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Triple helix formation at $(AT)_n$ adjacent to an oligopurine tract

Darren M. Gowers and Keith R. Fox*

Division of Biochemistry and Molecular Biology, School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton SO16 7PX, UK

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ABSTRACT

We have used DNase I footprinting to investigate the recognition of $(AT)_n$ tracts in duplex DNA using GT-containing oligonucleotides designed to form alternating G·TA and T·AT triplets. Previous studies have shown that the formation of these complexes is facilitated by anchoring the triplex with a block of adjacent T·AT triplets, i.e. using $T_{11}(TG)_6$ to recognize the target $A_{11}(AT)_6.(AT)_6T_{11}$. In the present study we have examined how the stability of these complexes is affected by the length of either the T·AT tract or the region of alternating G·TA and T·AT triplets, using oligonucleotides of type $T_x(TG)_y$ to recognize the sequence $A_{11}(AT)_{11}$. We find that successful triplex formation at $(AT)_n$ (n = 3, 6 or 11) can be achieved with a stabilizing tail of 11×T·AT triplets. The affinity of the third strand increases with the length of the $(GT)_n$ tract, suggesting that the alternating G·TA and T·AT triplets are making a positive contribution to stability. These complexes are stabilized by the presence of manganese or a triplex-specific binding ligand. Shorter oligonucleotides, such as $T_7(TG)_5$, bind less tightly and require the addition of a triplex-binding ligand. $T_4(GT)_5$ showed no binding under any conditions. Oligonucleotides forming a 3′-terminal T·AT are marginally more stable that those with a terminal G·TA. The stability of these complexes was further increased by replacing two of the T·AT triplets in the T_n tail region with two C^+·GC triplets.

INTRODUCTION

Although the discovery of three-stranded DNA structures dates from 1957 (1), interest in these complexes has recently intensified due to the realization that synthetic oligonucleotides can be used as antigene agents forming intermolecular DNA triplexes at specific DNA sequences (2–4). Two types of triplex have been characterized which differ in the orientation of the third strand. Pyrimidine-rich oligonucleotides bind parallel to the purine strand of the target duplex forming T·AT and C^+·GC triplets (5–7), while purine-rich third strands bind in an antiparallel orientation generating G·GC, A·AT and T·AT triplets (8,9). In each of these triplets the third strand bases make hydrogen bond contacts only to the purine base of the duplex. As a result, triplex formation is generally restricted to homopurine-homopyrimidine tracts; pyrimidine interruptions in the purine strand cause a large decrease in affinity.

There is, therefore, considerable interest in developing means for recognizing pyrimidine residues by triplex formation. Several synthetic compounds have been tested to fulfil this role, including deoxynebularine (10), azole-substituted bases (11) and acridine-conjugated oligonucleotides (12). Efforts to recognize the hydrogen bonding face of the TA or CG base pair, for example with *N*4-(6-amino-2-pyridinyl)deoxycytidine (13) or functionalized naphthimidazoles (14), have met with moderate success.

Another approach to triplex formation at pyrimidine interruptions is to use standard DNA bases which form non-canonical triplets, such as G·TA (15–23) or T·CG (24–25). G·TA triplets have been studied in different sequence contexts, either singly (16,17) or in clusters (18). Single isolated G·TA triplets produce triplexes which are less stable than those containing only T·AT and C^+·GC triplets, but are more stable than other triplet combinations at the TA inversion. Up to three consecutive TA base pairs can be recognized using G·TA triplets, but these complexes are less stable and require additional factors, such as the presence of a triplex-binding ligand (18). The stability of the G·TA triplet is affected by the nature of the flanking base pairs (triplets); surrounding T·AT triplets generate more stable complexes than C^+·GC (15,20,21). Previous work by Chandler and Fox (26) showed that under certain conditions alternating G·TA and T·AT triplets can be used as a means for recognizing tracts of alternating AT. They examined the binding of $T_{11}(TG)_6$ to the centre of a DNA fragment containing the sequence $(TA)_{11}T_{34}$. This formed a complex containing six G·TA triplets alternating with six T·AT triplets and a block of 11×T·AT triplets. These studies showed that although $(TG)_6$ alone could not form a stable complex, successful triplex formation at the $(AT)_n$ tract could be achieved by attaching a T_{11} tail, generating an anchoring block of T·AT triplets. This complex was stable in the presence of manganese or a triplex-binding ligand.

This paper extends these studies and examines how the lengths of both the stabilizing anchor of T·AT triplets and the block of alternating G·TA and T·AT triplets affect triplex stability at regions within the sequence $(TA)_{11}T_{34}$. These studies were performed at pH 7.5 in manganese or magnesium containing buffers, both in the

*To whom correspondence should be addressed. Tel: +44 1703 594374; Fax: +44 1703 594459; Email: krf1@soton.ac.uk

presence and absence of a triplex-binding ligand. Since recent studies have suggested that C^+·GC imparts a greater stability than T·AT at low pH (27–29), we have examined the effect of introducing C^+·GC triplets into the stabilizing anchor.

MATERIALS AND METHODS

Chemicals and enzymes

Oligodeoxynucleotides were purchased from Oswel DNA Service (UK). Alkaline phosphatase, pUC18 and DNA ligase were from Pharmacia. Bovine DNase I was purchased from Sigma and stored at –20°C at a concentration of 7200 U/ml. Restriction enzymes and reverse transcriptase were purchased from Promega. The naphthylquinoline triplex-binding ligand (30–33) was a gift from Dr L.Strekowski (Department of Chemistry, Georgia State University) and was stored at –20°C as a 20 mM solution in dimethylsulphoxide.

