Triple helix DNA alters nucleosomal histone–DNA interactions and acts as a nucleosome barrier

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ABSTRACT

Oligonucleotides which form triple helical complexes on double-stranded DNA have been previously reported to selectively inhibit transcription both *in vitro* and *in vivo* by physically blocking RNA polymerase or transcription factor access to the DNA template. Here we show that a 16mer oligonucleotide, which forms triple helix DNA by binding to a 16 bp homopurine segment, alters the formation of histone–DNA contacts during *in vitro* nucleosome reconstitution. This effect was DNA sequence-specific and required the oligonucleotide to be present during *in vitro* nucleosome reconstitution. Binding of the triple helix oligonucleotide on a 199 bp mouse mammary tumour virus promoter DNA fragment with a centrally located triplex DNA resulted in interruption of histone–DNA contacts flanking the triplex DNA segment. When nucleosome reconstitution is carried out on a longer, 279 bp DNA fragment with an asymmetrically located triplex site, nucleosome formation occurred at the border of the triple helical DNA. In this case the triplex DNA functioned as a nucleosome barrier. We conclude that triplex DNA cannot be accommodated within a nucleosome context and thus may be used to site-specifically manipulate nucleosome organization.

INTRODUCTION

Nucleosomes are compact protein–DNA complexes consisting of 144–146 bp of DNA forming 1.8 superhelical turns around a histone protein core complex (1,2). A growing body of evidence has shown that nucleosomes are important for many aspects of transcriptional gene regulation (for reviews see 3,4). An example of this is seen in the *Drosophila heat shock protein 26* promoter, where a positioned nucleosome flanked by nucleosome-free DNA is utilized to structurally preset this promoter for instant activation (5). Positioned nucleosomes may also serve to structure an active tissue-specific enhancer, such as the serum albumin enhancer in liver (6), and/or to bring different transcription factors into close proximity and thereby stimulate transcription, as is observed in the vitellogenin promoter (7). Furthermore,

nucleosomes have been shown to repress basal transcription in several inducible genes (8–10). This might occur by a histone octamer-mediated occlusion of certain regulatory DNA elements, such that recognition by their cognate transactive factors or basal transcription factors are prevented (11–13). Studies of the *PHO5* promoter in *Saccharomyces cerevisiae* have demonstrated the finely tuned balance between two pairs of positioned nucleosomes and an inducible transactive factor, Pho4. Here an active Pho4 protein is necessary for nucleosome disruption to occur, which in turn is a prerequisite for transcriptional activation of the *PHO5* promoter (14).

In the mouse mammary tumour virus (MMTV) long terminal repeat there is an array of six sequence-specifically positioned nucleosomes, one of which organizes four glucocorticoid receptor binding DNA motifs (15). This nucleosome is rapidly disrupted or reorganized upon glucocorticoid hormone induction (15–17). This event is accompanied by binding of nuclear factor 1 (NF-1) to its target sequence located at the 3'-side of the glucocorticoid-disrupted nucleosome. It implies that the histone octamer prevents binding of NF-1 in the uninduced state (11,18,19). The repressive role of histones on the MMTV promoter was also demonstrated in competition experiments in *Xenopus* oocytes *in vivo* (10). Here the co-injection of competing DNA, which titrates the histone protein pool in the oocytes and inhibits nucleosome formation on injected MMTV DNA, resulted in high constitutive MMTV transcription.

Taken together, there are many examples of positioned nucleosomes occurring at defined positions within regulatory DNA segments in the living cell (20). It would be of great interest to be able to site-specifically disrupt or rearrange an individual nucleosome(s) and thus address their functional significance. With this in mind, we have tested if oligonucleotide-directed triple helical DNA can be accommodated within a nucleosome. One problem is that the stability of a triple helix induced by an oligonucleotide made of natural nucleotides is dependent on slightly acidic pH or on the presence of a very GC-rich homopurine segment (21). However, recent progress in the design of synthetic oligonucleotide derivatives which are able to form triple helix DNA under physiological conditions (22) have improved the prospects of this strategy.

