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Modified curved DNA that could allow local DNA underwinding at the nucleosomal pseudodyad fails to position a nucleosome *in vivo*

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ABSTRACT

In competitive *in vitro* reconstitution experiments synthetic DNA composed of tandem repeats of the repetitive sequence $(A/T)_3NN(G/C)_3NN$, specifically the 20 bp 'TG sequence' (5′-TCGGTGTTAGAGCCTGTA-AC-3′), was reported to associate with the histone octamer with an affinity higher than that of nucleosomally derived DNA. However, at least two groups have independently shown that tandem repeats of the TG sequence do not accommodate a stably positioned nucleosome *in vivo*. It was suggested that the anisotropic flexibility of the TG sequence, governed by a 10 bp sequence periodicity, is incompatible with the required underwinding of the DNA helix at the nucleosome pseudodyad while maintaining a bending preference that can be accommodated in the remainder of the nucleosome. Here we test this hypothesis directly by studying the *in vivo* nucleosomal structure of modified TG sequences designed to accommodate underwinding at the pseudodyad. We show that these modifications are not sufficient to allow stable incorporation of the TG sequence repeat into a nucleosome *in vivo*, but do note invasion from one end of the TG heptamer of a translationally random but rotationally constrained nucleosome. We discuss possible reasons for the absence of nucleosomes from the TG sequence *in vivo*.

INTRODUCTION

The nucleosome is the structural unit of eukaryotic chromatin and is composed of 166 bp of DNA spooled in two turns of a left-handed superhelix onto an octameric histone complex composed of two (H2A–H2B) and two (H3–H4) dimers (1,2). The number of superhelical turns in a nucleosome core is inconsistent with the constrained topology of a reconstituted circular molecule ($\Delta L_k \approx -1.0$ per nucleosome) (3,4), suggesting that the average helical period of nucleosomal DNA is less than that of DNA free in solution (5,6). Studies of nucleolytic cleavage periodicities of both reconstituted and mixed sequence cores (6–10) and the distribution of UV-induced cyclobutane pyrimidine dimers in chromatin (11) suggest an average local helical period (winding number; see 12) of ~10.2 for nucleosomal DNA. This local helical period does not appear constant throughout the length of the nucleosome, but increases in the region centered on the pseudodyad axis (6–10). A clear demarcation of these regions of different local period is seen in the hydroxyl radical cleavage pattern of a reconstituted nucleosome core, where the three central helical turns have a local helical period of 10.7 bp/turn, as opposed to the flanking regions, where a period of 10.0 bp/turn was measured (10). A similar result was obtained by Gale *et al.* (11) employing photofootprinting. We stress, however, that these conclusions implicitly assume that the angle of hydroxyl radical attack and the angular orientation of the maxima in pyrimidine dimer formation remain constant relative to the nucleosome supercoil axis. Arents and Moudrianakis reported that an average period of 10.7 in the three central helical turns allows optimal alignment of the remainder of the DNA helix at a 10.0 bp period with the parallel β-bridges and paired end helixes which were proposed to define the docking path of the DNA on the octamer surface (13). Consistent with these studies, the sequence periodicity of nucleotide dimers in core DNA displays a discontinuity in the central two helical turns (14) and the distribution period of nucleotide trimers increases in the same region of the core (15).

The histone octamer does not associate randomly with DNA in a population of unique sequence molecules. Certain rotational and translational settings are preferentially occupied and nucleosomes thus placed are operationally defined as positioned (reviewed by Simpson; 16). Although a clear correlation between the rotational setting of the histone octamer and the sequence-dependent anisotropic flexibility of the nucleosomal DNA was demonstrated (14,17–21), no similar unique determinant governing the translational setting of a nucleosome has been described. Satchwell *et al.* reported the preferential exclusion of extended $d(A)_n \cdot d(T)_n$ tracts from the central region of the nucleosome core (14), but this sequence was not under-represented in the corresponding region of dinucleosomes (22) nor sufficient to exclude nucleosomes *in vivo* (23). Several trinucleotides also appear to be statistically over- or under-represented at the nucleosome pseudodyad (24) and although a correlation between the translational setting of nucleosomes *in vitro* and the distribution

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of some trinucleotides was reported (19) the relation does not appear general (H.-G.Patterton, unpublished results). Nucleosomes often preferentially associate with curved DNA fragments (25,26) and Hsieh and Griffith (27) demonstrated the preferential location of a single octamer on a curved section of a 1216 bp fragment *in vitro*.

Several synthetic DNA sequences, designed to position the histone octamer in a defined and predictable manner, have been published (28,29). A fragment composed of a tandemly repeated 20 bp sequence, denoted the 'TG sequence' (5′-TCGGTGTTAGAGCCTGTAAC-3′), was reported to associate with the histone octamer *in vitro* with an affinity 100-fold higher than nucleosomal DNA derived from chicken erythrocytes (28). However, in subsequent *in vivo* studies no evidence of a positioned nucleosome on the similarly repeated TG sequence was found (30,31). It was suggested that with the wider range of positioning frames available *in vivo*, the anisotropically flexible TG sequence with a sequence periodicity of 10 bp is excluded from the nucleosome due to the offset in the preferential direction of bending of the sequence on either side of the nucleosomal pseudodyad, where the local helical period does not match the sequence period of the TG sequence (30). Here we test this hypothesis directly by investigating the *in vivo* nucleosomal structure of a tandem heptamer of the TG sequence and similar heptamers containing either a 1 or 2 bp insert in the central TG monomer, designed to comply with underwinding in the center of the nucleosome.