DNA fragments

Preparation of the fragment containing the sequence $(TA)_{11}T_{34}$ has been previously described (26,30). Plasmid k2 (30), which contains a human genomic fragment inserted in the *Bam*HI site of pUC19, was digested with *Hin*dIII and *Fnu*4H1 and labelled at the 3′-end of the *Hin*dIII site with [α-^{32}P]dATP using reverse transcriptase. Since the insert contains an internal *Hin*dIII site, this yields two radiolabelled fragments of 105 and 161 bp, the longer of which contains the sequence $(TA)_{11}T_{34}$. In this fragment, designated k2, the strand containing the sequence $(TA)_{11}T_{34}$ is visualized, with the alternating AT tract running towards the top of the footprinting gel. In order to visualize the opposite strand and to simplify the purification procedure, this fragment was digested with the *Nla*III and *Sau*3A1 and subcloned into pUC18 which had been cut with *Bam*HI and *Sph*I. This plasmid was digested with *Hin*dIII and *Sac*I and labelled at the 3′-end of the *Hin*dIII site with [α-^{32}P]dATP using reverse transcriptase. This procedure generates a fragment, designated k2rev, visualizing the strand containing the sequence $A_{34}(TA)_{11}$ in which the alternating AT tract runs towards the bottom of the footprinting gels. The sequences of fragments k2 and k2rev are shown in Figure 1a. In order to investigate the effect of introducing C^+·GC triplets into the block of flanking T·AT triplets, a fragment was prepared containing the sequence $AAAGAGA(TA)_5.(TA)_5TCTCTTT$. This sequence was cloned into *Bam*HI-cut pUC18. This plasmid was cut with *Hin*dIII and *Sac*I and labelled at the 3′-end of the *Hin*dIII site with [α-^{32}P]dATP using reverse transcriptase. This insert was oriented so that the labelled strand contained the sequence $AAAGAGA(TA)_5$. The sequence of this fragment, designated TC-$(AT)_n$ is also shown in Figure 1a. The sequence of each clone was confirmed by dideoxy sequencing using a T7 sequencing kit (Pharmacia). Radiolabelled fragments for footprinting were separated on a 6% (w/v) polyacrylamide gel. The DNA fragments of interest were eluted from the gel and dissolved in 10 mM Tris–HCl, pH 7.5, containing 0.1 mM EDTA, so as to give about 10 c.p.s./µl, as determined on a hand-held Geiger counter (~1 nM).

DNase I footprinting

Radiolabelled DNA fragments (1.5 µl) were mixed with oligonucleotide (1.5 µl) and 1.5 µl buffer or triplex-binding ligand.

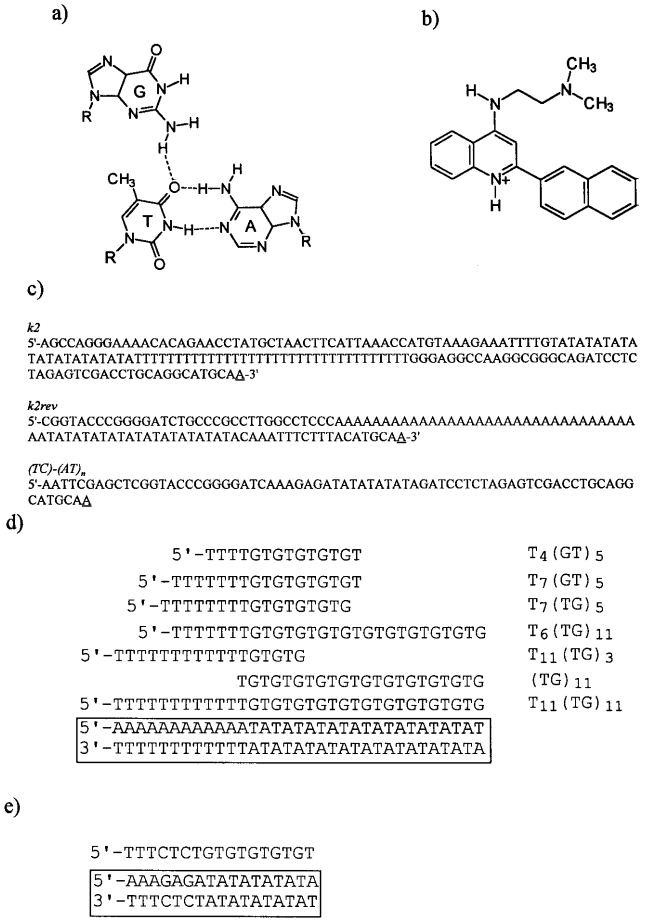

Figure 1. (**a**) Chemical structures of the G·TA triplet drawn as described in Radhakrishnan *et al*. (23) and (**b**) the naphthylquinoline triplex-binding ligand. (**c**) Sequence of fragments k2, k2rev and TC-$(AT)_n$. k2 is obtained by digesting with *Fnu*4H1 and *Hin*dIII while fragments k2rev and TC-$(AT)_n$ are *Sac*I–*Hin*dII fragments. The base bearing the radiolabel at the 3′-end is underlined. (**d**) Sequence of the target site (boxed) in fragments k2 and k2rev, together with the seven different oligonucleotides designed to interact with different portions of this site. (**e**) Sequence of the target site (boxed) in fragment TC-$(AT)_n$, together with the third strand oligonucleotide.