In this work we investigate the effects of triple helical DNA on the structure of *in vitro* reconstituted nucleosomes. Our results

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Figure 1. Detailed map of experimental probes (MTVth-58 and MTVth+19) and sequence of the triple helix oligonucleotide (see Materials and Methods for details). Probe MTVwt-58 is identical to MTVth-58 except for the absence of the three point mutations, necessary for triple helix oligonucleotide binding, shown here. Vector indicates the 58 bp of DNA originating from the pGEM-1™ *PvuII-SmaI* DNA segment (23). MMTV LTR indicates DNA originating from the mouse mammary tumour virus long terminal repeat promoter DNA (25). The numbers below the probe and mutation figures indicate the position of the nucleotide relative to the transcription initiation start site of the MMTV LTR promoter. The letters above the mutation sequence indicate the base that the wild-type sequence was mutated to.

demonstrate that a 16mer oligonucleotide-directed triple helix is excluded from the nucleosome when the oligonucleotide is present during nucleosome reconstitution. Conversely, a preformed nucleosome is able to prevent formation of oligonucleotide-directed triple helix DNA. Furthermore, we demonstrate that oligonucleotide-directed triple helix DNA may be utilized to direct a nucleosome to a defined translational position, since it behaves as a nucleosome barrier. We propose that triple helix-inducing oligonucleotides might be useful to site-specifically disrupt and/or reposition nucleosome(s) *in vivo*.

MATERIALS AND METHODS

Plasmids and oligonucleotide

The plasmid pMTVwt, which contains the -198 to -58 DNA segment of the MMTV promoter, has been described previously (23). It is referred to here as pMTVwt-58. Plasmid pMTVth-58 (th indicating triple helix) was constructed by selectively mutating positions -152, -157 and -159 in pMTVwt-58 to the bases shown in Figure 1 using a double-stranded oligonucleotide-directed mutagenesis strategy (Transformer Mutagenesis™; Clontech). Plasmid pMTVth+19 was created by exchanging the *SstI* fragment of pMTVth-58, which contains the three mutated bases (Fig. 1), with the same *SstI* fragment of pMTVwt-198/+19. pMTVwt-198/+19, which has been described previously (24), is a plasmid harbouring the MTV -198 to +19 DNA segment, with the exception of segment -57 to -52, where a hybrid *BamHI/BglII* site was introduced, along with a *BamHI* site placed at the +23 position (25).

The plasmids were first cut with either *BamHI* or *PvuII* and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim). The DNA was 5'-end-labelled as previously described (23) before cleavage with the second enzyme. The fragments were separated on 1.0% low melting point agarose gels (Seaplaque™; FMC BioProducts) and visualized by ethidium bromide staining fluorescence. The agarose was digested with Gelase™ (Epicentre Technologies) and the fragment purified by water-saturated isobutanol extraction and ethanol precipitation.

The triple helix-forming oligonucleotide (Fig. 1) was synthesised by Gilead Sciences (Foster City, CA) using the H-phosphonate method (26) and purified by denaturing acrylamide gel electrophoresis followed by Sephadex G-25 filtration. *N*⁶-Methyl-8-oxo-2'-deoxyadenosine was prepared as previously described (22).

Reconstitution of nucleosomes

Nucleosomes were reconstituted by a modified high salt exchange method (27) using long pieces of chromatin (28) from purified rat liver nuclei (29). This chromatin preparation yields long pieces of histone H1-depleted chromatin, ~30–60 nucleosomes in length. 5'-End-labelled DNA fragments were reconstituted as previously described (23). Briefly, the mixture was incubated for 30 min at 37°C and diluted stepwise with buffer containing no NaCl for several hours at room temperature to a final NaCl concentration of 0.1 M. For reconstitution in the presence of triple helix DNA the indicated concentration of triple helix-forming oligonucleotide was added only at the start of nucleosome reconstitution. No additional oligonucleotide was added during the salt dilution or at any later stage, thus diluting

the concentration of oligonucleotide by ~10-fold during the nucleosome reconstitution procedure. The reconstituted histone-DNA complexes were purified by sedimentation in a 5–30% glycerol gradient containing 50 mM Tris-HCl, pH 7.5, 1 mM Na₂EDTA and 0.1 mg/mg pork insulin in a Beckman SW 50.1 rotor at 35 000 r.p.m. for 15 h at 4°C. The purity of the reconstituted material was controlled by electrophoretic mobility shift assay (EMSA) in native 6% polyacrylamide gels (12).

