Alternate-strand DNA triple-helix formation using short acridine-linked oligonucleotides

Elinor WASHBROOK and Keith R. FOX*

Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

We have used DNAase I footprinting to examine the formation of intermolecular DNA triple helices at sequences containing adjacent blocks of purines and pyrimidines. The target sites $G_6T_6 \cdot A_6C_6$ and $T_6G_6 \cdot C_6A_6$ were cloned into longer DNA fragments and used as substrates for DNAase I footprinting, which examined the binding of the acridine (Acr)-linked oligonucleotides Acr- T_5G_5 and Acr- G_5T_5 respectively. These third strands were designed to incorporate both G·GC triplets, with antiparallel G_n strands held together by reverse Hoogsteen base pairs, and T·AT triplets, with the two T-containing strands arranged antiparallel to each other. We find that Acr- T_5G_5 binds to the target sequence $G_6T_6 \cdot A_6C_6$, in the presence of magnesium at pH 7.0, generating clear DNAase I footprints. In this structure

the central guanine is not recognized by the third strand and is accessible to modification by dimethyl sulphate. Under these conditions no footprint was observed with Acr- G_5T_5 and $T_6G_6 \cdot C_6A_6$, though this triplex was evident in the presence of manganese chloride. Manganese also facilitated the binding of Acr- T_5G_5 to a second site in the fragment containing the sequence $T_6G_6 \cdot C_6A_6$. This represents interaction with the sequence G_4ATCT_6 , located at the boundary between the synthetic insert and the remainder of the fragment, and suggests that this bivalent metal ion may stabilize triplexes that contain one or two mismatches. Manganese did not affect the interaction of either oligonucleotide with $G_6T_6 \cdot A_6C_6$.

INTRODUCTION

The formation of three-stranded nucleic acid structures was first suggested over 30 years ago [1–3]. The third strand lies within the DNA major groove and is held in place by the formation of hydrogen bonds to substituents on the DNA bases. Since 1987 it has been realized that these structures offer the possibility of designing agents with long-range sequence-recognition properties [4,5]. However, since most triplets involve contacts to the purine strand of the duplex, this strategy is largely restricted to recognition of homopurine stretches.

Two different types of triple helix have been identified, depending on the orientation of the third strand. Triplexes in which the third strand runs parallel to the purine strand of the duplex are generally pyrimidine-rich and include T·AT and C·GC triplets, which are stabilized by the formation of Hoogsteen base pairs [4–7]. In contrast, triplexes in which the third strand runs antiparallel to the duplex purine strand are generally purine-rich and are characterized by G·GC, A·AT and T·AT triplets, which contain reverse Hoogsteen base pairs [8–10]. Both these structures are stabilized by bivalent metal ions and polyamines [11–13].

There have been several attempts to expand the repertoire of triple helices to achieve recognition of more complex sequences. One strategy, enabling recognition across purine–pyrimidine or pyrimidine–purine junctions, is to synthesize oligonucleotides containing internal 3'–3' or 5'–5' linkages which incorporate a change in third-strand polarity [14–16]. An alternative strategy, with which the present study is concerned, is to use triplex-forming oligonucleotides which incorporate both types of triplex motif [17–19]. At purine–pyrimidine junctions the third strand binds to purines on alternate DNA strands, using opposite triplex motifs. For example, recognition across a $G_nT_m \cdot A_mC_n$

junction can, in principle, be achieved by forming antiparallel G·GC triplets in the left hand (G_n) end and parallel T·AT triplets in the right hand (T_m) end. The third-strand oligonucleotide would therefore require the sequence T_mG_n , as illustrated in Figure 1(a). Similarly, the target sequence $T_nG_m \cdot C_mA_n$ could be bound by the oligonucleotide G_mT_n (Figure 1b).

