Nucleosome core particles inhibit DNA triple helix formation

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We have used DNase I footprinting to examine the formation of DNA triple helices at target sites on DNA fragments that have been reconstituted with nucleosome core particles. We show that a 12 bp homopurine target site, located 45 bp from the end of the 160 bp *tyr*T(46A) fragment, cannot be targeted with either parallel (CT-containing) or antiparallel (GT-containing) triplex-forming oligonucleotides when reconstituted on to nucleosome core particles. Binding is not facilitated by the presence of a triplex-binding ligand. However, both parallel and antiparallel triplexes could be formed on a truncated DNA fragment in which the target site was located closer to the end of the DNA fragment. We suggest that intermolecular DNA triplexes can only be formed on those DNA regions that are less tightly associated with the protein core.

INTRODUCTION

Triple helix formation was first observed over 30 years ago [1,2], but its potential as a means of designing agents with considerable sequence recognition properties, with the ability artificially to control gene expression, was not realized until 1987 [3,4]. Two triple helix motifs have been characterized, depending on the orientation of the third strand, which can be positioned either parallel or antiparallel to the purine strand of the duplex. Parallel triplex formation is achieved using a pyrimidine-rich oligonucleotide and is characterized by the formation of T·AT and C^+·GC triplets [4–6]. Alternatively, purine-rich oligonucleotides bind antiparallel to the purine strand of the duplex, generating G·GC, A·AT and T·AT triplets [7–9]. Since the third strand usually binds by forming hydrogen bonds to purine residues in the duplex, the formation of triplex DNA is generally limited to homopurine·homopyrimidine sequences.

There have been numerous studies on triple helix formation to DNA fragments *in vitro*. However, cellular DNA is packed into higher-order structures, which may alter the local DNA conformation and/or mask potential binding sites. The basic unit of chromatin structure is the nucleosome, which consists of approx. 146 bp of DNA wound 1.8 times around the histone, forming a left-handed toroidal superhelix [10,11]. The protein core is an octamer containing two copies each of histones H2A, H2B, H3 and H4. Although nucleosomes are associated with many different DNA sequences, there is considerable evidence that they adopt well defined positions on DNA sequences both *in vivo* and *in vitro* [12–17]. There has been considerable progress in our understanding of DNA rotational positioning, which depends on DNA anisotropic bendability [18]. Since the double helix must bend as it wraps around the protein, sequences that facilitate bending have been implicated in directing nucleosome formation. In general, GC-rich regions are positioned with their wider-than-average minor grooves facing away from the protein core, while the narrow minor grooves of AT sequences face towards the protein [13,14]. Certain repetitive sequences, as well as double-stranded RNA, will not wrap around nucleosomes [19]. Within each DNA fragment it is not possible to satisfy all the local preferences; sequences at the centre of the nucleosome have a greater effect on rotational positioning than those towards the ends [20]. The helical repeat of DNA also varies along the nucleosome from about 10.0 bp per turn at the ends to 10.7 bp at the dyad, compared with a value of 10.5 bp for DNA free in solution [17]. It appears that the nucleosome cores are not only involved in DNA packaging, but also may play an important role in regulating the transcriptional activity of a gene [21,22].

The interaction of DNA with nucleosome core particles may present a problem for forming triplexes *in vivo*, since the third strand must wrap around the DNA duplex which is already wrapped around the protein core. If the third strand spans more than 10 bp it will have to thread between the protein surface to access the major groove. In addition, although little is known about the precise structure of triple-helical DNA, which may adopt an *A*-like [23] or *B*-like [24] configuration, the increase in DNA rigidity on triplex formation would suggest that there must be some distortion of the nucleosome structure. To date there has only been one report examining the formation of parallel triple helices on a target site on nucleosome-bound DNA [25]. This showed that triplex formation altered the histone–DNA contacts during nucleosome reconstitution, and functioned as a nucleosome barrier [25]. In this paper we examine the formation of both parallel and antiparallel triplexes at target sites within two fragments derived from *tyr*T DNA, when reconstituted with nucleosome core particles. Previous studies have demonstrated the formation of parallel triplexes at this target site [26], while the rotational position of the native fragment has been well documented [13].