Nuclease protection assays

Nucleosomes and free DNA were analysed using exonuclease III or DNase I footprinting assays as previously described (23), with the exception of double concentrations of DNase I for nucleosome reconstituted DNA and 1.5 U exonuclease III for both free and reconstituted DNA samples. G+A sequence marker lanes were prepared as previously described (30).

RESULTS

DNA fragments used for triple helix experiments

Two DNA segments were used to evaluate the effects of triple helix DNA on nucleosome formation *in vitro* (Fig. 1). A 199 bp *Pvu*II–*Bam*HI DNA fragment harbouring the –198 to –58 DNA segment of the MMTV promoter, here called MTVth–58, was created by introducing three point mutations at the indicated positions (Fig. 1). This produced a 16 bp homopurine segment to be used for oligonucleotide-directed triple helix formation (22). A longer 279 bp *Pvu*II–*Bam*HI fragment, MTVth+19, had the same upstream sequence as the shorter fragment and in addition the indicated downstream segment of the MMTV promoter (see Fig. 1; for further details see Materials and Methods). As a control for specificity of triple helix formation we used the corresponding 199 bp DNA fragment, MTVwt–58 (Fig. 1), which lacked the three mutations. This control DNA was previously shown to organize an *in vitro* reconstituted nucleosome into a defined rotational positioning and a major translational positioning covering the –219 to –76 DNA segment (23).

The triple helix-forming oligonucleotide GS373–82B (Fig. 1) contained the indicated modified nucleotides to stabilize the triple helix DNA under physiological conditions (22). All data shown here were performed at pH 7.5.

A triple helix oligonucleotide-induced change in the histone–DNA conformation is seen when the oligonucleotide is present during nucleosome reconstitution

Nucleosome reconstitution was followed by purification of the histone–DNA complex by glycerol gradient centrifugation and analysis by three methods: EMSA, DNase I footprinting and exonuclease III protection. The concentration of oligonucleotide required for formation of triple helix DNA was first evaluated on naked DNA by DNase I footprinting. This showed that 1 μ M oligonucleotide was insufficient, but that 10 μ M gave a complete DNase I footprint (22; data not shown). Standard nucleosome reconstitutions by the salt dilution method (27) were then performed in the presence of different concentrations (2.5–10 μ M) of triple helix-forming oligonucleotide and evaluated by DNase I footprinting. In these experiments a 2.5 μ M initial concentration of oligonucleotide, added at the start of nucleosome reconstitution, was sufficient for triple helix formation (data not

Figure 2. A triple helix oligonucleotide-induced reduction in sedimentation rate of the histone–DNA complex is detected by glycerol gradient centrifugation analysis. Gradient fractions were collected from the bottom of the tube. Nucleosome reconstitutions of DNA end-labelled at the *Bam*HI site (top strand DNA) were performed in the absence (–) and presence (+) of triple helix oligonucleotide. MTVwt–58 (top graph) and MTVth–58 (bottom graph). Nuc, nucleosomal DNA peak; free, free probe DNA peak.

shown). This concentration of oligonucleotide was thus used in the following experiments.