Oligonucleotide and ligand concentrations refer to the concentrations in this mixture. For fragments k2 and k2rev the oligonucleotides and ligand were dissolved in 10 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$. For fragment TC-$(AT)_n$ the third strand oligonucleotide was dissolved in 50 mM sodium acetate, pH 5.5, containing 10 mM $MgCl_2$. The complexes were left to equilibrate for at least 3 h at 20°C. DNase I digestion was initiated by adding 2 µl DNase I (0.4 U/ml) dissolved in 20 mM NaCl containing 2 mM $MnCl_2$ and 2 mM $MgCl_2$. The reaction was stopped after 1 min by adding 4 µl 80% formamide containing 10 mM EDTA and 0.1% (w/v) bromophenol blue.

Gel electrophoresis

The products of reaction were separated on 10% (w/v) polyacrylamide gels containing 8 M urea (National Diagnostics). Gels (40 cm long, 0.3 mm thick) were run at 1500 V for ~2 h. Gels were fixed in 10% (v/v) acetic acid before drying at 80°C and

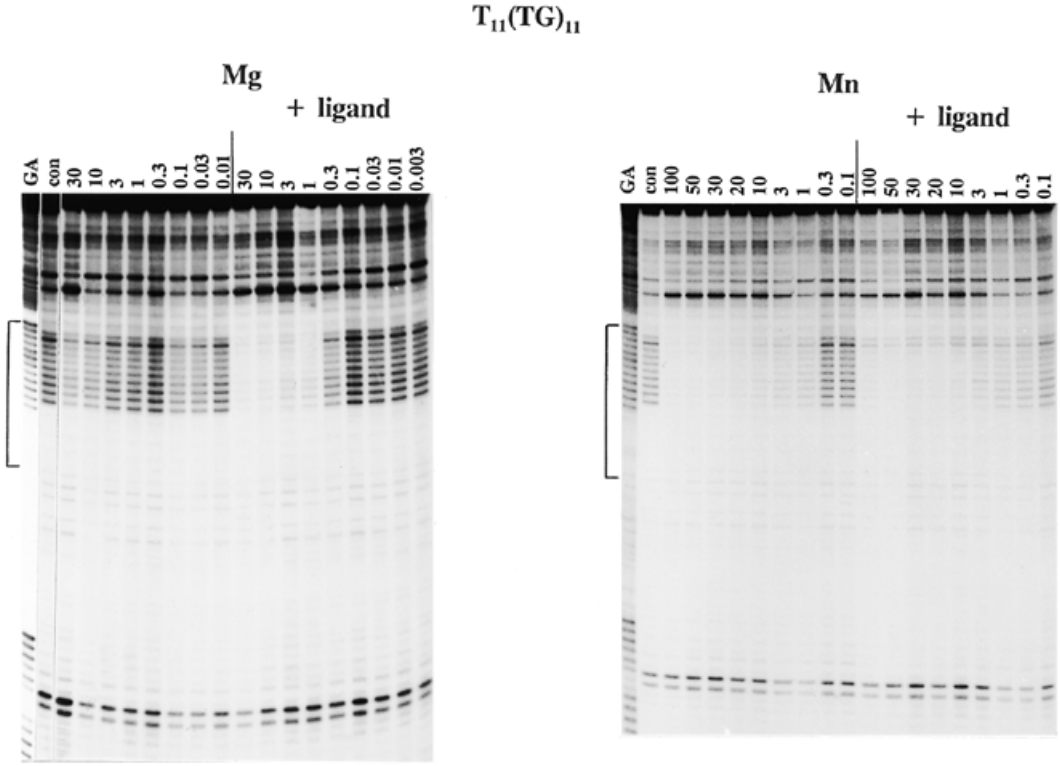

Figure 2. DNase I digestion of fragment k2 in the presence and absence of various concentrations of $T_{11}(TG)_{11}$. Reactions were performed in 10 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$. For each panel the right-hand lanes included 10 μM naphthylquinoline triple-binding ligand. The oligonucleotide concentration (μM) is shown at the top of each gel lane. Tracks labelled GA are Maxam–Gilbert markers specific for purines; con indicates cleavage of DNA in the absence of added oligonucleotide. The bracket indicates the position of the intended target site.

autoradiography at –70°C using an intensifying screen. Bands in the digestion pattern were assigned by comparison with Maxam–Gilbert sequencing lanes specific for adenine and guanine.

RESULTS

Previous studies have shown that six pairs of alternating G·TA and T·AT triplets can be stabilized by an adjacent block of 11×T·AT triplets (26). Although $(TG)_6$ did not produce a footprint, $T_{11}(TG)_6$ produced footprints which persisted to 1 μM in the presence of 10 mM $MnCl_2$ or 10 mM $MgCl_2$ together with 10 μM triplex-binding ligand. We have examined the binding of seven related oligonucleotides of sequence $T_x(TG)_y$, shown in Figure 1d, to fragments containing the target sequence $A_{34}(TA)_{11}.(TA)_{11}T_{34}$. These seven oligonucleotides were designed so as to alter the length of the stabilizing block of T·AT triplets or the region of alternating T·AT and G·TA.

