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Triple helix formation at $A_8XA_8 \cdot T_8YT_8$

Simon P. Chandler, Keith R. Fox*

Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton, SO9 3TU, UK

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We have examined the formation of DNA triple helices between the oligonucleotides T_8XT_8 (X = A,C,G,T) and DNA fragments containing the target sequences $A_8XA_8 \cdot T_8YT_8$ (X=T,C,G; Y=A,G,C), by DNase I footprinting. We find that $A_8GA_8 \cdot T_8CT_8$ yields a footprint with T_8CT_8 and shows a weaker interaction with T_{17} and T_8GT_8. $A_8CA_8 \cdot T_8GT_8$ yields a footprint with T_{17}, and shows weaker interaction with T_8CT_8. $A_8TA_8 \cdot T_8AT_8$ yields a footprint with T_8GT_8 and shows weaker interaction with T_{17}. Each of the successful complexes is characterised by enhanced DNase I cleavage at the 3′ end of the purine strand of the target, as well as protection at the 5′ end. We have been unable to form triplexes with third strands of the type A_8XA_8.

Triple helix; Sequence recognition; Stringency; DNase I footprinting

1. INTRODUCTION

One means of achieving long-range DNA sequence recognition is by oligonucleotide-directed triple helix formation [1,2]. To date the formation of these structures has been restricted to homopurine·homopyrimidine sequences. Two major types of intermolecular triplexes have been characterized. In one the third strand contains only pyrimidines (YYR type); AT is recognised by T while GC is recognised by C^+, requiring pH's less than 5.5 [3–5]. The other motif has a purine-containing third strand (RRY type); AT is recognised by A while GC is recognised by G [6,7]. In each case the identical strands run antiparallel to each other [3]. These two motifs cannot be easily combined within a single molecule [8]. In each case recognition is achieved by the formation of specific hydrogen bonds between the third strand bases and major groove substituents on the purines of the target site.

Although triple helices show exquisite sequence specificity we are interested to examine how single base changes, in both the target sequence and the third strand oligonucleotide, affect triplex formation. In this paper we investigate how single base changes are tolerated within triple helices containing T·AT triplets. We have prepared DNA fragments containing the target sites $A_8XA_8 \cdot T_8YT_8$ and examined their interaction with each oligonucleotide of the type T_8NT_8, where N = G,C,T or A, in turn.

*Corresponding author. Fax: (44) (703) 594 319.

2. MATERIALS AND METHODS

2.1. *Chemicals and enzymes*

Deoxyoligonucleotides were synthesised on an Applied Biosystems 380 B DNA synthesizer using standard phosphoramidite chemistry and used without further purification.

DNase I was purchased from Sigma and stored at −20°C at a concentration of 7,200 U/ml. Reverse transcriptase was purchased from Promega; restriction enzymes were purchased from Pharmacia or New England Biolabs. Plasmids (pUC19 and *Sma*I-cut, alkaline phosphatase-treated pUC18) were purchased from Pharmacia.

2.2. *DNA fragments*

The self-complementary oligonucleotides A_8NA_8 and T_8NT_8 (N = any base) were treated with polynucleotide kinase and ATP, annealed and ligated into *Sma*I (CCC/GGG)-cut pUC19 or pUC18. The ligation mixtures were transformed into *E. coli* TG2. White colonies were picked from X-gal, IPTG plates containing 100 μg/ml ampicillin. The sequences of successful clones were determined by dideoxy sequencing using a T7 sequencing kit (Pharmacia). The target sites $A_8GA_8 \cdot T_8CT_8$ and $T_8AT_8 \cdot A_8TA_8$ were obtained in pUC19 while $T_8GT_8 \cdot A_8CA_8$ was cloned into pUC18.

DNA fragments containing these inserts were obtained by cutting with *Hin*dIII, labelling at the 3′ end with α-[^{32}P]dATP using reverse transcriptase and cutting again with *Eco*RI. The radiolabelled fragments were separated from the rest of the plasmid on 8% polyacrylamide gels. In each case the DNA was also labelled at the opposite end by reversing the order of addition of *Hin*dIII and *Eco*RI.

2.3. *DNase I footprinting*

The radiolabelled DNA fragments (3 μl) containing the target sites were mixed with 3 μl oligonucleotide (10 μM and 100 μM), dissolved in 10 mM Tris-HCl containing 5 mM $MgCl_2$, and left to equilibrate at 4°C for 1 h. The samples were then digested by adding 2 μl DNase I (0.01 U/ml dissolved in 1 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl; samples were removed at 1 and 5 min and stopped by adding 3 μl of 80% formamide containing 10 mM EDTA. The products of the reactions were separated on 10% (*Hin*dIII labelled) or 13% (*Eco*RI labelled) polyacrylamide gels, containing 8 M urea, and run at 1,500 V for 2 h. Gels were fixed in 10% acetic acid before drying at 80°C and subjecting to autoradiography at −70°C using an intensifying screen.