When looked at in three dimensions, however, the transition of the third strand from one duplex strand to the other is not facile [17]. Although the duplex base pairs are arranged perpendicular to the helix axis, they are inclined relative to the phosphodiester backbone as it wraps around the helix. This effect is illustrated in Figures 1(c) and 1(d), which show the base pairs viewed looking along the DNA major groove. As a result of this helical twist, recognition of every purine in the target R_nY_m results in an overlap of two bases in the centre of the third strand (Figure 1d), so that it is possible to construct a continuous third strand which skips 2 bp at the centre of the junction. In contrast, for recognition across Y_nR_m junctions (Figure 1c), although a triplet is formed at every base pair, there is a discontinuity in the third strand at the junction, so that a linker, equivalent to an additional one or two bases, is required to bridge the gap [17].

The binding of triplex-forming oligonucleotides to their target sites can be increased by incorporating a strong DNA-binding agent, such as acridine (Acr) or psoralen, to one or other end of the third strand [20–25]. In this study we examine the formation of specific complexes at R_nY_m and Y_nR_m junctions, using short Acr-linked oligonucleotides. We have attempted to form triple helices at the target sequences $G_6T_6 \cdot A_6C_6$ and $T_6G_6 \cdot C_6A_6$ using the Acr-linked oligonucleotides 5'-Acr- T_5G_5 and 5'-Acr- G_5T_5 respectively. These target sites were designed to be slightly longer than the third-strand oligonucleotides to allow for the possibility of skipping one or two bases in the centre. In each case the target

Abbreviation used: Acr, acridine.

* To whom correspondence should be addressed.

Figure 1 Schematic representation of alternate strand triple-helix formation across YR and RY junctions

(a) Binding of T_5G_5 to the duplex $G_5T_5 \cdot A_5C_5$. (b) Binding of G_6T_6 to the duplex $T_5G_5 \cdot C_5A_5$. In both structures the duplex is boxed and recognition is achieved by the formation of antiparallel G-C and parallel T-A triplets. In (c) and (d) the DNA helix has been opened out and is viewed from along the major groove. Dashed lines indicate the Hoogsteen base pairs. Third-strand pyrimidines are positioned closer to the duplex purine strand, whereas third-strand purines are located in the centre of the major groove. Recognition of the YR junction (c) requires a linker between the two halves of the third strand, whereas for recognition across the RY junction (d) the central two base pairs can be omitted.

sequences were cloned into longer DNA fragments and used as substrates for DNAase I footprinting.

MATERIALS AND METHODS

Oligonucleotides

The Acr-linked oligonucleotides Acr- G_5T_5 and Acr- T_5G_5 were gifts from Dr. M. J. McLean (Cambridge Research Biochemicals, Cambridge, U.K.). In these compounds the [2-methoxy-6-chloro-9-amino]acridine is linked to the terminal phosphate group via a pentamethylene chain. These compounds were stored at -20°C in water at a concentration of $360\ \mu\text{M}$. All other oligonucleotides, used for preparing target sequences, were prepared on an Applied Biosystems 380B DNA synthesizer and used without purification.

Plasmids

The oligonucleotides $GATCG_6T_6$ and $GATCA_6C_6$ or $GATCT_6G_6$ and $GATCC_6A_6$ were treated with polynucleotide kinase and ATP and cloned into the *Bam*HI site of pUC18. The

ligation mixture was transformed into *Escherichia coli* TG2 and successful clones were picked in the usual way as white colonies from agar plates containing 5-bromo-4-chloro-3-indolyl β -D-galactoside and isopropyl β -D-thiogalactopyranoside. The sequences were checked using a T7 sequencing kit (Pharmacia). Several multimeric clones were obtained; the following clones were used in this work: pGT1, a monomeric insert of G_6T_6 oriented so that the A_6C_6 -containing strand is visualized by labelling the 3'-end of the *Hind*III site; pGT2, which contains a dimeric insert of G_6T_6 , with the two halves in opposite orientation, i.e. $GATCA_6C_6GATCG_6T_6$; and pTG2, a dimer of T_6G_6 which lacks a single guanine in one half, so that the sequence $GATCT_6G_5GATCC_6A_6$ is visualized by labelling the 3'-end of the *Hind*III site.