$T_{11}(TG)_{11}$

Figure 2 shows the results of DNase I footprinting experiments examining the interaction of $T_{11}(TG)_{11}$ with fragment k2. This oligonucleotide should form a complex containing 11 T·AT triplets flanking a block of 11 alternating T·AT and G·TA triplets. This extends the region of the $(AT)_n$ tract targeted by triplex formation, compared with our previous studies with $T_{11}(TG)_6$ (26). As expected, no changes in DNase I digestion were observed with $(TG)_{11}$ under any conditions (not shown), confirming that alternating G·TA and T·AT triplets alone do not produce a stable triplex. The first panel shows results in the presence of 10 mM $MgCl_2$, for which no clear footprint is evident, though there is a slight reduction in band intensity at the highest oligonucleotide concentrations (>10 μM). Addition of 10 μM naphthylquinoline triplex-binding ligand (30–33) (right-hand lanes) induces a footprint, which covers the entire $(TA)_{11}$ tract and which persists to an oligonucleotide concentration of ~1 μM. As previously noted, there is almost no DNase I cleavage within the long T_n tract and oligonucleotide binding can only be assessed by changes in cleavage of bands within the $(AT)_n$ tract. Although it is not possible to use DNase I footprinting to detect binding of the T_n tail to the $A_n.T_n$ tract, successful formation of a block of T·AT triplets can be inferred from the induced binding of $(TG)_{11}$ to the $(AT)_{11}$ tract. The second panel of Figure 2 shows a similar experiment performed in the presence of 10 mM $MnCl_2$. In this case a clear footprint covering the entire $(AT)_n$ tract can be seen, which persists to ~1 μM even in the absence of the triplex-binding ligand. In the presence of the triplex-binding ligand the footprinting persists to 0.3 μM.

Figure 3 shows the interaction of $T_{11}(TG)_{11}$ with fragment k2rev, which reveals the opposite DNA strand, positioning the $(AT)_n$ tract towards the bottom of the gel. Once again, the oligonucleotide has little effect in the presence of $MgCl_2$ alone (left-hand panel), though a footprint is induced on addition of 10 μM triplex-binding ligand which persists to ~1 μM oligonucleotide. In this case, enhanced cleavage is also evident at the bottom (3′-end) of the target site, close to the triplex–duplex boundary (indicated by the arrow). The right-hand panel of Figure 3 shows the interaction of $T_{11}(TG)_{11}$ with this fragment in the presence of

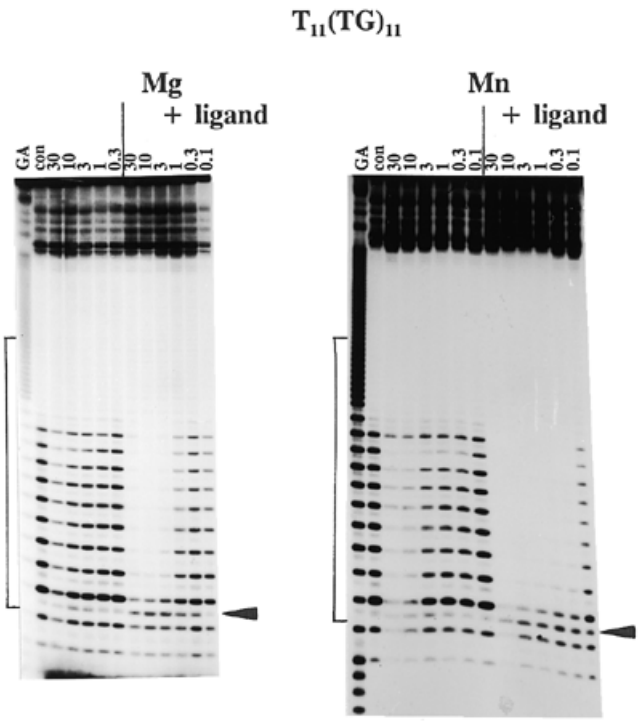

Figure 3. DNase I digestion of fragment k2rev in the presence and absence of various concentrations of $T_{11}(TG)_{11}$. Reactions were performed in 10 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$. For each panel the right-hand lanes included 10 μM naphthylquinoline triple-binding ligand. The oligonucleotide concentration (μM) is shown at the top of each gel lane. Tracks labelled GA are Maxam–Gilbert markers specific for purines; con indicates cleavage of DNA in the absence of added oligonucleotide. The bracket indicates the position of the intended target site. The arrow indicates enhanced cleavage observed in the presence of the oligonucleotide.

10 mM $MnCl_2$. The results are similar to those seen with fragment k2, with footprints evident to 10 and 0.3 μM in the absence and presence of 10 μM triplex-binding ligand respectively.

These results show that a block of 11 consecutive T·AT triplets can be used to support recognition of $(AT)_{11}.(AT)_{11}$ by $(TG)_{11}$.

$T_{11}(TG)_3$

Figure 4 shows the results of DNase I footprinting experiments of this oligonucleotide with fragments k2 and k2rev. The first panel shows the interaction with k2. The left-hand side of this panel reveals that, in the presence of magnesium, $T_{11}(TG)_3$ only affects the cleavage pattern on addition of triplex-binding ligand, for which the footprint persists to 1 μM. As expected, only the lower part of the $(TA)_n$ tract is protected from cleavage, since the third strand only covers the 5′-end of the $(TA)_{11}$ tract. In this instance the footprints are accompanied by enhanced cleavage within the $(AT)_{11}$ tract about three bases above the triplex–duplex boundary. We presume that this enhancement reflects oligonucleotide-induced changes in the local DNA structure at the triplex–duplex junction, which provides further evidence for successful triplex formation. The right-hand side of this panel shows the results of similar experiments performed in manganese containing buffers. No footprint is seen in the absence of the triplex-binding ligand, in contrast to the results with $T_{11}(TG)_{11}$ and $T_{11}(TG)_6$. This suggests that the region of alternating G·TA and T·AT triplets makes a positive contribution to stability of the complexes, since shortening this region appears to lower the affinity. Once again, a clear footprint is evident in the presence of the ligand, which persists to ~1 μM.