Bands were assigned by comparison with Maxam–Gilbert sequencing lanes specific for guanine.

3. RESULTS

3.1. $A_8GA_8 \cdot T_8CT_8$

Fig. 1 presents DNase I digestion of the fragment containing the insert $A_8GA_8 \cdot T_8CT_8$ in the presence of each of the four oligonucleotides. Although DNase I cleavage of the insert is extremely poor in the control lanes, clear changes are evident in the flanking sequences with certain oligonucleotides. Looking first at the DNA labelled at the *Hin*dIII end (the A_8GA_8 strand), bands are missing at the 5′ (upper) end of the insert in the presence of T_8CT_8, but with none of the other oligonucleotides. This effect was still present at concentrations below 10 μM. At the 3′ (lower) end of the insert cleavage of the ApG step at the triplex–duplex boundary is markedly enhanced. This effect can also be seen with T_{17} and, to a lesser extent, with T_8GT_8. This enhancement will be considered further in section 4, but we assume that it indicates some, albeit weaker, interaction with these sequences. When this DNA is labelled at the *Eco*RI end (T_8CT_8 strand) a footprint is evident, at the upper end of the insert, with T_8CT_8. Weaker protection can also be seen with T_{17} and T_8GT_8. No enhancements are evident at the 3′ end.

3.2. $A_8TA_8 \cdot T_8AT_8$

Fig. 2 presents DNase I digestion of a fragment containing the insert $A_8TA_8 \cdot T_8AT_8$ in the presence of each of the four oligonucleotides. Once again cleavage of the insert is poor in the control lanes. It can be seen that T_8GT_8 protects from enzyme cleavage at the upper (5′) end of the insert; no changes are evident with any of the other oligonucleotides. Similar effects were observed at concentrations below 10 μM. In addition T_8GT_8 generates a weak enhancement of the ApG step at the 3′ end of the purine strand (*Eco*RI labelled). A similar weak enhancement is also produced by T_{17}, suggesting that there is some interaction with this oligonucleotide.

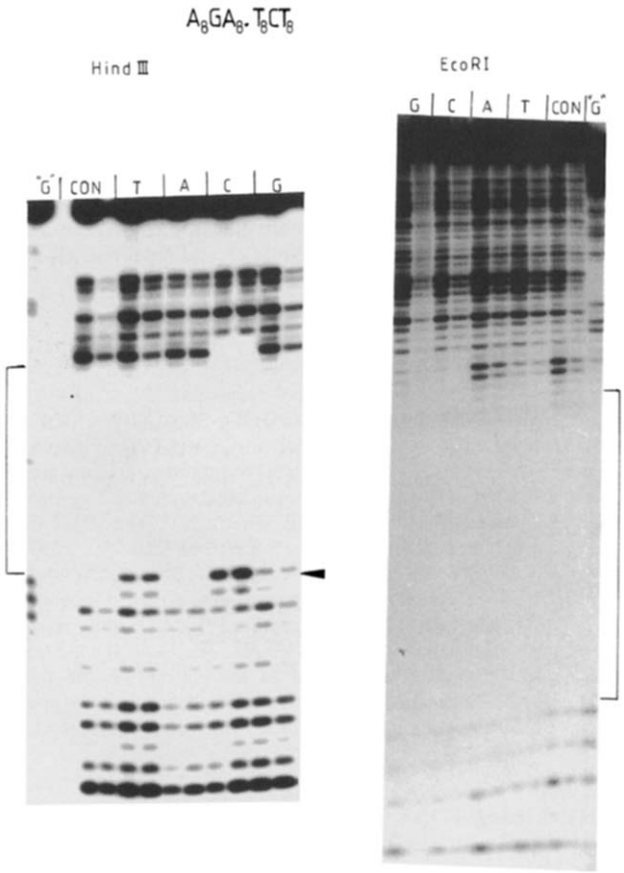

Fig. 1. DNase I digestion of a DNA fragment containing the sequence, A_8GA_8, in the presence and absence of the four oligonucleotides at a concentration of 100 μM. The position and length of the insert is indicated by the square brackets. The DNA was labelled at the 3′ end of the *Hin*dIII or *Eco*RI site as indicated. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. Con, control; T, T_{17}; A, T_8AT_8; C, T_8CT_8; G, T_8GT_8. Tracks labelled 'G' are dimethylsulphate piperidine markers specific for guanine. The enhanced bands are indicated by the arrow.