MATERIALS AND METHODS

Plasmid constructions

Pairs of complementary oligonucleotides which form the monomeric 'TG sequence' (28) [5′-TCGGTGTTAGAGCCTGTAAC-3′ (TG0W) and 5′-CCGAGTTACAGGCTCTAACA-3′ (TG0C)], the TG sequence with a 1 bp insert [5′-TCGGTGTTAGAGCC**G**TGTAAC-3′ (TG1W) and 5′-CCGAGTTACA**C**GGCTCTAAC-3′ (TG1C)] and the TG sequence with two 1 bp inserts [5′-TCGGTG**A**TTAGAGCCTG**A**TAAC-3′ (TG2W) and 5′-CCGAGTTA**T**CAGGCTCTAA**T**CA-3′ (TG2C)] were synthesized on a DNA synthesizer (Applied Biosystems) and purified on a 10% polyacrylamide–8 M urea gel by established procedures (32). Although the latter pair of oligonucleotides contains two 1 bp inserts spaced 10 bp apart we refer to this construct as containing a 2 bp insert to avoid ambiguity. The A+T-rich regions in the oligonucleotide sequences shown above are underlined (28) and the additional nucleotides inserted into the TG sequence are shown in bold for the latter two pairs of oligonucleotides. The purified oligonucleotides were phosphorylated with T4 polynucleotide kinase and annealed as previously described (33). A trimer and heptamer of the TG fragment were isolated from a 10% polyacrylamide gel following ligation with T4 DNA ligase (33).

The symmetrical *Ava*I site in the polycloning region of the yeast shuttle vector pRS424ΔZ (33) was converted to an asymmetrical *Ava*I site of sequence CTCGGG by a two-step polymerase chain reaction procedure (34). The TG heptamer was ligated into this asymmetric *Ava*I site of plasmid pRS424ΔZ and the resulting plasmid denoted pRS424ΔZ-TG7. Constructs containing a similar heptameric TG repeat, but differing at the central TG monomer which contained either a 1 or 2 bp insert, were prepared by a three-step ligation procedure. A trimeric TG fragment was first ligated into the asymmetric *Ava*I site of pRS424ΔZ, regenerating the single asymmetric *Ava*I site. A monomer of the TG duplex containing either a 1 or 2 bp insert was then ligated into this *Ava*I site, followed, finally, by the ligation of another TG trimer into the regenerated *Ava*I site. The resulting plasmids were denoted pRS424ΔZ-TG3.1.3 (central TG monomer with a 1 bp insert) and pRS424ΔZ-TG3.2.3 (containing a 2 bp insert in the central TG monomer). A corresponding pRS425 (35) series was constructed by ligation of the *Spe*I–*Pst*I fragment from the pRS424ΔZ series, containing the heptameric TG sequence, into *Spe*I/*Pst*I-cut pRS425 and the resulting plasmids denoted pRS425-TG7, pRS425-TG3.1.3 and pRS425-TG3.2.3. The sequences of all inserts were verified by chain terminating nucleotide sequencing (United States Biochemicals). The purified plasmids were transformed into yeast strain FY23 (**MATa** *ura3-52 trp1Δ63 leu2Δ1*) by a dimethyl sulfoxide-enhanced lithium chloride procedure (36).

Probing, indirect end-label mapping and primer extension footprinting of nucleosomes

Transformed yeast cells were grown at 30°C to an OD_{600} of 1.0 in 100 ml SC medium lacking the appropriate amino acid (37). Permeabilized yeast spheroplasts were prepared and the chromatin and purified free DNA digested with micrococcal nuclease (MNase) as described by Kent *et al.* (38). Appropriately digested samples were cut to completion with the restriction endonuclease *Ngo*MI and electrophoresed on a 1.5% agarose gel in 1× TBE (90 mM Tris–borate, pH 8.3, 2 mM EDTA). The DNA was transferred to a nylon membrane (Duralon-UV, Stratagene), UV cross-linked and hybridized after Church and Gilbert (39). A 202 bp *Ngo*MI–*Pvu*II fragment of pRS425, random prime labeled with [α-^{32}P]dCTP to a specific activity of ~10^9 c.p.m./μg, was used as a probe.

To investigate the nucleosomal location of the TG7 sequence and the adjacent region in the plasmid, chromatin and free DNA were prepared, electrophoretically resolved and transferred to a nylon membrane as above, but without the restriction endonuclease treatment. The membrane was hybridized with a 152 bp *Pst*I–*Bam*HI fragment from pRS425-TG7 (TG7 sequence), a 162 bp *Hin*dIII–*Pvu*II fragment from pRS425-TG7 (sequence adjacent to the TG7 insert) or a 196 bp *Bam*HI–*Hin*cII fragment from the *STE6* yeast gene. The probes were random prime labeled with [α-^{32}P]dCTP to specific activities of ~10^9 c.p.m./μg.

Purification of yeast nuclei and primer extension footprinting following MNase or DNase I digestion of chromatin and free DNA were performed as previously described (33). A [γ-^{32}P]ATP end-labeled 32 nt oligonucleotide, annealing with the 5′ nucleotide aligned at position 3342 in pRS425, was used in all the primer extensions reported in this study.

Curvature of the native and modified TG heptamers

Curvature of *Spe*I–*Pst*I fragments from the pRS425 plasmid containing the TG7, TG3.2.3 and TG3.1.3 motifs were determined by electrophoresis on a 10% polyacrylamide gel (29:1, acrylamide:bisacrylamide) in 1× TBE at 4°C (40).