MATERIALS AND METHODS

Chemicals and enzymes

Oligonucleotides used for triplex formation, PCR and site-directed mutagenesis were purchased from Genosys or Oswel. These were dissolved in ultra-pure water from a Millipore Milli-Q Plus system at a stock concentration of 1 mM and stored at −20 °C. Plasmid pUC18 was purchased from Promega. DNase I was purchased from Sigma and stored at −20 °C at a concentration of 7200 units/ml. Restriction enzymes and reverse transcriptase were purchased from Promega. The triplex-binding

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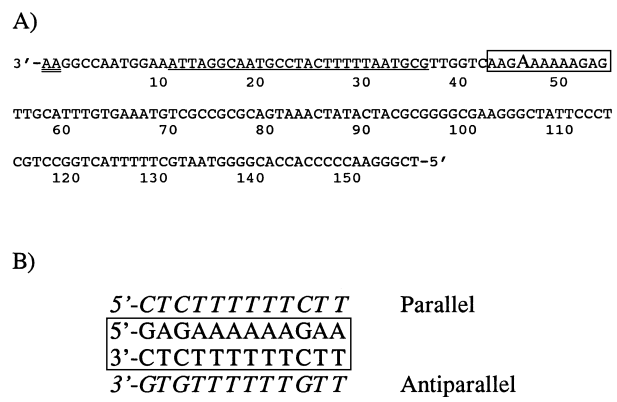

Figure 1 (A) Sequence of the *tyr*T(46A) DNA fragment, and (B) target site present in *tyr*T(46A) and *tyr*T(46A)(Δ11–36)

(**A**) Bases in the sequences are numbered as in previous studies [13,30–33]. The underlined region, between positions 11 and 37, has been deleted in *tyr*T(46A)(Δ11–36). The triplex target site is boxed; the nucleotide at position 46, which has been changed to adenine, is indicated in larger type. Note that the sequence is written from 3′ to 5′. The adenines at the 3′-end bearing the ^{32}P are double-underlined. Previous studies [13] have shown that bases 0–145 interact with the nucleosome core particle. (**B**) The target site present in *tyr*T(46A) and *tyr*T(46A)(Δ11–36) is boxed, showing the interaction with the oligonucleotides forming parallel and antiparallel triplexes. The third-strand oligonucleotides are shown in italics.

naphthoquinoline derivative [27–29] was a gift from Dr. L. Strekowski, Department of Chemistry, Georgia State University, Atlanta, GA, U.S.A. This was stored as a 20 mM stock solution in DMSO at −20 °C, and diluted to working concentrations immediately before use.

DNA fragments

We used the *tyr*T DNA fragment for these studies on triplex formation on DNA fragments complexed with nucleosome core particles, since this fragment has been extensively studied [13,30–33]. Although this DNA fragment is prokaryotic in origin, it forms stable complexes with nucleosome core particles, and its position on the nucleosome core has been fully characterized [13,30,31], revealing that the first 145 bp is closely associated with the nucleosome core particle [13]. The native *tyr*T DNA fragment contains a run of purines between positions 43 and 54, interrupted by a single thymine at position 46. We changed this residue to adenine by site-directed mutagenesis using the PCR [26], generating *tyr*T(46A), whose sequence is shown in Figure 1(A). This produces a fragment containing 12 consecutive purine residues, which can be used as a target site for triplex formation using the oligonucleotides shown in Figure 1(B). During the preparation of this mutated fragment we isolated a clone missing bases 11–36 of the original sequence, but containing the mutated adenine at position 46. The sequence of this truncated version, designated *tyr*T(46A)(Δ11–36), is shown in Figure 1(A).

Radiolabelled fragments containing the cloned triplex sites were obtained by cleaving the plasmids with *Eco*RI, labelling at the 3′-end using reverse transcriptase and [α-^{32}P]dATP and then cutting again with *Ava*I. The radiolabelled fragments were separated from the rest of the plasmid on 6% (w/v) non-denaturing polyacrylamide gels.