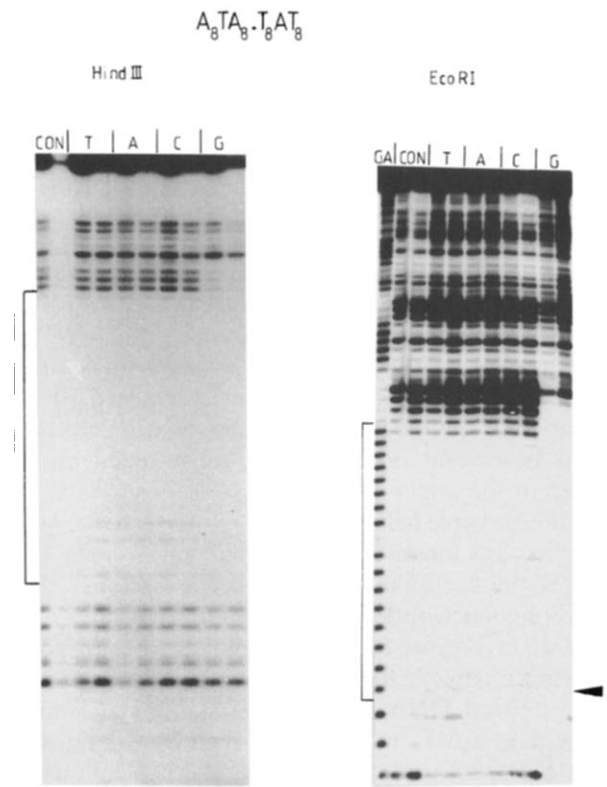

Fig. 2. DNase I digestion of a DNA fragment containing the sequence, A_8TA_8, in the presence and absence of the four oligonucleotides at a concentration of 100 μM. The position and length of the insert is indicated by the square brackets. The DNA was labelled at the 3′ end of the *Hin*dIII or *Eco*RI site as indicated. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. Con, control; T, T_{17}; A, T_8AT_8; C, T_8CT_8; G, T_8GT_8. The enhanced bands are indicated by the arrow. The track labelled GA is a Maxam–Gilbert sequencing lane specific for purines.

3.3. $A_8CA_8 \cdot T_8GT_8$

Fig. 3 presents DNase I digestion of a fragment containing the insert $A_8CA_8 \cdot T_8GT_8$ in the presence of each of the four oligonucleotides. Once again cleavage of the insert is poor in the control lanes. Looking first at the fragment labelled at the *Hin*dIII site (labelled pyrimidine strand), it can be seen that T_{17} protects from DNase I cleavage at the upper (5′) end of the insert; no changes are evident with the other oligonucleotides. When this fragment is labelled at the 3′ end of the *Eco*RI site (labelled purine strand) both T_{17} and T_8CT_8 can be seen to protect from DNase I cleavage. Both these protections are accompanied by enhanced DNase I cleavage of the ApG step at the 3′ end of the triplex–duplex boundary. Similar effects were observed at concentrations below 10 μM.

3.4. A_8NA_8 as the third strand

We have also examined the formation of triple helices containing a purine-rich third strand at each of these target sites. In each case we failed to detect any evidence for triplex formation; there were no footprints or 3′ enhancements. Changing the divalent metal ion from Mg^{2+} to Co^{2+} or Mn^{2+} had no effect. We would have expected A_8GA_8 to form a stable triplex with $A_8GA_8 \cdot T_8CT_8$, employing standard A·AT and G·GC triplets. We presume that this must be a consequence of this particular sequence context. However, in other studies we have shown that the A·AT triplet is much less stable than the G·GC triplet (Washbrook and Fox, unpublished observations).