Calculation of the difference in DNase I cleavage extent in chromatin compared with free DNA

The difference in the DNase I cleavage extent in chromatin versus free DNA in the heptameric TG regions in pRS425-TG7 and

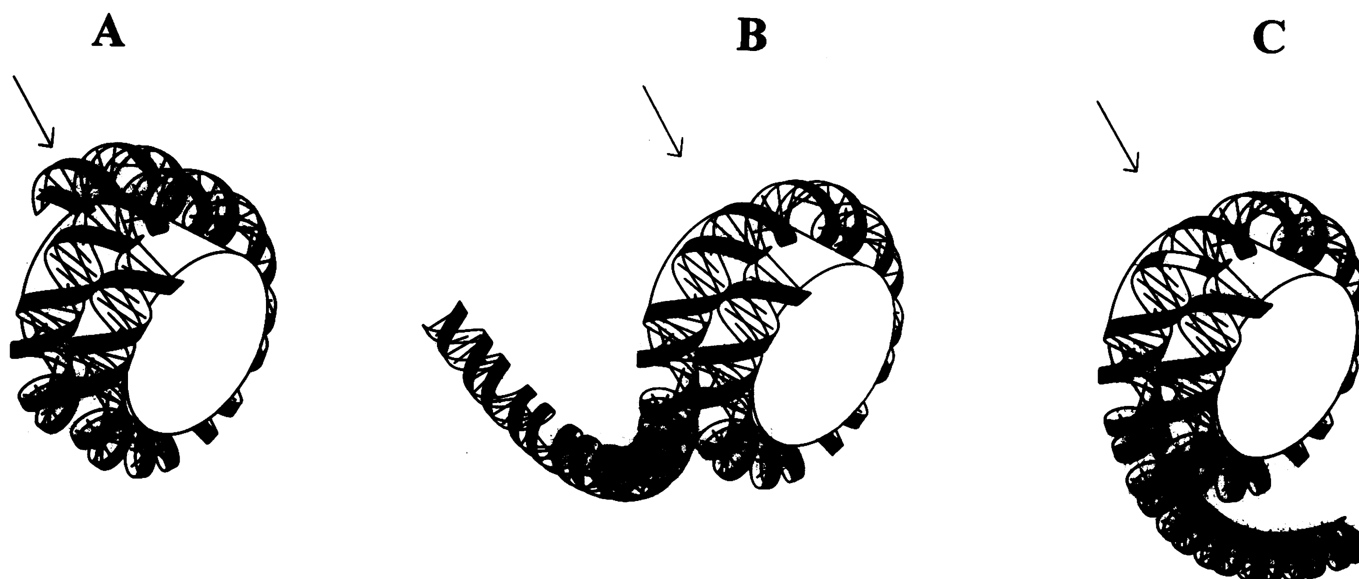

Figure 1. (A) Schematic presentation in perspective of a nucleosome core containing two full turns (166 bp) of DNA in a negative supercoil. The local helical period (winding number) of the three helical turns centered at the dyad axis (co-linear with the arrow) on the side of DNA entry and exit is 10.7 bp/turn and that of the flanking DNA on either side is 10.0 bp/turn. In an anisotropically flexible molecule with a sequence periodicity of 10 bp, underwinding of the DNA molecule over the central three helical turns at the dyad axis will offset the preferential direction of bending on either side of the dyad. This is indicated in (B), where the length of DNA unassociated with the octamer surface has a bias to bend in a plane that is not perpendicular to the supercoil axis. (C) The introduction of 2 bp (represented by the white strip in the helix backbone) into the central underwound region re-aligns the direction of bending with that required to follow the surface of the histone octamer.

pRS425-TG3.2.3 was calculated from the densitometric scan of a primer extension gel, described above. The area under each peak that corresponded to individual bands on the gel was determined and the extent of DNase I cleavage at a given position calculated as the ratio of the area of the relevant peak to that of all the peaks above it on the gel (including itself), as described by Drew and Travers (17). The difference in the extent of DNase I cleavage in chromatin compared with free DNA was calculated by subtracting the cleavage extent at a given bond in the free DNA from the corresponding bond in the chromatin.

RESULTS

The hypothesis

In a nucleosome core 166 bp of DNA is wound onto a histone octamer in two negative supercoils, as schematically shown in Figure 1A. The local helical period of the nucleosomal DNA is not constant throughout the entire length of the core (6,7,9), but appears to be underwound to 10.7 bp/turn over the three central helical turns compared with the flanking regions, which have a local helical period of 10.0 bp/turn (10). The favored direction of bending of an anisotropically flexible DNA molecule with a sequence periodicity of 10 bp, such as the TG sequence (28), can thus be accommodated in the flanking regions of the nucleosome core. However, if the 10.0 bp sequence periodicity extends for the length of the nucleosome the underwinding of the DNA duplex over the central three helical turns will offset the preferential direction of bending between the two sides of the DNA molecule on either side of the dyad axis. This is illustrated in Figure 1B, where the preferred direction of bending, exaggerated as a planar curve, is offset from that required to follow the surface of the histone octamer. It has been suggested that this absence of a single direction of favored bending compatible with smooth winding of the DNA around the histone octamer forms the basis of the inability of the TG sequence to be accommodated in a nucleosome *in vivo* (30).