Nucleosome reconstitutions performed in the absence and presence of a 2.5 μ M initial concentration of triple helix-forming oligonucleotide were purified by glycerol gradient centrifugation as shown in Figure 2. The radioactivity profile of ³²P-end-labelled DNA from the fractionated glycerol gradients showed a slight but consistent triple helix oligonucleotide-dependent shift of the peak corresponding to the histone–DNA complex for the MTVth–58 DNA fragment. No effect of the oligonucleotide was seen for the MTVwt–58 control DNA fragment (Fig. 2). The

Figure 3. Gel shift analysis of MTVth-58 shows a shift in nucleosome mobility in the presence of triple helix oligonucleotide. Nucleosomes reconstituted in the absence (–) and presence (+) of triple helix oligonucleotide (TH oligo) were analysed on 6% non-denaturing polyacrylamide gels. Free, free probe DNA; nuc, reconstituted nucleosomal DNA.

sedimentation rate of the MTVth-58 histone–DNA complex corresponded to a sedimentation coefficient of 9.5–10 S using the sedimentation coefficient for the MTVwt-58 mononucleosome of 11 S (23) as a reference. The oligonucleotide-induced reduction in sedimentation rate of the MTVth-58 histone–DNA complex indicates a change in its conformation or content. There was no effect on the sedimentation rate of the free DNA due to the presence of the triple helix oligonucleotide in either DNA fragment (Fig. 2).

Analysis of the histone–DNA complexes and the free DNA peaks from the glycerol gradients by EMSA demonstrated an oligonucleotide-dependent increase in electrophoretic mobility of the MTVth-58 histone–DNA complex when compared with the MTVwt-58 control complex (Fig. 3, compare lanes 3 and 4 with lanes 7 and 8). Free DNA peaks from glycerol gradients of both MTVwt-58 and MTVth-58 nucleosome reconstitutions migrated at the same rate on EMSA, regardless of the presence or absence of triple helix oligonucleotide (Fig. 3, lanes 1 and 2 and 5 and 6). The slightly higher electrophoretic mobility of the MTVth-58 DNA–histone complex in the presence of oligonucleotide again indicates a change in conformation or content of this histone–DNA complex.

Nuclease protection assays show triple helix-induced interference with histone–DNA contacts

DNase I footprinting and exonuclease III protection analysis were applied to evaluate the structural changes that occur in the presence and absence of the triple helix oligonucleotide by nucleosome reconstitution of MTVth-58 DNA and the corresponding control DNA, MTVwt-58. DNase I footprinting revealed a nucleosomal organization of both DNA fragments in the absence of oligonucleotide, as seen by the characteristic 10 bp ladder of DNase I-sensitive sites with intervening segments of protection when compared with free DNA (Fig. 4, compare lanes 2 with 5, 7 with 10 and 12 with 15). These DNase I patterns show that the two DNA fragments MTVth-58 and MTVwt-58 have an identical and defined rotational setting on the histone octamer surface (Fig. 4, compare lanes 5 and 10).

A distinct difference in the nucleosomal DNase I cleavage pattern of the MTVth-58 DNA can be seen when comparing

Figure 4. DNase I footprinting of MTVth-58 DNA in the presence of triple helix oligonucleotide shows the absence of nucleosome-specific cleavages 5' but not 3' to the triple helix site. Nucleosomes (Nuc) reconstituted in the absence (–) or presence (+) of triple helix oligonucleotide (TH oligo) and free DNA (free) were purified on glycerol gradients and then digested with DNase I and analysed on 6% polyacrylamide sequencing gels. G+A, sequencing lane; ▲, nucleosome-specific cut resistant to the presence of triple helix oligonucleotide; △, nucleosome-specific cut in the absence but not in the presence of triple helix oligonucleotide; Top, top strand DNA; Bottom, bottom strand DNA.

nucleosome reconstitutions performed in the presence versus the absence of triple helix oligonucleotide (Fig. 4, compare lanes 9 with 10 and 14 with 15). These changes to a DNase I pattern more similar to free DNA were most pronounced on the 5'-side of the triple helix site, where no nucleosome-specific DNase I cuts were detected in the presence of the triple helix oligonucleotide (Fig. 4, lanes 13 and 14). The nucleosomal DNase I pattern on the 3'-side of the triple helix was also converted to a pattern more similar to free DNA, although some nucleosome-specific cuts remain on this side (Fig. 4, lanes 9 and 14). In addition, a clear triple helix oligonucleotide-induced footprint can be seen in both the free DNA and nucleosomal DNA lanes when triple helix oligonucleotide is added to the MTVth-58 probe (Fig. 4, lanes 8 and 9 and 13 and 14), but not for the MTVwt-58 probe (Fig. 4, lanes 3 and 4). This confirms that the oligonucleotide does indeed bind specifically to the 16 bp polypurine segment and forms a DNA triple helix (22).