DNA reconstitution on to nucleosome core particles

Nucleosome core particles derived from chicken erythrocytes were prepared as previously described [13,14] and stored at −20 °C in 50% glycerol. The DNA fragments of interest were reconstituted on to the nucleosomes at pH 7.5 by a salt-exchange method as previously described [12–14], by incubating the labelled DNA (approx. 1 nmol of base pairs) with 50 μg of nucleosome core particles in a high-salt buffer. The salt concentration was slowly decreased to 100 mM by stepwise additions of 5 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 0.1% (v/v) Nonidet P40. Incorporation of the DNA into nucleosomes was checked by retardation on agarose gels.

DNase I footprinting

Reconstituted core particles

Nucleosome core particles were digested with DNase I using a method adapted from that of Drew and Travers [13] to allow conditions for triplex formation. A sample of 10 μl of the reconstituted nucleosomes was mixed with 20 μl of various concentrations of oligonucleotide dissolved in 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl and 10 mM $MnCl_2$ for antiparallel triplex formation, or 50 mM sodium acetate, pH 5.5, containing 100 mM NaCl and 10 mM $MgCl_2$ for parallel triplexes. This mixture was allowed to equilibrate for at least 1 h. Higher concentrations of DNase I are required for digesting the nucleosome-bound DNA fragments than for the free DNA due to the presence of the large amount of unlabelled DNA from the chicken nucleosomes; the enzyme concentration was chosen so that about 60–70% of the DNA remained uncut. The digestion was stopped after 1 min by the addition of 100 μl of phenol. The reaction volume was increased to 100 μl with the appropriate buffer and extracted twice with phenol, followed by two extractions with ether. The DNA was precipitated with ethanol, washed twice with 70% ethanol and dried under vacuum.

Free DNA

The free DNA was taken through the same stages as the reconstituted DNA, but the nucleosome cores were replaced with 10 mM Tris/HCl, pH 7.5, containing 10 mM NaCl. A 2.5 μl sample of this stock was mixed with 5 μl of buffer containing various concentrations of oligonucleotide, left to equilibrate at room temperature and then digested for 1 min with DNase I. The reaction was stopped by ethanol precipitation. The pellet was washed twice in 70% ethanol and dried.

Gel electrophoresis

The pellets produced were dissolved in 80% formamide containing 10 mM EDTA, 10 mM NaOH and 0.1% Bromophenol Blue. These were boiled for 3 min before loading on to 8% polyacrylamide gels containing 8 M urea. These were then run at 1500 V for 2 h. Gels were 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 the DNase I digests were assigned by comparison with Maxam–Gilbert markers specific for guanine and adenine.

RESULTS

Triplex formation with *tyr*T(46A)

Figure 2 shows the DNase I digestion of *tyr*T(46A) in the presence of various concentrations of 5′-CTCTTTTTTCTT, an oligonucleotide designed to form a parallel triple helix containing $9 \times T \cdot AT$ and $3 \times C^+ \cdot GC$ triplets, as shown in Figure 1(B). The experiment was performed at pH 5.5 so as to stabilize the $C^+ \cdot GC$ triplets. Figure 2(a) shows the interaction with free DNA, in

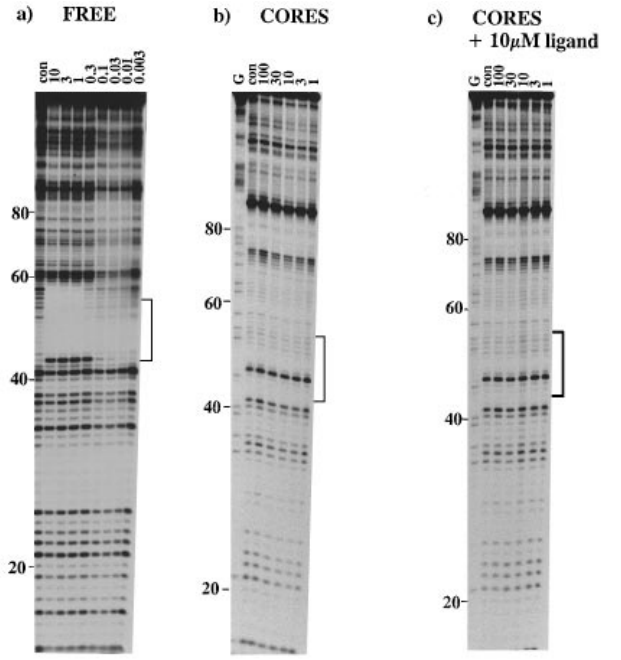

Figure 2 DNase I footprints showing parallel triplex formation on *tyr*T(46A)