This proposed structural solution implies that the central overwinding of the DNA helix by 30 bp $\times$ 360° [(1/10.0) − (1/10.7)] = 71°, or insertion of ~2 bp, will re-align the preferential direction of bending on either side of the dyad axis, as schematically shown in Figure 1C, thereby facilitating continuous winding of the DNA molecule onto the octamer surface.

We have tested this suggestion *in vivo* by comparing the nucleosomal structure of a tandem heptamer of the 20 bp TG sequence with a similar heptamer containing a 1 or 2 bp insertion in the central TG monomer. The inserted nucleotide and position of insertion were chosen to comply with the anticipated bending requirement of the DNA in the nucleosome core. Specifically, the hydroxyl radical footprint of the TG sequence reconstituted into nucleosome cores *in vitro* indicated that the DNA molecule assumes a rotational orientation placing the dinucleotides C_2G_3 and $G_{12}C_{13}$ at positions where the minor groove points away from the octamer surface (28; the subscripts refer to sequence positions in the TG0W oligonucleotide; see Materials and Methods). Thus for the 1 bp insert the nucleotide G was inserted between nucleotides C_{14} and T_{15}, thereby introducing the two dinucleotide steps CG and GT bordering the inserted nucleotide. The dinucleotide step CG is found more often at positions in the core where the minor groove is oriented away from the octamer surface, whereas the GT dinucleotide step has a less pronounced angular preference (14,19). Therefore, the location of the inserted G is expected to be compatible with the local bending requirements in the nucleosome core.

The TG sequence containing the 2 bp insert was constructed by insertion of an A between nucleotides G_6 and T_7 and also between

G_{16} and T_{17}, again keeping with the preferred angular orientation of the formed dinucleotides relative to the direction of bending of the nucleosomal DNA. The two 1 bp inserts were introduced 10 bp apart to change the preferential direction of bending in a gradual manner and although this construct contains 2 × 1 bp inserts, it is referred to as a 2 bp insert in the text for reasons of clarity. If the absence of a positioned nucleosome on the native TG sequence *in vivo* is due solely to the offset between the preferred direction of bending between the two halves of the sequence in a nucleosome, the insertion of 1 or 2 bp into the center of the motif is expected to generate a frame that would closely fit the required bending properties of nucleosomal DNA and therefore represent a very favorable positioning frame.

Prior to cloning and investigating the nucleosomal structure of the modified sequences *in vivo* the suitability of the modified sequences to accommodate a nucleosome was examined theoretically with an algorithm based on that of Drew and Calladine (19). In this treatment the ability of a 120 bp stretch of DNA composed of three contiguous regions of 44, 32 and 44 bp with local helical periods of 10.0, 10.7 and 10.0 bp/turn, respectively, to be wrapped onto the surface of a cylinder was tested. The cumulative preference of each dinucleotide present in this 120 bp window to be placed at the angular orientation determined by the direction of bending of the molecule onto the cylinder was calculated with the center of the window consecutively placed at each position along the DNA sequence of interest. We assumed an identical angular preference for each dinucleotide step in a helix with a periodicity of 10.7 as opposed to 10.0 bp/turn, even though the twist angle (ω) between the constituent base pairs will differ by ~2°. The result is shown in Figure 2. Although this theoretical treatment strictly gives an indication of the likelihood of a specific rotational as opposed to a translational setting of a nucleosome, any inherent inability of the modified TG sequences to uniformly wrap onto the surface of an octamer, incorporating underwinding of the DNA in the central region of the nucleosome, should be evident.

Looking first at the trace of the native TG sequence (labeled TG7 in Fig. 2), an oscillation of significant amplitude is visible in the signal, indicating the presence of a well-defined rotational positioning frame on one side of the DNA helix (19). This is expected for a sequence designed to be anisotropically flexible (28). Examining the trace of the TG sequence containing a 2 bp insert in the center of the 140 bp motif next (labeled TG323 in Fig. 2), it is seen that several settings that incorporate the entire TG motif into a nucleosome are more favorable in the modified TG sequence relative to the native TG sequence. Positioning frames placing the region containing the 2 bp insert close to the pseudodyad axis appear particularly favored. This is directly due to the modified TG sequence more closely fitting the idealized bending requirement that is tested. Although it is not clear whether the numeric difference between the likelihood of placing a nucleosome over the TG3.2.3 sequence as opposed to the TG7 sequence is significant, this result does show that modification of the TG sequence did not compromise the anisotropic flexibility of this sequence, judged by the current understanding of sequence-directed bending preferences of DNA.

Curvature of the modified TG fragments

The *in vitro* reconstitution of the histone octamer with DNA suggests that the octamer has a higher affinity for curved DNA