Exonuclease III protection analysis was also used to analyse histone–DNA contacts, since this 3'-specific exonuclease is partially inhibited at such sites, giving a 10 bp repetitive pattern

Figure 5. Exonuclease III analysis of MTVth-58 DNA confirms the absence of a nucleosome structure 5' but not 3' of the triple helix site. Nucleosomes (Nuc) reconstituted in the absence (–) or presence (+) of triple helix oligonucleotide (TH oligo) and free DNA (free) were digested for increasing amounts of time with exonuclease III (exo III) and analysed on a 6% polyacrylamide sequencing gel. G+A, sequencing lane; $\blacktriangleleft$, triple helix oligonucleotide-resistant nucleosome border stops; $\triangleleft$, triple helix oligonucleotide-induced removal of nucleosome border stops; $\bullet$, triple helix oligonucleotide-resistant nucleosome-specific stops; $\circ$, triple helix oligonucleotide-induced alteration of nucleosome-specific stops; Top, top strand DNA; Bottom, bottom strand DNA.

of relative inhibition when digesting DNA on a histone octamer surface (31). Free MTVth-58 DNA showed a triple helix oligonucleotide-dependent block of exonuclease III activity at the border of the oligonucleotide binding site (Fig. 5, lane 12), demonstrating that triple helical DNA inhibits exonuclease III activity. Exonuclease III digestion of nucleosome reconstituted MTVth-58 and MTVwt-58 DNA in the absence of triple helix oligonucleotide results in protection of the probe from exonuclease III activity at sites corresponding to histone–DNA contact. The first distinct histone-induced exonuclease III stops were located at positions –219 and –209 on the bottom strand and –76 and –66 on the top strand (Fig. 5, lanes 16 and 17 and 20 and 21). This suggests that we have two preferred translational positions of the histone octamer, –219 to –76 and –209 to –66, and that the dyad of the 144 bp nucleosomal DNA segment is located at positions –148 and –138, respectively. The –164 to –149 polypurine segment forming the triple helical DNA in MTVth-58 is thus located close to the nucleosomal dyad, but on the 5'-side of it. Nucleosome reconstitution of the MTVth-58 DNA in the presence of triple helix oligonucleotide produced an exonuclease III pattern similar to that of free DNA. In fact, all histone-specific contacts flanking the triple helix DNA disappeared, while some contacts (positions –66, –76 and –86) near the 3'-end remained (Fig. 5, lanes 23 and 24, black arrows and circle). No oligonucleotide-induced change could be seen for the MTVwt-58 control (Fig. 5, lanes 4–7), again confirming the binding specificity of the triple helix oligonucleotide. Taken together, the exonuclease III analysis confirms the DNase I footprinting results for the MTVth-58 DNA, that histone–DNA contacts are perturbed by triplex DNA.

We conclude that nucleosome reconstitution of a DNA fragment harbouring a segment of triple helical DNA results in disturbed histone–DNA contacts not only over the segment of triple helical DNA, but also 55 bp away, as seen here on the 5'-side

but also 50 bp away on the 3'-side of this *in vitro* reconstituted nucleosome. The 199 bp long MTVth-58 has a centrally located segment of triple helical DNA leaving only 92 and 91 bp respectively of flanking double-stranded DNA on either side. Since a histone octamer organizes 144 bp of DNA into a nucleosome, this strategy of using a short DNA segment and a centrally located triple helix DNA segment should promote inclusion by the histone octamer of the triplex DNA within the bound DNA segment. However, our experiments demonstrate that this triple helix DNA-containing histone–DNA complex has grossly disturbed histone–DNA contacts, to the extent that such contacts are lacking on its 5'-side.