(**a**) Free DNA; (**b**) and (**c**) DNA that has been reconstituted with nucleosome core particles. The reactions were performed in 50 mM sodium acetate containing 100 mM NaCl and 10 mM $MgCl_2$. The oligonucleotide concentration (μM) is shown at the top of each lane. Tracks labelled 'con' show digestion in the absence of added oligonucleotide. Complexes in (**c**) contained 10 μM of the naphthoquinoline triplex-binding ligand. Tracks labelled 'G' correspond to Maxam–Gilbert sequencing lanes specific for guanines. The brackets indicate the position of the target site. Numbers correspond to the sequence shown in Figure 1(A).

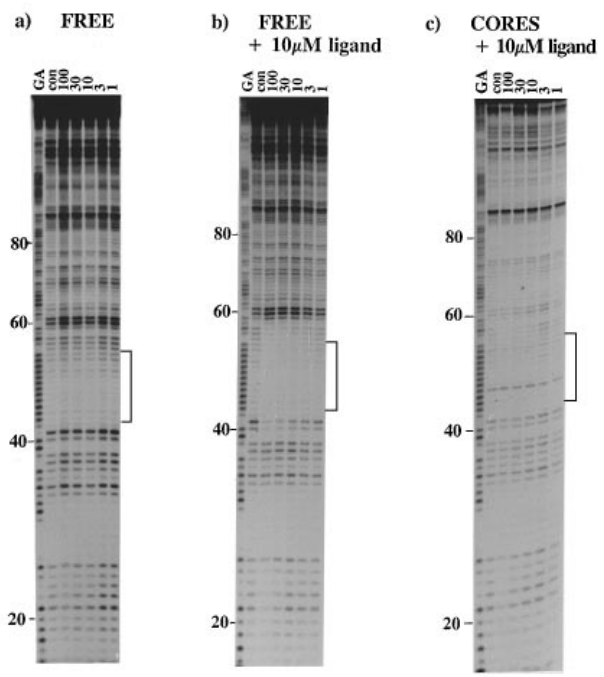

Figure 3 DNase I footprints showing antiparallel triplex formation on *tyr*T(46A)

Panels (**a**) and (**b**) correspond to free DNA in the absence (**a**) and presence (**b**) of 10 μM triplex-binding ligand. Panel (**c**) corresponds to DNA that has been reconstituted with nucleosome core particles and also contains 10 μM triplex-binding ligand. The reactions were performed in 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl and 10 mM $MnCl_2$. The oligonucleotide concentration (μM) is shown at the top of each lane. Tracks labelled 'con' show digestion in the absence of added oligonucleotide. Tracks labelled 'GA' correspond to Maxam–Gilbert sequencing lanes specific for purines. The brackets indicate the position of the triplex target site. Numbers correspond to the sequence shown in Figure 1(A).

which a clear footprint can be seen covering the entire target site. This footprint persisted to oligonucleotide concentrations as low as 0.3 μM and was accompanied by enhanced cleavage at the 3′-(lower) side of the site, at the triplex–duplex junction. Figure 2(b) shows the results obtained when the DNA was reconstituted with nucleosome core particles. It can be seen that cleavage in the control was modified by interaction with the nucleosomes, and was not the same as for free DNA. Several bands were present in the free DNA but absent from the core DNA (see, for example, positions 37/38), while others can be seen in core but not free DNA (positions 46 and 89). These changes are consistent with previous studies on the wrapping of the *tyr*T fragment around nucleosome core particles [13,30,31], although the exact pattern is modified as a result of working at the lower pH. In contrast to the results with free DNA, the oligonucleotide did not affect the DNase I digestion pattern. Bands were still clearly evident within the target site, most notably the nucleosome-specific band at position 46, and no enhancement was found at the 3′-end. Figure 2(c) shows that the naphthoquinoline triplex-binding ligand had no effect on the interaction. This ligand has been shown to stabilize weak complexes with free DNA by as much as 100-fold [28,29]. The lack of interaction, even in the presence of the ligand, emphasizes that the nucleosome has severely diminished the ability of the third strand to interact with its target site. Interestingly, the ligand had no effect on the DNase I cleavage pattern at a concentration of 10 μM, confirming that it has little interaction with duplex DNA.