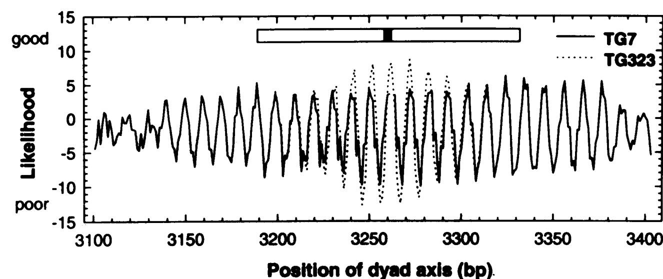

Figure 2. Plot of the predicted rotational preference of nucleosomes on the native (solid line) and modified (dotted line) TG sequences in the pRS425 plasmid, based on the algorithm of Drew and Calladine (19). The likelihood (*y* axis) for the location of the nucleosomal dyad axis at each position in the sequence (*x* axis) was calculated by adding the natural logarithm of the preference of each dinucleotide in a 119 base step window, centered on the dyad axis for the angular orientation defined by each particular setting of the dyad axis (19,51). The angular orientation of each base step in the 120 bp window was calculated in a model where the three central helical turns centered on the dyad axis have a helical period of 10.7 bp/turn and the flanking DNA has a 10.0 bp/turn period. The numbering origin of the parental plasmid pRS425 is retained in the sequence numbers shown in the figure. The location of the 140 bp TG sequence is indicated by the blank rectangle in the top of the figure and the location of the 2 bp insert in the TG motif by the solid rectangle. The plots of both TG7 and TG3.2.3 are shown superimposed to aid comparison. This necessitates the break in the plot of TG7 at the position where TG3.2.3 contains the first of the additional 2 bp.

(25–27), raising the possibility that if a 1 or 2 bp insert in the center of the 140 bp tandemly repeated TG sequence changes the curvature of the sequence, the affinity of the histone octamer for the sequence may change. In order to discriminate between such an altered curvature and an offset between the direction of preferential bending of the molecule on the ability of the sequence to accommodate a nucleosome *in vivo*, we investigated the curvature of the native and modified TG sequences.

The electrophoretic mobilities of *Spe*I–*Pst*I fragments of 158, 159 and 160 bp containing the TG7, TG3.1.3 and TG3.2.3 sequences, respectively, were determined on a 10% polyacrylamide gel at 4°C and are shown in Figure 3. The apparent size of the bands resolved in lanes 2–4 were calculated as 187, 183 and 185 bp with a third-order polynomial fitted to the size standards in lanes 1 and 5. These values correspond to a nearly identical 'R factor' (ratio of the apparent to the true size in base pairs) of 1.2 for TG7, TG3.1.3 and TG3.2.3. As a control of the ability of this gel to discriminate curved fragments from fragments that have a time-averaged linear conformation note the significantly retarded mobility of the 281 and 872 bp fragments of *Hae*III-digested ΦX174 DNA (lanes 1 and 5 of Fig. 3; these fragments were omitted from the size calculation).

This result shows that the native TG sequence is only modestly curved under the conditions of electrophoresis, although it is not known to what extent the anisotropic flexibility of this sequence contributes to this apparent curved conformation. The basis of sequence-dependent curvature may be distinct from that of anisotropic flexibility (reviewed in 41,42). It is also clear that the 1 and 2 bp insertions in the TG sequence cause little deviation in electrophoretic behavior of these fragments, demonstrating that the insertion(s) did not produce a detectable change in the magnitude of the apparent curvature of the TG sequence. Although this type of experiment does not discriminate between equal magnitudes of curvature in different directions, the inserted

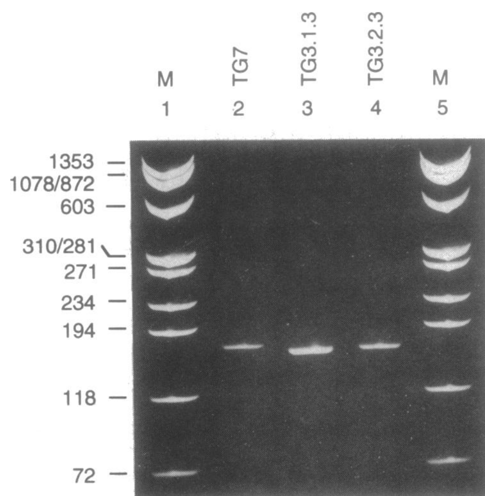

Figure 3. Curvature of the native and modified heptameric tandem repeats of the TG sequence. Fragments of 158, 159 and 160 bp containing, respectively, the heptameric repeat of the TG sequence (TG7; lane 2) and the heptameric repeat in which the central TG monomer contains an insert of 1 (TG3.1.3; lane 3) or 2 bp (TG3.2.3; lane 4) were purified from *SpeI/PstI*-digested plasmids and electophoresed on a 10% polyacrylamide gel at 4°C. ΦX174 DNA digested with *HaeIII* was used as a standard (M; lanes 1 and 5) with the size of individual fragments indicated in base pairs to the left of the figure.

