainder of the third strand dangling free in solution, rather than straddling across the entire target sequence, and would not involve the central base pair in triplet recognition. We do not favor this possibility since it does not readily account for the differences observed between the various oligonucleotides, though it may explain why no enhancements are observed at the highest concentration of ligand and oligonucleotide (Figure 3).

Since these compounds stabilize triple-helical DNA and have little or no effect on duplex structures, they have potential for enhancing the activity of oligonucleotides as anti-gene agents. However, since they relax the stringency of triple helix formation, and stabilize complexes containing unfavorable triplet mismatches, they may promote binding to sequences other than the desired target site. Triplex-forming oligonucleotides will therefore need to be carefully designed so as to minimize this strong secondary interaction. However, this reduced stringency suggests a further use for these agents. Since most base triplets involve recognition of the purine strand of the duplex, the formation of these structures is generally restricted to homopurine·homopyrimidine sequences. These triplex-binding ligands may be useful for stabilizing oligonucleotide binding across sequences for which there are no good triplet matches, and could therefore broaden the range of potential triplex target sites.

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