Several experiments were conducted where the triple helix-forming oligonucleotide was added after nucleosome reconstitution on the MTVth-58 DNA. We did not achieve any alteration in histone–DNA contacts or any formation of triple helix DNA by this approach (data not shown). These experiments were conducted at both 25 and 37°C using physiological pH (7.2) and ionic strength conditions. We conclude that prebound histone octamer inhibited oligonucleotide binding and triplex DNA formation.

Triple helical DNA acts as a nucleosome barrier

What are the effects of triple helical DNA on nucleosome positioning and how close to a triple helical DNA segment can a nucleosome be positioned? To answer these questions we used a longer, 279 bp DNA fragment, MTVth+19 (Fig. 1). This fragment contains 168 bp of double-stranded MMTV DNA on the 3'-side of the triplex DNA site, enough space for a histone octamer to bind. The MTVth+19 DNA fragment was reconstituted in the absence and presence of triple helix oligonucleotide as above. Glycerol gradient and gel shift analysis yielded no

Figure 6. The triple helix oligonucleotide redirects a positioned nucleosome 3' of the triple helix site on MTVth+19 DNA. Nucleosomes reconstituted (Nuc) in the absence (-) or presence (+) of triple helix oligonucleotide (TH oligo) and free DNA (free) were digested with DNase I and analysed on a 6% sequencing gel. Empty bars indicate protection against DNase I cleavage in the presence of the th oligonucleotide. G+A, sequencing lane; ◀, nucleosome-specific cut in the absence of the triple helix oligonucleotide; ◁, triple helix oligonucleotide-induced nucleosome-specific cuts; Top, top strand DNA; Bottom, bottom strand DNA.

obvious differences in the absence and presence of the triple helix oligonucleotide (data not shown).

DNase I footprinting and exonuclease III protection assays of the glycerol gradient purified 11 S histone-DNA complex showed a triple helix oligonucleotide-dependent shift in positioning of the nucleosome (Figs 6 and 7). In the absence of triple helix oligonucleotide the DNase I pattern of the histone-DNA complex resembled that of free DNA over large parts of the fragment, with some nucleosome-specific DNase I cuts present (see black triangles in Fig. 6, lanes 4 and 8). However, when triple helix oligonucleotide was present during the nucleosome reconstitution a much more extended and distinct 10 bp DNase I nucleosomal ladder could be seen on the 3'-side of the triplex DNA (Fig. 6, lane 3; white triangles signify triplex DNA-induced nucleosome-specific DNase I cut sites), while the 5'-side was identical to the naked DNA pattern (Fig. 6, lane 7). The 3'-boundary of the nucleosomal 10 bp ladder of DNase I cuts with intervening protections was extended from around position -58 to position -14 by the presence of the triple helix oligonucleotide (Fig. 6, compare lanes 3 and 4). This indicated that the nucleosome positioning was shifted towards the 3'-side of the

Figure 7. Exonuclease III analysis of MTVth+19 DNA shows the redirection of a positioned nucleosome at the 3' border of the triple helix site in the presence of triple helix oligonucleotide. Nucleosomes reconstituted (Nuc) in the absence (-) or presence (+) of triple helix oligonucleotide (TH oligo) and free DNA (free) were digested for increasing amounts of time with exonuclease III (exo III) and analysed on a 6% sequencing gel. G+A, sequencing lane; ◀, proposed triple helix oligonucleotide-induced nucleosome border stop; ●, triple helix oligonucleotide-resistant exonuclease III stops; ○, triple helix oligonucleotide-induced alteration of nucleosome-specific stops; Top, top strand DNA; Bottom, bottom strand DNA.

DNA fragment by the formation of triple helical DNA. Furthermore, the triple helical DNA caused a shift in helical setting of the nucleosomal DNA segment flanking the 3'-side of the triple helix DNA (Fig. 6, compare black and white triangles in lanes 3 and 4; best seen between the -113 and -86 DNA segment).