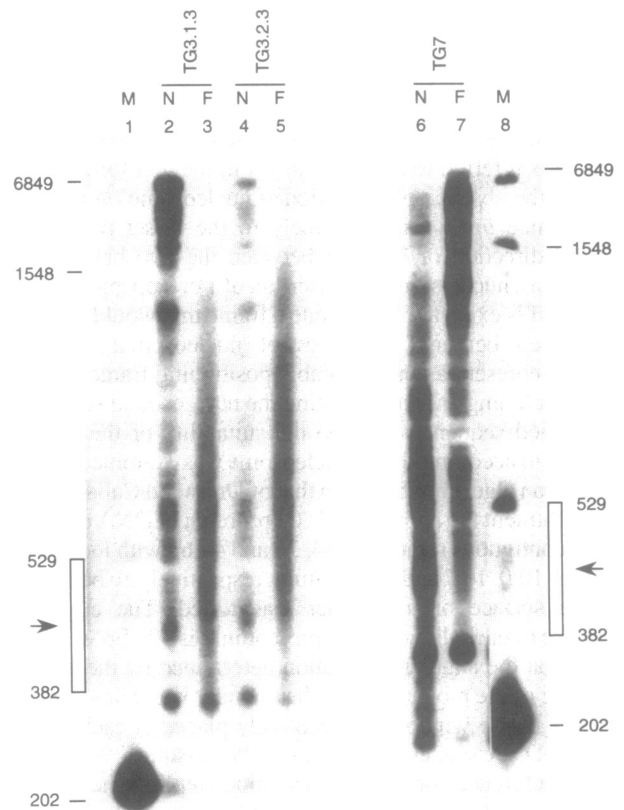

Figure 4. The chromatin structure of the native and modified TG sequence repeats determined by indirect end-labeling. The cleavage pattern obtained by MNase digestion of chromatin in permeabilized spheroplasts (N, lanes 2, 4 and 6) and purified DNA (F, lanes 3, 5 and 7) of pRS425-TG3.1.3 (TG3.1.3, lanes 2 and 3), pRS425-TG3.2.3 (TG3.2.3, lanes 4 and 5) and pRS425-TG7 (TG7, lanes 6 and 7) are shown. The purified MNase-digested DNA was electrophoresed on a 1% agarose gel following cleavage with the restriction endonuclease *NgoMI*, the DNA transferred from the gel to a nylon membrane, UV cross-linked and probed with a 202 bp *NgoMI–PstI* [α-^{32}P]dCTP random prime labeled fragment. A mixture of pRS425 digested with *NgoMI* and either *PvuII*, *PstI*, *AvaI*, *AflIII* or *BspHI* was used as a standard. The size of individual fragments are labeled in base pairs to the sides of the figure. The location of the TG heptamer in the sequence of each of the probed plasmids is indicated by the rectangle to the side of each panel. The arrows indicate the position of enhanced cleavage in the chromatin compared with the free DNA in the TG heptameric repeat. The two panels represent autoradiographs of two different gels.

nucleotides in TG3.1.3 and TG3.2.3 were chosen to comply with the direction of anisotropic flexibility of the TG sequence (see above) and are unlikely to result in a curvature in a direction opposite to the apparent curvature in the TG7 sequence. We therefore conclude that possible differences in the ability of the modified versus the native TG sequences to position nucleosomes *in vivo* are not expected to result from a change in the curvature of these sequences.

The nucleosomal organization of the native and modified TG sequences *in vivo*

The nucleosomal organization of the heptameric repeat of the TG sequence (TG7) and also the repeats where the central monomeric TG sequence contains a 1 (TG3.1.3) or 2 bp (TG3.2.3) insert was investigated by indirect end-labeling of DNA isolated from MNase-treated yeast spheroplasts. These sequences were present upstream of the *LEU2* selectable marker in the polylinker of the multi-copy yeast plasmid pRS425. The result is shown in Figure 4. Looking first at the MNase digestion pattern of the TG7-containing plasmid (lanes 6 and 7 of Fig. 4), it is seen that the central region of the TG7 sequence, indicated by the rectangle to the right of the figure, is readily cleaved in chromatin (arrow to the right of Fig. 4; lane 6). The extent of cleavage at this position is significantly higher than in the corresponding region in free DNA (lane 7), suggesting that the central region of the TG7 sequence is not incorporated into a nucleosome in the majority of plasmid molecules *in vivo*. On either side of the TG7 motif the distribution of MNase cleavage sites in chromatin closely mirrors that of free DNA, suggesting that nucleosomes are essentially randomly positioned in these regions.

Looking next at the digestion patterns of the TG3.1.3 (lanes 2 and 3) and TG3.2.3 (lanes 4 and 5) sequences it is seen that the central regions of these modified TG motifs are similarly exposed to MNase in chromatin (arrow to the left of Fig. 4). The enhanced cleavage seen in the center of the modified TG regions in chromatin is not caused by a change in the MNase susceptibility of this region due to the change in the nucleotide sequence, since the free DNA does not show a comparable increase in the extent of cleavage. There is no evidence of a nucleosome-length region including the modified TG motif that is protected from MNase digestion in chromatin but not in free DNA. This result shows that the introduction of 1 or 2 bp into the central TG monomer does not allow the tandemly repeated TG sequence to be incorporated into a stably positioned nucleosome *in vivo*.

To further address incorporation of the native and modified TG sequences into nucleosomes *in vivo* MNase-digested chromatin prepared from yeast transformed with pRS425-TG7 or pRS425-TG3.2.3 was electrophoretically resolved into a nucleosome ladder on an agarose gel, transferred to a nylon membrane

and probed with nucleosome-length probes that contain the TG7 sequence or vector sequences directly adjacent to the TG7 insert. The result, shown in Figure 5, indicates that the TG repeat motif is incorporated into a particle of approximately mononucleosome size in both pRS425-TG7 and pRS425-TG3.2.3 (compare equally digested samples in lanes 3 and 11 of Fig. 5B). The region in the plasmid immediately adjacent to the inserted TG repeat is also included in a mononucleosome in both the plasmid containing the native TG7 repeat and the modified TG3.2.3 repeat (compare lanes 4 and 10 of Fig. 5C). In both cases, however, the resolution of the probed area into nucleosome multimers is poor. This is not due to imperfect transfer of the DNA to the nylon membrane, since if the blot shown in Figure 5C is stripped and re-probed with a fragment complementary to the *STE6* gene in the yeast genome a more extensive nucleosome ladder similar to that obtained with the ethidium bromide stained gel (Fig. 5A) is visible (Fig. 5D). The dispersed appearance of the nucleosome ladders in Figure 5B and C therefore suggests that the nucleosomes in the region of both the TG7 and TG3.2.3 repeats are not uniquely phased for a distance of even a few nucleosomes and that a range of linker lengths are present between nucleosomes resolved in this region in the population of DNA molecules. The appearance of both the native and modified TG sequence in a fragment of mononucleosome length is not inconsistent with the enhanced MNase cleavage in the central region of these sequences in chromatin seen in the indirect end-labeling experiment above: the combination of these two results suggests that nucleosomes may incorporate the flanking regions of the TG repeat motif, but are seldom placed over either the entire native or modified TG repeat.