DNA fragments

The polylinker fragments containing the cloned triplex target sites were obtained by cutting the plasmids with *Hind*III, labelling at the 3'-end using reverse transcriptase and [α - ^{32}P]dATP and cutting again with *Eco*RI. In some cases, the fragments were labelled at the opposite end by reversing the order of addition of *Hind*III and *Eco*RI. The radiolabelled fragments were separated from the rest of the plasmid on 6% (w/v) polyacrylamide gels.

DNAase I footprinting

Radiolabelled DNA ($2\ \mu\text{l}$) was mixed with $4\ \mu\text{l}$ of oligonucleotide at the concentration indicated in the text and left to equilibrate for at least 30 min at 20°C . The oligonucleotides were diluted in 10 mM Tris/HCl, pH 7.5, containing 5 mM MgCl_2 (or 5 mM MnCl_2 in some experiments). In some experiments, 0.1 mM spermidine was also included, but this was subsequently shown not to affect triplex formation. The complexes were digested by adding $2\ \mu\text{l}$ of DNAase I (0.01 Kunitz units/ml). Samples were removed at 1 and 5 min and the reaction stopped by adding $4\ \mu\text{l}$ of formamide containing 10 mM EDTA.

Dimethyl sulphate protection

Radiolabelled DNA ($2\ \mu\text{l}$) was mixed with $10\ \mu\text{l}$ of oligonucleotide, dissolved in an appropriate buffer and left to equilibrate for at least 30 min at 20°C . The complexes were then reacted with $1\ \mu\text{l}$ of dimethyl sulphate for 1 min. The reaction was stopped by the addition of β -mercaptoethanol and the DNA precipitated with ethanol. The DNA pellets were then boiled in 10% (v/v) piperidine and lyophilized. Samples were redissolved in $6\ \mu\text{l}$ of formamide loading buffer and subjected to electrophoresis.

Gel electrophoresis

The products of digestion were separated on 9% (w/v) (*Hind*III-labelled) or 12% (w/v) (*Eco*RI-labelled) polyacrylamide gels containing 8 M urea. These were run at 1500 V for ~ 2 h. Gels were then fixed in 10% (v/v) acetic acid, transferred to Whatman 3MM paper, dried under vacuum at 80°C and autoradiographed at -70°C with an intensifying screen. Bands in DNAase I digests were assigned by comparison with Maxam-Gilbert dimethyl sulphate markers specific for guanine. Gels were scanned with a Hoefer GS365W scanning microdensitometer. Differential cleavage plots were constructed from these scans, expressing the intensity of each band in the oligonucleotide-treated sample relative to that in the control, normalized with respect to the total intensity in each lane.

Figure 2 DNAase I digestion of fragments containing (a) dimeric (pGT2) and (b) monomeric (pGT1) inserts of the sequence $G_6T_6 \cdot A_6C_6$ in the absence (CON) and presence of the acridine-linked oligonucleotides $5'$ -Acr- T_5G_5 (AcrTG) and $5'$ -Acr- G_5T_5 (AcrGT)

Each pair of lanes corresponds to digestion by the enzyme for 1 (left) and 5 min (right). Oligonucleotide concentrations (μ M) are shown at the top of each pair of lanes. All complexes were formed in 10 mM Tris/HCl, pH 7.5, containing 5 mM $MgCl_2$, except for the lanes labelled —Mg, in which the magnesium was omitted. The square brackets indicate the position and sequence of the triplex target sites. Tracks labelled G correspond to dimethyl sulphate-piperidine markers specific for guanine.

RESULTS

$G_6T_6 \cdot A_6C_6$

Figure 2 presents DNAase I digestion patterns for monomeric (pGT1) and dimeric (pGT2) inserts of fragments containing the insert $G_6T_6 \cdot A_6C_6$ in the presence of the Acr-linked oligonucleotide $5'$ -Acr- T_5G_5 . In each case the oligonucleotide $5'$ -Acr- G_5T_5 , which possesses the wrong orientation to form a triple helix, is included as a control.