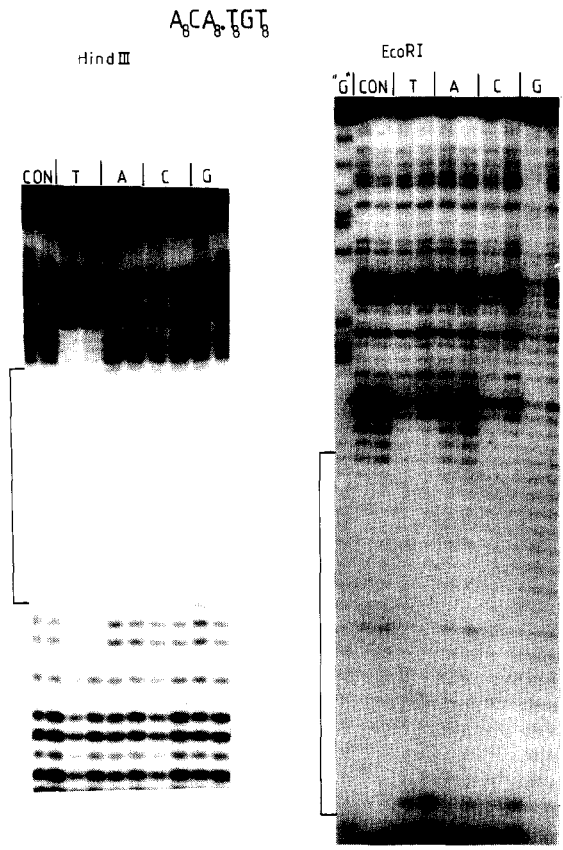

Fig. 3. DNase I digestion of a DNA fragment containing the sequence, A_8CA_8, in the presence and absence of the four oligonucleotides (100 μM for *Hin*dIII label, 10 μM for *Eco*RI label). The position and length of the insert is indicated by the square brackets. The DNA was labelled at the 3′ end of the *Hin*dIII or *Eco*RI site as indicated. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. Con, control; T, T_{17}; A, T_8AT_8; C, T_8CT_8; G, T_8GT_8. The track labelled 'G' is a dimethylsulphate piperidine marker specific for guanine. The enhanced bands are indicated by an arrow. Note that the lanes for T_8GT_8 are underdigested, relative to the other samples, and show no changes in relative band intensity.

4. DISCUSSION

4.1. *Sequence recognition*

The results presented in this paper show that, at pH 7.5 in the context of T·AT triplets, G, C and T in the A_n strand can be recognized by C, T and G, respectively. A weaker interaction, detected by the presence of enhanced DNase I cleavage, is found for certain other third strand bases. In each case an adenine in the third strand (i.e. T_8AT_8) abolishes triple helix formation. These results are summarised in Table I.

Recognition of G by C is similar to the well-characterised $C^+ \cdot GC$ triplet, except that our results were obtained at pH 7.5, at which we would not expect the cytosine to be protonated. The G·TA triplet has been previously described in different sequence contexts by several groups [9,10], while there is one other report of T·CG triplet [11]. Each of these base substitutions need not necessarily represent the formation of novel base triplets, but may instead be 'null-bases' which, although not actively contributing to triplex stability, do not abolish its formation. In this regard it is interesting to note that a third strand T can be tolerated opposite any base. The presence of a third strand A abolishes triplex formation, whereas G and C are forbidden opposite C and T, respectively, under these conditions.

These rules may aid the design of oligonucleotides targeted against sequences which are not strict runs of purines. In addition this study indicates secondary binding sites that may be occupied by triplex-forming oli-

Table I

Stability of triple helices formed by T_8YT_8 at $A_8XA_8 \cdot T_8XT_8$ target sites

Target (A_n strand)	Third strand			
	A	G	C	T
C	X	X	W	S
G	X	W	S	W
T	X	S	X	W

S indicates strong binding, yielding a DNase I footprint; W indicates a weaker interaction characterised by 3′ enhancement but no footprint; X indicates no detectable interaction.

gonucleotides. For example T_{17} can bind tightly to A_{17} and A_8CA_8, and shows a weaker but significant interaction with A_8GA_8 and A_8TA_8.

4.2. *DNase I enhancements*

Each successful DNase I footprint is accompanied by enhanced DNase I cleavage at the 3′ end of the purine strand, at the triplex–duplex boundary. This enhancement is also observed with several other oligonucleotides which fail to produce footprints, and has been taken as indicative of a weaker interaction. We presume that these enhancements represent a DNA structural change caused by the binding of the oligonucleotide. Indeed, since DNase I and the third strand bind in different DNA grooves, the footprints themselves can not arise from steric occlusion, but must result from changes in DNA structure and/or rigidity. A previous study has also shown enhanced sensitivity to diethylpyrocarbonate modification at a triplex–duplex boundary [12]. These enhancements are evident in some conditions in which footprints are not produced. This may be because they represent an increase in cleavage against a very low background in the control and so may be evident when only a small proportion of the target sites are occupied. In contrast, the formation of a DNase I footprint requires that a large proportion of the sites are occupied to significantly reduce the band intensity.

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