The second panel of Figure 4 shows the results of similar experiments with fragment k2rev in magnesium containing buffers. A footprint is evident in the presence of 10 μM triplex-binding ligand, covering the upper two strong bands from the $(AT)_n$ tract, which persists to an oligonucleotide concentration of 1 μM. In this case, some changes can also be seen at the upper end of the fragment, above the 5′ (upper)-end of the A_{34} tract, in which a few bands are protected from DNase I cleavage in the presence of the oligonucleotide. This is far removed from the intended target site and must represent non-specific binding of $T_{11}(TG)_3$ within the A_{34} tract. We presume that this is caused by the 12 consecutive T·AT triplets, which can form at any position within the $A_{34}.T_{34}$ tract, leaving the remaining five bases of the oligonucleotide either hanging free in solution or forming three mismatched G·AT triplets. The latter suggestion seems unlikely, since other studies have shown that G·TA is much more stable than G·AT (15).

$T_6(TG)_{11}$

The results presented above demonstrate that 11 consecutive T·AT triplets can anchor the interaction between $(TG)_{11}$ and $(AT)_{11}.(AT)_{11}$. We next decreased the length of the stabilizing T·AT tail and examined whether six T·AT triplets were sufficient to stabilize the interaction with $(AT)_{11}$. No binding of this oligonucleotide was observed in the presence of 10 mM $MgCl_2$, even after adding 10 μM triplex-binding ligand (not shown). Figure 5 shows the interaction of this oligonucleotide with fragment k2 in the presence of 10 mM $MnCl_2$. In the absence of the triplex-binding ligand (left lanes) a footprint is evident at high oligonucleotide concentrations (<30 μM) which covers the entire $(TA)_n$ tract. It therefore appears that shortening the stabilizing anchor from 11 to six T·AT triplets decreases the affinity by ~30-fold. Addition of 10 μM triplex-binding ligand (right lanes) potentiates formation of this triplex and a clear footprint can be seen which persists to 1 μM.

$T_7(TG)_5$ and $T_7(GT)_5$

These two oligonucleotides were designed to compare the effect of T·AT and G·TA as the terminal triplets. Both oligonucleotides are capable of interacting with the centre of the target site, generating complexes containing the same number of T·AT and G·TA triplets, but in different configurations. $T_7(TG)_5$ will generate a block of eight consecutive T·AT triplets followed by nine triplets which alternate between T·AT and G·TA, terminating in a G·TA. $T_7(GT)_5$ produces a stabilizing tail which is shorter by one T·AT triplet and is followed by 10 triplets which alternate between T·AT and G·TA, terminating in a T·AT. The interaction of these oligonucleotides with k2 is presented in the first two panels of Figure 6. Neither oligonucleotide shows any interaction with the target site in the presence of $MgCl_2$, even after adding 10 μM triplex-binding ligand, and no interaction is seen with 10 mM $MnCl_2$ alone. This is similar to the behaviour of $T_6(TG)_{11}$. However, in the presence of 10 mM $MnCl_2$ and 10 μM triplex-binding ligand, $T_7(TG)_5$ produces a footprint which persists to an oligonucleotide concentration of ~10 μM (Fig. 6, left-hand panel). Similarly, $T_7(GT)_5$, which possesses a shorter