Figure 3 shows the interaction with 5′-TTGTTTTTTGTG, an oligonucleotide designed to form an antiparallel triple helix containing $9 \times T \cdot AT$ and $3 \times G \cdot GC$ triplets, as shown in Figure 1(B). Since these triplets are stable at physiological pH values, this reaction was performed at pH 7.5; however, we changed the bivalent metal ion to manganese, since this has been shown to stabilize antiparallel triplets [34,35]. Figure 3(a) shows the interaction with free DNA, in which we were unable to detect triplex formation at the target site. We presume that this lack of interaction is due to the high proportion of antiparallel $T \cdot AT$ triplets, which are much less stable than $G \cdot GC$ triplets. However, on adding the triplex-binding ligand (Figure 3b) a footprint was evident at the target site, and is most clearly seen by the reduced intensity of the bands at positions 40/41 and 55–57. When these experiments were repeated with DNA that had been reconstituted on to nucleosome core particles, we observed no change in the DNase I pattern in both the presence (Figure 3c) and the absence of the ligands. Although cleavage in this region is weak in the drug-free control, it can be seen that the nucleosome-specific band at position 46 is unaffected by the oligonucleotide. Once again, cleavage of core-bound DNA is different from that of free DNA, consistent with previous digestion studies, but this pattern is not affected by the presence of the triplex-forming oligonucleotide.

Triplex formation with *tyr*T(46A)(Δ11–36)

The DNA sequence *tyr*T(46A)(Δ11–36) was cloned by accident and is an unusual product from the PCR. It contains the correct

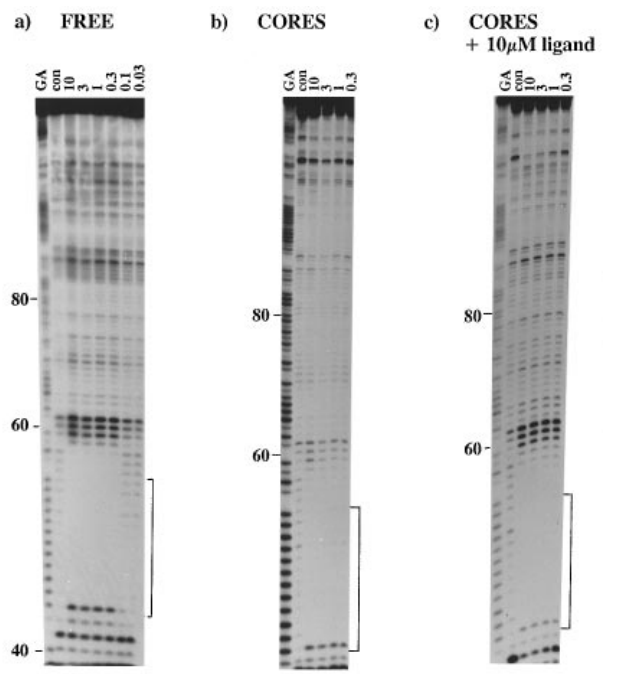

Figure 4 DNase I footprints showing parallel triplex formation on *tyr*T(46A)(Δ11–36)

(**a**) Free DNA; (**b**) and (**c**) DNA that has been reconstituted with nucleosome core particles. The reactions were performed in 50 mM sodium acetate, pH 5.5, containing 100 mM NaCl and 10 mM $MgCl_2$. The oligonucleotide concentration (μM) is shown at the top of each lane. Tracks labelled 'con' show digestion in the absence of added oligonucleotide. Complexes in (**c**) contained 10 μM of the naphthoquinoline triplex-binding ligand. Tracks labelled 'GA' correspond to Maxam–Gilbert sequencing lanes specific for guanine and adenine. The brackets indicate the position of the target site. Numbers correspond to the sequence shown in Figure 1(A).