Nucleosomal organization of the native and modified TG sequences at single nucleotide resolution

In order to obtain a more detailed view of the packaging of the TG sequences in chromatin, primer extensions of MNase- and DNase I-digested chromatin in yeast nuclei were performed. We also investigated the nucleosomal organization of the parental plasmid, pRS425, to address the possibility that the placement of the TG heptamer disrupted a strong positioning sequence in pRS425 at the point of insertion and that the remnant of this positioning sequence influences the local chromatin structure in the plasmid containing the native and modified TG repeats. However, neither the MNase- (lanes 5 and 6 of Fig. 6A) nor DNase I-digested (lanes 7 and 8 of Fig. 6A) chromatin show a protected area or cleavage modulation indicative of a stably positioned nucleosome. Similarly, MNase digestion of the plasmid containing the TG7 (Fig. 6B) or TG323 (Fig. 6C) insert does not show stably positioned nucleosomes. The absence of enhanced cleavage in the center of the TG motif, in contrast to that found for the double-stranded cleavages discussed above, is most likely due to differences in the rate of appearance of double-strand breaks as opposed to single-strand nicks.

The continuous accessibility of DNase I to the TG7 repeat in chromatin and free DNA confirms the absence of a positioned nucleosome incorporating the entire TG heptamer (lanes 7 and 8 of Fig. 6B). Interestingly, towards the *Pst*I side of the TG sequence a weak modulation at a period of ~10 bp (indicated by triangles to the right of Fig. 6B) is visible in the DNase I cleavage pattern in chromatin compared with free DNA (lanes 7 and 8 of Fig. 6B). This weak cleavage modulation, which rapidly disappears towards the center of the TG7 heptamer, is more distinct in Figure 7, where the extent of DNase I cleavage at each phosphodiester bond in free DNA is subtracted from the extent of cleavage of the corresponding bond in chromatin. This treatment compensates for the sequence specificity of DNase I and accentuates the effect of chromatin on the accessibility of the nuclease to the DNA. The difference in the extent of DNase I cleavage in pRS425-TG7 (Fig. 7, top plot) clearly shows a cleavage modulation at a period of ~10 bp, reflecting occlusion of the DNase I molecule from one side of the DNA helix in chromatin, most likely due to close association of the DNA with an octamer surface. The sites of preferential DNase I cleavage in chromatin, represented by the maxima in Figure 7, align within 1 nt with the hydroxyl radical cleavage maxima of a nucleosome core reconstituted onto a tandem repeat of the TG sequence *in vitro* (28). It is further clear from Figure 7 that the amplitude of this cleavage modulation diminishes towards the other end of the TG repeat motif. Since both the low resolution and single nucleotide resolution MNase digests of pRS425-TG7 in chromatin suggested the absence of a translationally positioned nucleosome in this region, the DNase I result is most likely due to translationally randomly placed nucleosomes becoming rotationally constrained when a sufficient length of the anisotropically flexible TG heptamer is associated with the octamer surface. The weak 10 bp period does not extend for more than one helical period beyond the *Pst*I side of the TG heptamer into the pRS425 sequences (lanes 7 and 8 of Fig. 6B), as expected for the superimposed signal of nucleosomes that incorporate little or no fraction of the TG heptamer and are randomly positioned in several rotational frames on the vector sequences.

A similar weak cleavage periodicity at ~10 bp is also detectable in the DNase I-digested chromatin of the TG heptamer containing a central 2 bp insert (Fig. 6C, lanes 7 and 8) and is readily visible in the plot of the difference in the extent of DNase I cleavage in chromatin compared with free DNA (Fig. 7, bottom plot). This cleavage modulation is again more pronounced on one side of the TG repeat, suggesting that only the flanking region of the TG heptameric sequence is incorporated into rotationally positioned nucleosomes. An identical result was obtained with the pRS425-TG3.1.3 plasmid, in which the central TG monomer contains a single inserted base pair (data not shown).

DISCUSSION

We have shown above that the introduction of 1 or 2 bp into the center of a 140 bp region composed of a tandemly repeated TG sequence is not sufficient to allow the stable positioning of a nucleosome on this sequence *in vivo*. This result demonstrates that the absence of a stably positioned nucleosome on this sequence *in vivo* is not exclusively due to a requirement for underwinding of the nucleosomal DNA at the pseudodyad while maintaining an overall bending preference compatible with the smooth winding of the DNA onto the octamer surface. The result further suggests that any favorable change in the free energy of nucleosome formation associated with introduction of the central 1 or 2 bp is not sufficient to compensate for an unfavorable energy change that appears to result in the absence of nucleosomes from the TG sequence *in vivo*.