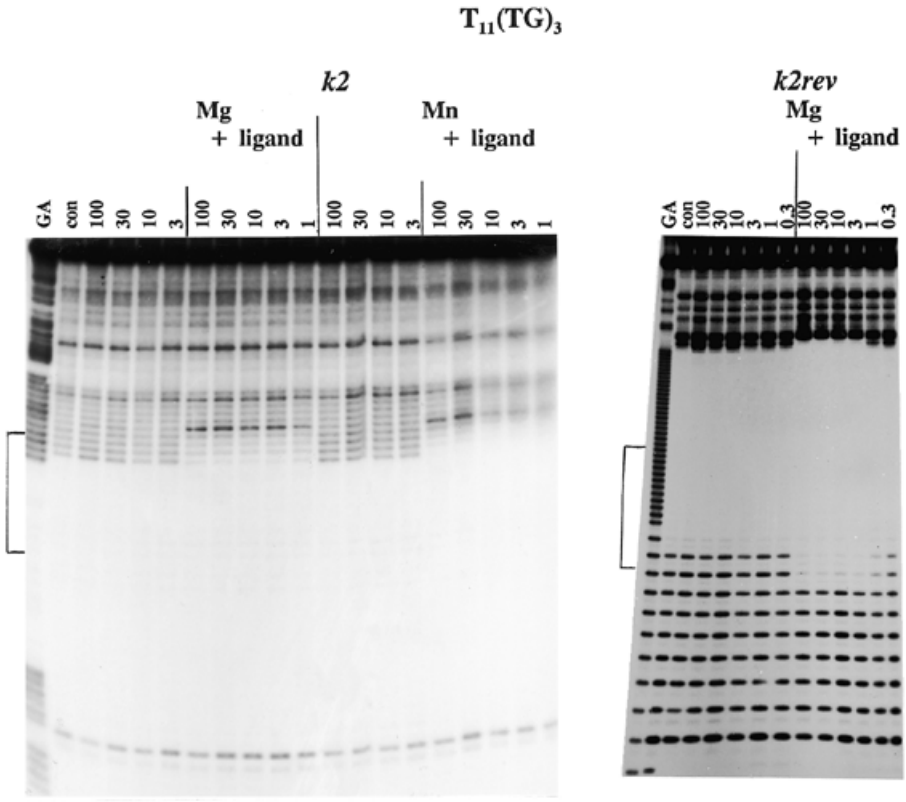

Figure 4. DNase I digestion of fragments k2 and k2rev in the presence and absence of various concentrations of $T_{11}(TG)_3$. Reactions were performed in 10 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and either 10 mM $MgCl_2$ or 10 mM $MnCl_2$. For each set of conditions the right-hand lanes included 10 μM naphthylquinoline triple-binding ligand. The oligonucleotide concentration (μM) is shown at the top of each gel lane. Tracks labelled GA are Maxam–Gilbert markers specific for purines; con indicates cleavage of DNA in the absence of added oligonucleotide. The bracket indicates the position of the intended target site.

stabilizing T·AT tail, generates a footprint in the presence of manganese and the triplex-binding ligand which persists to an oligonucleotide concentration of ~3 μM (Fig. 6, centre panel). It therefore appears that the complex containing a terminal T·AT triplet is slightly more stable than that with a terminal G·TA. These oligonucleotides produce footprints which cover only the lower half of the $(TA)_{11}$ tract and, as expected, the footprint with $T_7(GT)_5$ protects one more bond than $T_7(TG)_5$. The results of similar experiments with fragment k2rev are presented in the third panel of Figure 6. Once again, footprints are only evident in the presence of the triplex-binding ligand and persist to 10 and 3 μM for $T_7(TG)_5$ and $T_7(GT)_5$ respectively. Since the $(AT)_n$ tracts runs towards the bottom of the gel in this fragment, it can be clearly seen that the footprint with $T_7(GT)_5$ is one base longer than that with $T_7(TG)_5$, as expected.

$T_4(GT)_5$

This shorter oligonucleotide should generate a complex with only four consecutive T·AT triplets anchoring 10 triplets which alternate between G·TA and T·AT. This oligonucleotide did not alter the DNase I cleavage pattern of fragment k2, even at a concentration of 100 μM in the presence of 10 mM $MnCl_2$ and 10 μM triplex-binding ligand (not shown). This lack of binding is presumably due to an inadequate length of both the stabilizing T·AT tail and the block of T·AT/G·TA triplets.

Inclusion of C^+·GC triplets in the T·AT tail

In the complexes described above, the regions of alternating T·AT and G·TA triplets were stabilized by tails consisting of only T·AT triplets. Since several recent reports have suggested that C^+·GC imparts a greater stability to triplexes at low pH than T·AT (27–29), we designed a sequence in order to examine whether an anchor containing both T·AT and C^+·GC triplets could form a better anchor for a block of alternating G·TA and T·AT triplets. For these studies we prepared fragment TC-$(AT)_n$ and examined its interaction with $TTTCTCT(GT)_5$. This should form a complex with a seven triplet anchor of 5×T·AT and 2×C^+·GC triplets adjacent to a block of 10 triplets alternating between G·TA and T·AT. Because of the need for protonation of the third strand cytosines, these experiments were performed at pH 5.5.

The results of these DNase I footprinting experiments are presented in Figure 7. This experiment was performed in the presence of 10 mM $MgCl_2$, without addition of the triplex-binding ligand, conditions under which all the $T_x(TG)_y$ oligonucleotides failed to produce a DNase I footprint. It can be seen that the oligonucleotide produces a clear footprint, covering the entire target site, which persists to a concentration of 3.0 μM, with some attenuated bands still evident at 0.3 μM. This oligonucleotide is directly comparable with $T_7(GT)_5$ (Fig. 6), for which binding was only detected in the presence of manganese and 10 μM triplex-binding ligand. Both third strands are 17 bases long and differ only in the introduction of two C^+·GC triplets in the stabilizing tail. This footprint is also accompanied by enhanced

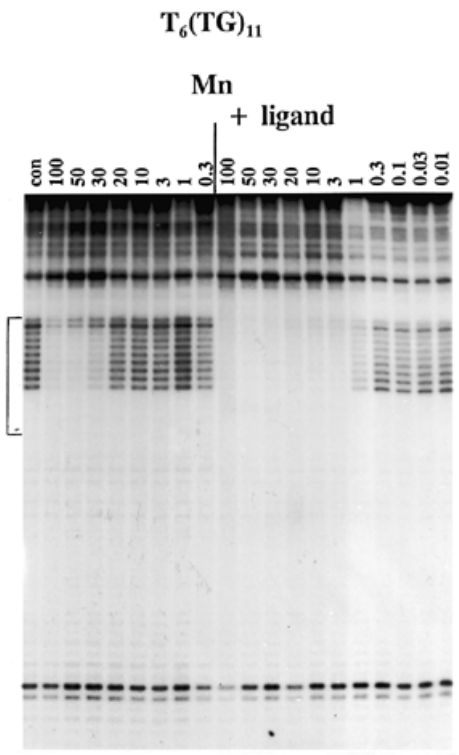

Figure 5. DNase I digestion of fragment k2rev in the presence and absence of various concentrations of $T_6(TG)_{11}$. Reactions were performed in 10 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and 10 mM $MnCl_2$. The right-hand lanes included 10 μM naphthylquinoline triple-binding ligand. The oligonucleotide concentration (μM) is shown at the top of each gel lane. The track labelled GA is a Maxam–Gilbert marker specific for purines; con indicates cleavage of DNA in the absence of added oligonucleotide. The bracket indicates the position of the intended target site.

cleavage of two bands at the 3′ (lower)-end of the target site. The lower of these corresponds to the triplex–duplex junction and is in a similar location to that observed with other triplex footprints. The upper of these two bands is one base within the target site. If these enhancements indicate structural changes at the triplex–duplex junction, this may suggest that a proportion of the triplexes are one base shorter, i.e. the terminal G·TA triplet may be transiently fraying from the target site.