It can be seen that, as predicted, $5'$ -Acr- T_5G_5 produces clear footprints around each of the A_6C_6 and G_6T_6 tracts and that no changes are evident with the $5'$ -Acr- G_5T_5 . The interaction can be seen to require bivalent metal ions since no footprint is produced in the absence of magnesium. Since the DNAase I digestion buffer contains both magnesium and manganese (at a final concentration of ~ 0.5 mM) this implies that the rate of formation of these triplexes must be slow, compared with the DNAase I digestion time. Examination of the results for the dimeric insert, which are also presented as a differential cleavage plot in Figure 3, reveals that the footprint in the A_6C_6 tract extends beyond the insert by 3–4 bases in the $5'$ (upper) direction whereas in the G_6T_6 the footprint is staggered in the $3'$ (lower) direction. These

differences correspond to the location of the acridine moiety, which should be intercalated towards the end of the AT tracts.

It should be remembered that these footprints do not arise from direct steric occlusion of the enzyme since the triplex-forming oligonucleotide is positioned in the DNA major groove, whereas DNAase I cuts from the minor groove; instead, these must result from triplex-induced changes in DNA structure and/or flexibility. Figure 3 reveals that cleavage of the third TpT step in the G_6T_6 tract is not affected by the oligonucleotide; indeed, this cleavage is slightly increased. This enhanced band is clearer in the presence of manganese (see Figure 6 below). There is no enhancement at the equivalent position in the A_6C_6 tract of either the monomeric or dimeric inserts. This band is located 3 bases from the purine–pyrimidine junction. Since this is the distance by which DNAase I cleavage is generally staggered across the two DNA strands, it may be consistent with a model in which the central bases at the junction are not bound by the oligonucleotide [17].

The interaction of this sequence with $5'$ -Acr- T_5G_5 was further investigated by studying its ability to protect guanine residues from dimethyl sulphate methylation. The results of this experiment are presented in Figure 4. It can be seen that in the

Figure 3 Effect of 5'-Acr-T₅G₅ on DNAase I cleavage of a fragment containing a dimeric insert of the sequence A₅C₆·G₆T₆

The points, derived from densitometer scans of the data presented in Figure 2, represent the cleavage of each bond in the presence of the oligonucleotide relative to that in the control.

Figure 4 Effect of Acr-linked oligonucleotides on dimethyl sulphate modification of a fragment containing a dimeric insert of the sequence G₆T₆·A₅C₆

(a) Autoradiograph of the products of dimethyl sulphate/piperidine modification. 0, no oligonucleotide added (control); GT, 5'-Acr-G₅T₅; Acr-TG, 5'-Acr-T₅G₅. The oligonucleotide concentration (μM) is shown at the top of each lane. The track labelled —Mg was performed in the absence of magnesium. (b) Effect of 5'-Acr-T₅G₅ on dimethyl sulphate/piperidine modification of G₆T₆. The bars, derived from densitometer scans of the data presented in (a), correspond to the cleavage of each guanine in the presence of the oligonucleotide, relative to that in the control. (c) Schematic representation of the interaction of 5'-Acr-T₅G₅ with G₆T₆·A₅C₆.

presence of the oligonucleotide the upper (5') five guanines are protected from methylation, while the guanine residue in the centre is unaffected. This interaction also requires the presence of magnesium. No changes are apparent with the reverse oligonucleotide (5'-Acr-G₅T₅), confirming the specificity of the interaction. A schematic representation of the interaction of 5'-Acr-T₅G₅ with this target sequence is shown in Figure 4(c).