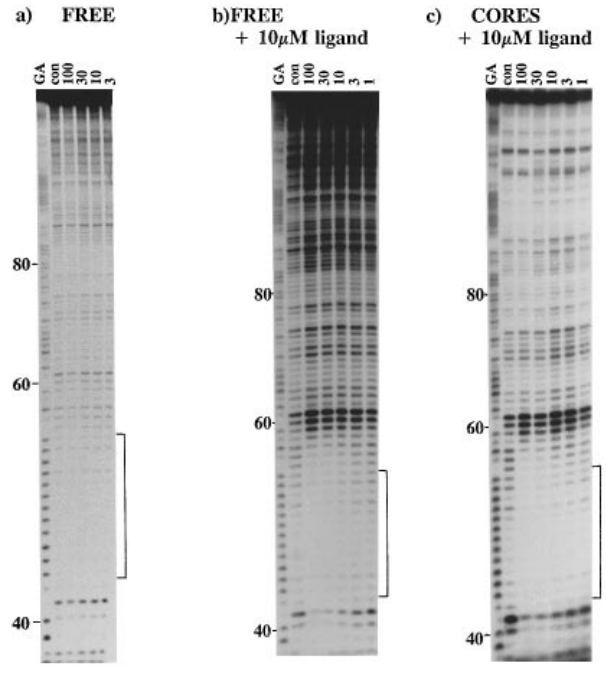

Figure 5 DNase I footprints showing antiparallel triplex formation on *tyr*T(46A)(Δ11–36)

Panels (**a**) and (**b**) correspond to free DNA in the absence (**a**) and presence (**b**) of 10 μM triplex-binding ligand. Panel (**c**) corresponds to DNA that has been reconstituted with nucleosome core particles and also contains 10 μM triplex-binding ligand. The reactions were performed in 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl and 10 mM $MnCl_2$. The oligonucleotide concentration (μM) is shown at the top of each lane. Tracks labelled 'con' show digestion in the absence of added oligonucleotide. Tracks labelled 'GA' correspond to Maxam–Gilbert sequencing lanes specific for guanine and adenine. The brackets indicate the position of the triplex target site. Numbers correspond to the sequence shown in Figure 1(A).

altered target site, with an adenine at position 46, but is missing all the bases between positions 11 and 36. We have examined triplex formation on this truncated sequence, since the target site lies closer to the end of the fragment than in *tyr*T(46A). The results are shown in Figures 4 and 5 for the formation of parallel and antiparallel triplexes respectively. Figure 4(a) shows a clear footprint at the target site in free DNA (see especially the reduced cleavage at positions 55–57), which persisted down to a concentration of 0.3 μM. This was again accompanied by enhanced cleavage at the 3′-(lower) end of the target, at the triplex–duplex junction (position 43). Note that the site is lower in the gel than in Figures 2 and 3, since the target site is located closer to the 3′-end of the strand. Figures 4(b) and 4(c) examine triplex formation on this fragment after reconstituting on to nucleosome core particles in the absence and presence respectively of the triplex-binding ligand. In contrast to *tyr*T(46A), a clear footprint can be seen, which was still evident at 0.3 μM ligand. The enhanced cleavage at the 3′-end of the target site is also evident. The integrity of the reconstituted nucleosomes complexed with the oligonucleotide was checked by agarose gel electrophoresis, which showed that the oligonucleotide had not displaced the DNA from the protein core. In addition, hydroxyl radical digestion of nucleosome core particles reconstituted with this fragment (results not shown) generated a phased cleavage pattern, revealing that the triplex target site adopted the same rotational setting as determined for *tyr*T DNA. It appears that the presence of nucleosome core particles has not prevented triple helix formation on this truncated DNA fragment, in contrast to the results with the full-length sequence. This difference will be considered further in the Discussion section, but probably arises because the target site is situated closer to the end of the DNA fragment, where it may be less tightly bound to the nucleosome core.