Exonuclease III analysis of MTVth+19 DNA labelled on either the bottom strand or the top strand showed many histone-DNA-specific contact points (Fig. 7, lanes 8 and 9 and 16 and 17). This finding, in combination with the DNase I footprinting result, is compatible with a variable translational positioning of the mononucleosome in this 279 bp DNA fragment. This is not unexpected for such a long DNA fragment when used in *in vitro* nucleosome reconstitution. Exonuclease III analysis of MTVth+19 DNA in the presence of triple helix oligonucleotide showed that all histone-DNA contacts on the 5'-side of the triplex DNA were interrupted (Fig. 7, bottom strand, lanes 6 and 7), while only two major histone-induced exonuclease III protection sites positioned at -15 and -5 remain on the 3'-side (Fig. 7, lanes 14 and 15, white arrow and circles). These positions correspond to 134 and 144 bp of DNA, respectively, when measured from the 3'-border (-149) of the triple helix site (see Fig. 1). Exonuclease III is only partly inhibited at a nucleosome border and is able to cut further into the nucleosome, generating a 10 bp pattern as seen here (Fig. 7, lanes 14 and 15; 31). We are thus unable to determine whether all histone octamers are located with their 3'-border at position -5 or if some are located at position -15. The exonuclease III protection analysis is in perfect agreement with the DNase I footprinting of the MTVth+19 reconstituted mononucleosome and shows that triplex DNA can direct the

nucleosome to the 3'-side, i.e. where there is enough DNA to accommodate a nucleosome. Furthermore, our results show that a nucleosome can be located very close to triple helical DNA. We conclude that triplex DNA functions as a nucleosome barrier.

DISCUSSION

Our results demonstrate that an oligonucleotide-directed DNA triple helix locally interferes with histone-DNA contacts, preventing nucleosomal organization at the site of the triplex DNA. The sequence specificity of the triple helix-forming oligonucleotide was demonstrated by the absence of a triple helix DNA footprint on the corresponding wild-type DNA fragment, which differed by only 3 bp in the 16 bp homopurine triple helix binding segment (Fig. 1). The specificity and the localization of the oligonucleotide-induced protection seen by DNase I footprinting and exonuclease III protection analysis confirm that triple helical DNA is indeed formed (22).

The triple helix-induced inhibition of histone-DNA contacts was extensive on the 199 bp pMTVth-58 DNA fragment. Here the complete absence of a nucleosome-like DNase I pattern was seen on the 5'-side of the triple helix DNA segment, which was corroborated by the exonuclease III protection analysis. However, a partial 10 bp DNase I pattern and exonuclease III pattern, typical of nucleosomal DNA, remained on the 3'-side of the triple helix DNA, demonstrating that some histone-DNA contacts were retained (summarized in Fig. 8A). It should be pointed out that these remaining histone-DNA contacts rendered a complex stable enough for purification on glycerol gradients. The triple helix induced-structural change in the histone-DNA complex was confirmed by glycerol gradient centrifugation and EMSA. We do not know what is contained within this altered histone-DNA complex, since attempts to analyse the relative stoichiometry of core histones by silver stained SDS-PAGE and radioactive labelling experiments have so far been inconclusive.

Triplex DNA functions as a nucleosome boundary, as demonstrated using the 279 bp long DNA fragment, MTVth+19. This DNA fragment has 167 bp of double-stranded DNA downstream of the triple helical segment, allowing enough space for a nucleosome to form. These experiments also showed that a nucleosome can be located very close to a DNA triple helix. However, our results cannot rule out the possibility that some histone octamers incorporate the triplex DNA into the first 10 bp of nucleosomal DNA structure. Nevertheless, the proximity of the histone octamer to the triplex DNA is in agreement with previous data showing the co-existence of UL9 protein binding to herpes simplex virus type I promoter DNA immediately adjacent to a triplex DNA structure (32). In our study the formation of triple helix oligonucleotides directed the histone octamer into one preferred translational position, while this DNA segment in the absence of triple helix would have many different translational positions of the histone octamer (Figs 7 and 8B). We also note that the altered positioning of the histone octamer by the triple helical DNA is accompanied by a changed rotational setting of the DNA close to the triplex DNA border (Fig. 6, lanes 3 and 4). The simplest explanation is that the DNA segment which directs the major rotational frame of the nucleosome in the absence of triplex DNA becomes covered or located on the 5'-side of the triplex DNA segment in its presence.