Figure 5 DNAase I digestion of a fragment containing a dimeric insert of the sequence T₅G₅·C₆A₆ in the absence (CON) and presence of the Acr-linked oligonucleotides 5'-Acr-T₅G₅ (AcrTG) and 5'-Acr-G₅T₅ (AcrGT)

Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. Oligonucleotide concentrations (μM) are shown at the top of each pair of lanes. The square brackets indicate the position and sequence of the triplex target sites. Note that a single guanine is missing from the upper half of the dimer. The track labelled G corresponds to a dimethyl sulphate-piperidine marker specific for guanine.

Although we can be confident about the location of the G₅ portion of the third strand, there is some ambiguity as to the position of Acr-T₅ and whether or not the central thymine is also skipped.

Figure 6 DNAase I digestion of fragments containing the dimeric inserts $G_6T_5 \cdot A_6C_6$ (pGT2) and $T_6G_5 \cdot C_6A_6$ (pTG2) in the presence of 5 mM $MnCl_2$

CON, control; AcrTG, 5'-Acr- T_5G_5 ; AcrGT, 5'-Acr- G_5T_5 . Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. Oligonucleotide concentrations (μM) are shown at the top of each pair of lanes. The square brackets indicate the position and sequence of the triplex target sites. Note that a single guanine is missing from the upper half of the dimer in pTG2.

$T_6G_5 \cdot C_6A_6$

Figure 5 shows the results of similar DNAase I footprinting experiments examining the interaction of these oligonucleotides with a fragment containing the target sequence $T_6G_5 \cdot C_6A_6$. No changes in the cleavage pattern are evident with either oligonucleotide, even though 5'-Acr- G_5T_5 has the correct sequence to form a stable triplex. It has been suggested that the change in position of the third strand across the YR junction requires a linker of ~ 2 bp between the two halves of the molecule [17] (Figure 1c). As a result, only 8 of the 10 possible triplets will be able to form. Even the presence of the intercalating acridine moiety is not sufficient to stabilize this short triplex. A further destabilizing factor for this triplex is that there will be a loss of base stacking in the third strand at the junction.

Effect of manganese

It has recently been reported that the nature of the bivalent cation can affect triplex stability and that manganese and cobalt may have a greater stabilizing effect than magnesium [13]. We have therefore repeated these experiments replacing the magnesium with manganese. The results of this are shown in Figures 6 and 7. The results obtained with the RY target sequence ($G_6T_6 \cdot A_6C_6$ -pGT2, Figure 6) are similar to those seen using magnesium as the counterion (Figure 2). A clear protection can be seen with 5'-Acr- T_5G_5 , centred on each of the target sites in the dimer. This protection is staggered towards the upper (5') end of each site, consistent with the location of the intercalating acridine moiety.

In contrast, the presence of manganese dramatically alters the patterns produced with the fragment bearing the YR junction (pTG2, Figure 6). We had anticipated that the increased binding in the presence of manganese might stabilize the interaction of Acr- G_5T_5 with this target site. Although this does appear to be the case, the results show that both oligonucleotides produce footprints under these conditions, though at different positions within the fragment. These results are presented as differential cleavage plots in Figure 7. Looking first at the patterns for 5'-Acr- G_5T_5 , a large footprint can be seen around the sequence T_6G_5 , extending in the 3' (lower) direction into the second half of the dimer. This is what we might expect with this oligonucleotide, since the acridine moiety will be located at the 3' (lower) end of the block of guanines. Although cleavage in the second (lower) half of the dimer is reduced, this does not appear to be such a good binding site. The possible reasons for this will be considered further in the Discussion section.