In the presence of 10 μM triplex-binding ligand (not shown) this oligonucleotide produces a similar footprint which persists to a concentration of 1 μM. This poor potentiation may suggest that the ligand does not bind to this block of seven stabilizing triplets, possibly because it is known to prefer T·AT over C^+·GC triplets (33).

DISCUSSION

The results presented in this paper demonstrate that, under certain conditions, it is possible to form specific triplexes at $(AT)_n$ tracts generating blocks of alternating G·TA and T·AT triplets.

Oligonucleotide length

These results indicate that triplex stability increases with the length of both the T·AT anchor and the block of alternating G·TA and T·AT triplets. As a result of poor DNase I cleavage of the A_n-T_n tracts it is not possible to comment directly on binding of the T_n tails of the third strand oligonucleotides. However, the observation that neither $(TG)_6$ nor $(TG)_{11}$ alone form stable triplexes, but generate clear footprints when attached to the T_n tails, provides compelling evidence for successful formation of the block of T·AT triplets as expected. These blocks of alternating G·TA and T·AT triplets can be stabilized by attaching them to a block of 11 or six canonical T·AT triplets. In general, those oligonucleotides which contain long blocks of T·AT or T·AT/G·TA or both form the most stable complexes. For example, the three oligonucleotides of type $T_{11}(TG)_n$ (n = 3, 6 or 11) show that as n increases the structure becomes more stable. If the regions of alternating T·AT and G·TA triplets were not contributing to binding, oligonucleotides with long $(TG)_n$ tails would be expected to form less stable complexes. In contrast, it appears that the region of alternating T·AT and G·TA triplets makes a positive contribution to stability of the complexes. Comparing the third strands $T_{11}(TG)_{11}$ and $T_6(TG)_{11}$, it can be seen that reducing the length of the $(T·AT)_n$ block by five triplets reduces binding affinity by ~10- to 30-fold. This effect is most obvious in the presence of the triplex-binding ligand, consistent with the suggestion that it is preferentially binding to the T·AT region. It should also be noted that increasing the length of the $(TG)_n$ portion increases the size of the DNase I footprint, consistent with the suggestion that the entire triplex is forming properly and that this region is not hanging free in solution.

Divalent metal ion

Each of the complexes described in this paper is more stable with manganese as the divalent cation. Indeed, with the exception of the triplex formed on fragment TC-$(AT)_n$, none of the complexes are stable in the presence of magnesium without the addition of the triplex-binding ligand. This is consistent with previous reports that manganese has a greater stabilizing effect than magnesium (35). Since manganese alone permits binding of $T_{11}(TG)_{11}$ and $T_{11}(TG)_6$ but not $T_{11}(TG)_3$, it is possible that the cation preferentially interacts with the alternating T·AT and G·TA triplets. It has been suggested that manganese acts by polarizing the bases, thereby increasing the strength of Hoogsteen hydrogen bonds (35), similar to the effect seen with duplex DNA (36). Although we have no experimental evidence for the location of this metal ion, one possibility is that it might be bound by guanine N7 and O6, in a similar fashion to that observed for barium in the crystal structure of $(CG)_3$ (37). A further possibility is that the metal ion preferentially binds to the junction between the T·AT and G·TA/T·AT triplets.

Terminal triplet

Comparison of $T_7(TG)_5$ with $T_7(GT)_5$ (Fig. 6) shows that the latter binds tighter than the former, even though the block of T·AT triplets is one shorter. This suggests that the stability of these triplexes is affected by the nature of the 3′-terminal triplet. Placing the weaker G·TA triplet at the end of the structure may result in some fraying at the end of the third strand, as is also suggested by the unusual pattern of enhancements seen in Figure 7.

Triplex-binding ligand

Since the ligand does not induce binding of $(TG)_{11}$ but facilitates interaction with shorter oligonucleotides such as $T_7(TG)_5$, it seems reasonable to suppose that it is preferentially located within