One interesting aspect of this work is the requirement for the triplex DNA-forming oligonucleotide to be present during

Figure 8. Proposed model system for the effects of triple helix oligonucleotide formation during nucleosome reconstitution. (A) MTVth-58 DNA in the absence (top) and presence (bottom) of triple helix oligonucleotide ($\blacktriangle$). The dotted line surrounding the area 5' of the probe represents an unknown histone-DNA structure that may exist in the presence of the triple helix oligonucleotide, although the data indicates that no histone-DNA contacts are present. (B) MTVth+19 DNA in the absence (top) and presence (bottom) of triple helix oligonucleotide ($\blacktriangle$). Ellipsoids represent nucleosome boundaries; solid lines represent MTVth DNA; stippled bars indicate the triple helix oligonucleotide binding site.

nucleosome assembly in order for histone-DNA contacts to be inhibited. Conversely, preassembly of the nucleosome blocks formation of triplex DNA. In our experiment with MTVth-58 DNA the 3'-border of the triplex DNA segment was located 1–10 bp from the nucleosome dyad (Fig. 5). The capacity of a histone octamer to inhibit triple helix formation might vary with different translational positions within the nucleosome and might also depend on the local sequence context, since this can influence the stability of the histone-DNA contacts (33). However, the nucleosome barrier effect (Fig. 7), where essentially all histone octamers were positioned close to but outside the triplex helix DNA, argues for a strong inhibition of triplex DNA formation all over the nucleosome. In this context we note that in a recent review van Holde and Zlatanova (34) have proposed that oligonucleotide-induced DNA triple helices most likely occur at internucleosomal regions, since in their opinion it is highly unlikely that such a different structure as a DNA triplex would be accommodated on the specialized surface of the histone core. They also note within the same review that other non-B DNA forms, such as Z-DNA and cruciform structures, have been shown to be incapable of forming nucleosomes (34), although the

results are as yet controversial. Our experimental results confirm the hypothesis by van Holde and Zlatanova concerning triple helix DNA and thus resemble the situation seen for Z-DNA and cruciform structures (34).

Whether triplex DNA may play a role in the *in vivo* regulation of gene activity remains to be seen. Experimental data on inhibition of gene activation both *in vitro* and *in vivo* suggests that the only requirement for inhibition is the overlapping of DNA recognition elements for both the DNA binding protein and the triple helix oligonucleotide (35). However, the fact that nucleosomes are important determinants in promoter structure and transcriptional regulation suggest that triple helix-forming oligonucleotides might be used to direct nucleosomes into certain translational positions or to create nucleosome-free regions, provided that these reagents are stable and functional in the living cell. The increasing knowledge of how nucleosomes affect transcriptional regulation thus suggest that triplex DNA may be used not only as a repressor, as suggested before (35), but also as an activator, by removal of a repressive nucleosome, thereby exposing a transactive factor binding-site (see Introduction). It will be interesting to see if triple helix-forming oligonucleotides are able to compete with nucleosome assembly when present during DNA replication in the living cell.

In conclusion, the data presented here suggest that a triple helix oligonucleotide can be used to inhibit nucleosome formation *in vitro*. The use of the triple helix oligonucleotide in nucleosome positioning may provide a means to specifically direct nucleosome presence (or absence) over a regulatory region and thus may help to dissect the different actions of transcriptional activators, such as GR. In addition, the use of triple helix oligonucleotides should be evaluated as agents which overcome the repressive effects of nucleosomes, altering promoter structure and possibly inducing transcription of specific genes.

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