Figure 6 also shows the unexpected result that, in the presence of manganese, Acr- T_5G_5 produces a footprint on fragment pTG2. This is in a different position to that produced by Acr- G_5T_5 , located higher up the gel, towards the 5' end of the labelled strand. The differential cleavage plot for this interaction is presented in Figure 7(b). It appears that the oligonucleotide is binding towards the 5' end of the insert, probably recognizing the sequence GGGGATCTTTT formed at the junction with the remainder of the fragment. By analogy with the interaction of this oligonucleotide with the correct target sequence G_6T_6 , described above, we suggest that two bases in the centre (probably the TC step) will be skipped by the third strand. This complex will then contain four antiparallel G·GC triplets, five parallel

Figure 7 Effect of Acr-linked oligonucleotides on DNAase I digestion of a fragment containing a dimeric insert of the sequence $T_6G_6 \cdot C_6A_6$

The points, derived from densitometer scans of the data presented in Figure 6, represent the cleavage of each bond in the presence of the oligonucleotide relative to that in the control. (a) 5'-Acr-G₅T₅ (5 μM). (b) 5'-Acr-T₅G₅ (10 μM).

T·AT triplets, and an unusual antiparallel G·AT mismatch close to the centre. Attempts to probe the nature of the triplexes formed in the presence of manganese with dimethyl sulphate proved unsuccessful; we could detect no changes in the pattern of guanine modification.

DISCUSSION

The results presented in this paper demonstrate that it is possible to achieve sequence-specific DNA recognition across RY and YR junctions using short Acr-linked oligonucleotides designed to generate triplexes which contain both parallel and antiparallel triplet motifs. The results are consistent with a model in which recognition across an R_nY_m junction is achieved without specific interaction with two base pairs at the centre, whereas recognition across a Y_nR_m junction requires extra bases in the DNA third strand [17]. By using 5'-Acr-T₅G₅ to recognize G₆T₆·A₆C₆, 10 triplets are formed across 12 bp, with uninterrupted base stacking within the third strand. In contrast, the interaction between Acr-G₅T₅ and the target sequence T₆G₆·C₆A₆ generates a structure containing eight triplets and a probable discontinuity in the third strand, where two bases are used to bridge the gap (see Figure 1c). As a result, recognition across the Y_nR_m junction is less stable than recognition across an R_nY_m junction.

Previous studies have shown that manganese and cobalt can stabilize certain intermolecular triplexes [13], especially those

containing A·AT triplets. The present results show that manganese can stabilize recognition across the YR junction, and permits binding across the RY junction with an oligonucleotide incorporating at least one third-strand mismatch. For the unexpected interaction of Acr-T₅G₅ with pTG2, it is worth noting that the footprint is evident at the upper but not the lower target site. Even though the insert is symmetrical, it is cloned into a longer fragment so that the boundary at the other end has the sequence AAAAAAGCTCCTCT. If this were to be bound by Acr-G₅T₅ in a similar fashion, skipping the central AG, then this would create at least two G·AT triplets. It therefore appears that manganese may be able to stabilize nine triplets with one mismatch, but not eight triplets with two mismatches. Since recognition across the T₆G₆ junction with G₅T₅ involves the formation of eight triplets, it is tempting to speculate that the energy change on replacing magnesium with manganese is equivalent to the formation of an extra base triplet. An alternative explanation for the effect of manganese is that it stabilizes recognition across RY or YR junctions. In this regard, it may be relevant that in the secondary interaction of Acr-T₅G₅ with pTG2 the G·AT mismatch is located close to the junction and so may not have a strong destabilizing effect.

The binding of 5'-Acr-G₅T₅ to pTG2 in the presence of manganese is unusual in that, although the fragment contains two approximately equivalent sites, the upper site (T₆G₆) produces a much better footprint than the lower one (C₆A₆). This

effect is difficult to explain, but may be related to the position of the acridine moiety in this complex. Since dimethyl sulphate modification under these conditions was unsuccessful, we cannot be certain about the exact position of the complex, though the most likely structure would use two thymines to form this bridge, leaving five G·GC and three T·AT triplets. If the acridine is intercalated at the triplex–duplex junction then this will be located at GpA (upper) or CpC (lower). Perhaps these sites have different binding affinities. Alternatively, if intercalation occurs one base from the triplex–duplex boundary, then the acridines from the two sites will be situated at adjacent base steps (ApT and TpC). Simultaneous binding to both sites will therefore be forbidden by the neighbour exclusion principle.

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