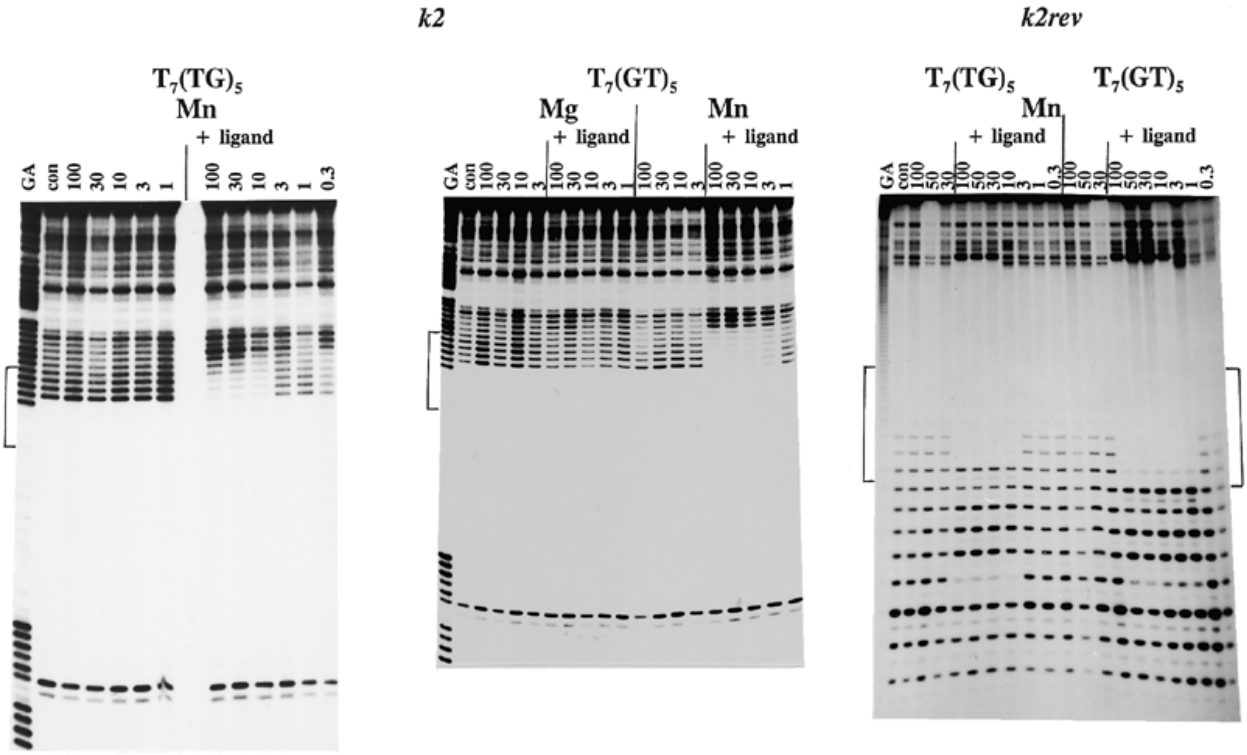

Figure 6. DNase I digestion of fragments k2 and k2rev in the presence and absence of various concentrations of $T_7(TG)_5$ and $T_7(TG)_5$. Reactions were performed in 10 mM Tris–HCl, pH 7.5, containing 50 mM NaCl and 10 mM $MnCl_2$. For each set of conditions the right-hand lanes included 10 μM naphthylquinoline triple-binding ligand. The oligonucleotide concentration (μM) is shown at the top of each gel lane. Tracks labelled GA are Maxam–Gilbert markers specific for purines; con indicates cleavage of DNA in the absence of added oligonucleotide. The bracket indicates the position of the intended target site.

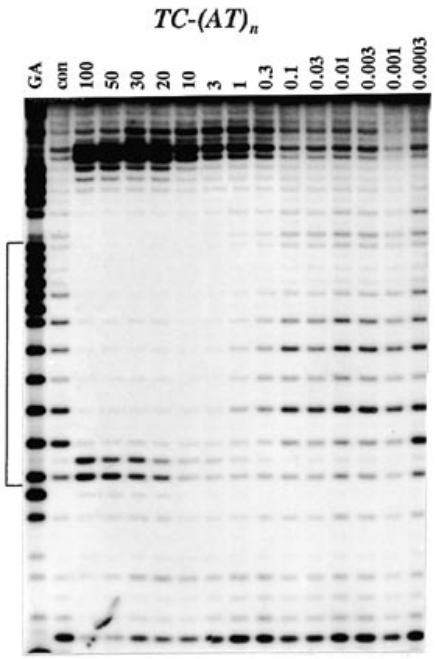

Figure 7. DNase I digestion of fragment TC-$(AT)_n$ in the presence and absence of various concentrations of TTTCTCT$(GT)_5$. Reactions were performed in 50 mM sodium acetate, pH 5.5, containing 10 mM $MgCl_2$. The oligonucleotide concentration (μM) is shown at the top of each gel lane. The track labelled GA is a Maxam–Gilbert marker specific for purines; con indicates cleavage of DNA in the absence of added oligonucleotide. The bracket indicates the position of the intended target site.

the block of T·AT triplexes. We presume that base stacking between the G·TA and T·AT triplets does not generate a favourable intercalation site for the ligand.

Structural effects

There have been several reports of enhanced DNase I cleavage at the triplex–duplex junction. These are usually observed at the 3′-end of the duplex purine strand. Similar enhancements are seen with fragment k2rev for the interaction with $T_{11}(TG)_{11}$ (Fig. 3) and triplex formation with TC-$(AT)_n$. These are each found at the end of the block of alternating G·TA and T·AT triplets and provide yet further evidence for specific binding of these regions. Surprisingly, no such enhancement is seen with $T_{11}(TG)_3$ (Fig. 4), though enhanced cleavage is seen at the 5′-end of the triplex, within the $(AT)_n$ tract in fragment k2.

C^+·GC vs T·AT triplets

The results presented in Figure 7 show that inclusion of a few isolated C^+·GC triplets within the stabilizing tail significantly increases the strength of the interaction, so that the complex is stable in the presence of magnesium alone. Since T·AT and C^+·GC are isomorphous, the increase in binding strength must be attributed to the positive charge on the protonated cytosine. This effect has recently been noted in other studies (27–29). The greater affinity of this complex, compared with those with anchors containing only T·AT triplets, suggests that selective recognition of pyrimidine interruptions may be realistically achieved with natural bases, generating triplets such as G·TA and

T·CG. The stability of complexes containing these triplets may be further increased by designing novel base analogues which increase the strength of the canonical T·AT and C^+·GC triplets.

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