Figure 5 shows the results of similar experiments investigating the formation of antiparallel triplexes on nucleosome cores reconstituted with this truncated fragment. Once again no footprint was evident with the free DNA (Figure 5a). Addition of the triplex-binding ligand promoted triplex formation, producing a footprint which extends over the entire target site (Figure 5b) and can be clearly seen from the reduced cleavage at positions 40/41 and 55–57. Repeating this experiment with DNA complexed to the nucleosome core particles produced the DNase I digestion patterns shown in Figure 5(c). A clear footprint is evident at the target site (see the reduced cleavage at positions 40/41 and 55–57), which shows a similar concentration-dependence to that with the free DNA.

DISCUSSION

The results presented here show that it is not possible to form either parallel or antiparallel triple helices at a 12-base target site in *tyr*T(46A) when this fragment is complexed with nucleosome core particles. However, triplex formation occurs on nucleosomes reconstituted with a truncated fragment [*tyr*T(46A)(Δ11–36)] containing the same site in an identical sequence context, but

located closer to the end of the fragment. The only factor which might explain the different behaviour of these two fragments is the location of the triplex target sites within the two fragments. The position and orientation of *tyr*T DNA has been well documented [13,30,31] and shows clear differences between the nucleosome-bound and free DNA, from which we can be sure that the region of interest of *tyr*T(46A) is wrapped around the protein [13]. Hydroxyl radical digestion of the truncated sequence suggests that the target site adopts the same rotational positioning on the nucleosome core particles, although we cannot be sure of its translational setting. The similarity in rotational positioning for the full-length and truncated fragments is to be expected, since sequences at the centre of the core particles, which are the same in both fragments, exert the greatest influence on nucleosome positioning [20]. However, deleting 26 bp from the *Eco*RI end of the sequence will bring the triplex target site closer to the end of the fragment. It is clear that this change has altered the way in which the target site interacts with the protein core and permits the formation of a stable triplex. It is worth noting that agarose gel electrophoresis confirmed the integrity of the nucleosome cores under all the conditions examined, confirming that the oligonucleotide had not affected the stability of the reconstituted core particles.

These results suggest that triplex formation cannot be targeted to DNA that is tightly associated with nucleosome cores, but reveal that regions closer to the edge of the nucleosome, which may be less tightly bound, can form intermolecular triplexes. It is possible that this arises from end-effects causing the fraying or breathing of the duplex ends. Further experiments with related full-length fragments are required to characterize fully the effect of target site position and sequence composition on the ability to form stable triplexes on nucleosome core particles. At first sight this inability to target nucleosomal DNA by intermolecular triple helix formation appears to present a limitation to the usefulness of oligonucleotides in controlling gene expression. However, the sequences that are most tightly associated with histone proteins in the most highly condensed chromatin are likely to be those that are not being actively transcribed. Sequences that are being actively transcribed must have a looser association with the protein and should, therefore, be transiently exposed for targeting by triple helix formation. Experiments examining the effect of transcription on triple helix formation are currently in progress. It therefore appears that oligonucleotides will be especially useful for targeting genes that are being transcribed, exactly as required for an efficient anti-gene agent.

An additional problem *in vivo* is that the linker DNA between the nucleosomes is associated with histone H1 (H5). This is thought to seal the DNA–protein complex. This protein is not present in our preparations, and it is not clear how it will affect triplex formation. However, this may not present a problem *in vivo*, as it has been shown that histone H1 is not present on actively transcribing DNA [21,22].

The inability to form stable triplexes at this target site using GT-containing oligonucleotides which were designed to bind in an antiparallel configuration, except in the presence of a triplex-binding ligand, was surprising, but is probably because of the short length of the target and because of the large number of the less stable T·AT triplets. However, the formation of longer triplexes may be even more difficult, as the third strand has to wrap around the nucleosome-bound DNA. In addition, target sequences containing a greater proportion of GC base pairs are less suitable for recognition by parallel (CT-containing) oligonucleotides as a result of the requirement for cytosine protonation.

In summary, the results presented in this paper indicate that DNA that is tightly bound on nucleosome core particles cannot be targeted with triplex therapy, but suggests that regions towards the edges of the nucleosome core particles can form successful intermolecular triplexes.

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