The significant difference in the ability of a DNA sequence to form a nucleosome *in vitro* as opposed to *in vivo* is surprising, since it was previously shown that the histone octamer positioned

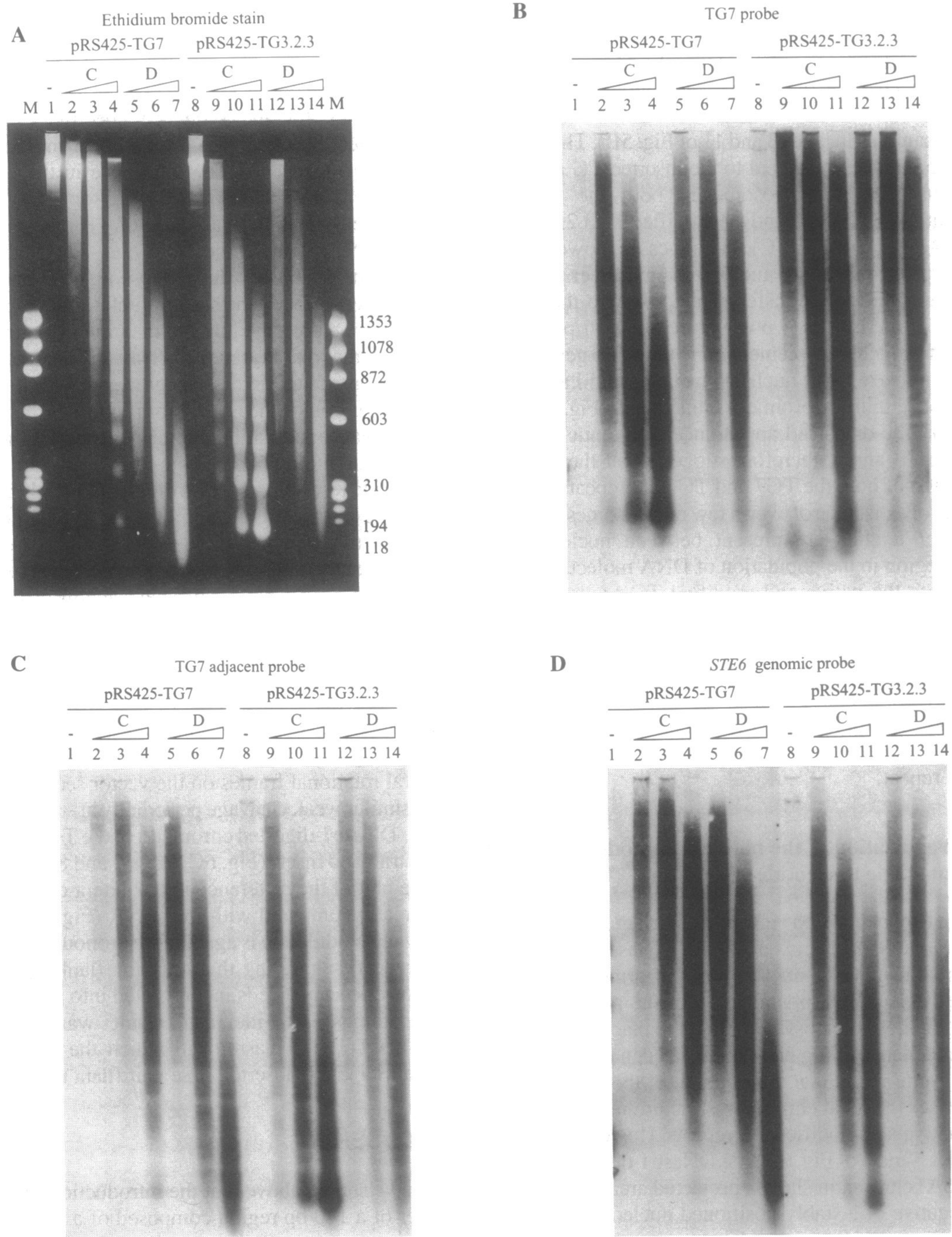

Figure 5. Incorporation of the native and modified TG repeat sequences and the adjacent plasmid sequence into nucleosomal DNA. The chromatin in permeabilized yeast spheroplasts (C, lanes 2–4 and 9–11) and purified DNA (D, lanes 5–7 and 12–14) from yeast transformed with pRS425-TG7 (lanes 1–7) or pRS425-TG3.2.3 (lanes 8–14) were digested with MNase, electrophoretically resolved on an agarose gel and the DNA transferred to a nylon membrane. Lanes 1 and 8 in each panel represent chromatin samples that had not been treated with MNase. (**A**) The ethidium bromide stained gel. The lanes labeled M represent *Hin*dIII-digested λ DNA size standards, with the size in base pairs of selected fragments indicated to the right of the panel. The transferred DNA, cross-linked to the nylon membrane, was hybridized with a 152 bp probe containing the TG7 heptameric repeat (**B**), a 162 bp probe containing the plasmid sequence adjacent to the TG7 heptameric repeat in pRS425-TG7 (**C**) and a 196 bp probe complementary to a region of the *STE6* gene in the yeast genome (**D**).

in similar frames *in vitro* and *in vivo* on a mouse satellite repeat unit (43), as well as on the sea urchin 5S DNA sequence (44). In the presence of a tandem repeat of the 5S DNA sequence, however, nucleosomes adopted different positions on the two repeats, demonstrating that although there is sufficient positioning information inherent in the DNA–histone interaction, additional factors are present *in vivo* that can affect the position of nucleosomes (44). Such factors may include: (i) the participation of the DNA molecule in processes such as transcription; (ii) the interaction of sequence-specific DNA binding proteins; (iii) the

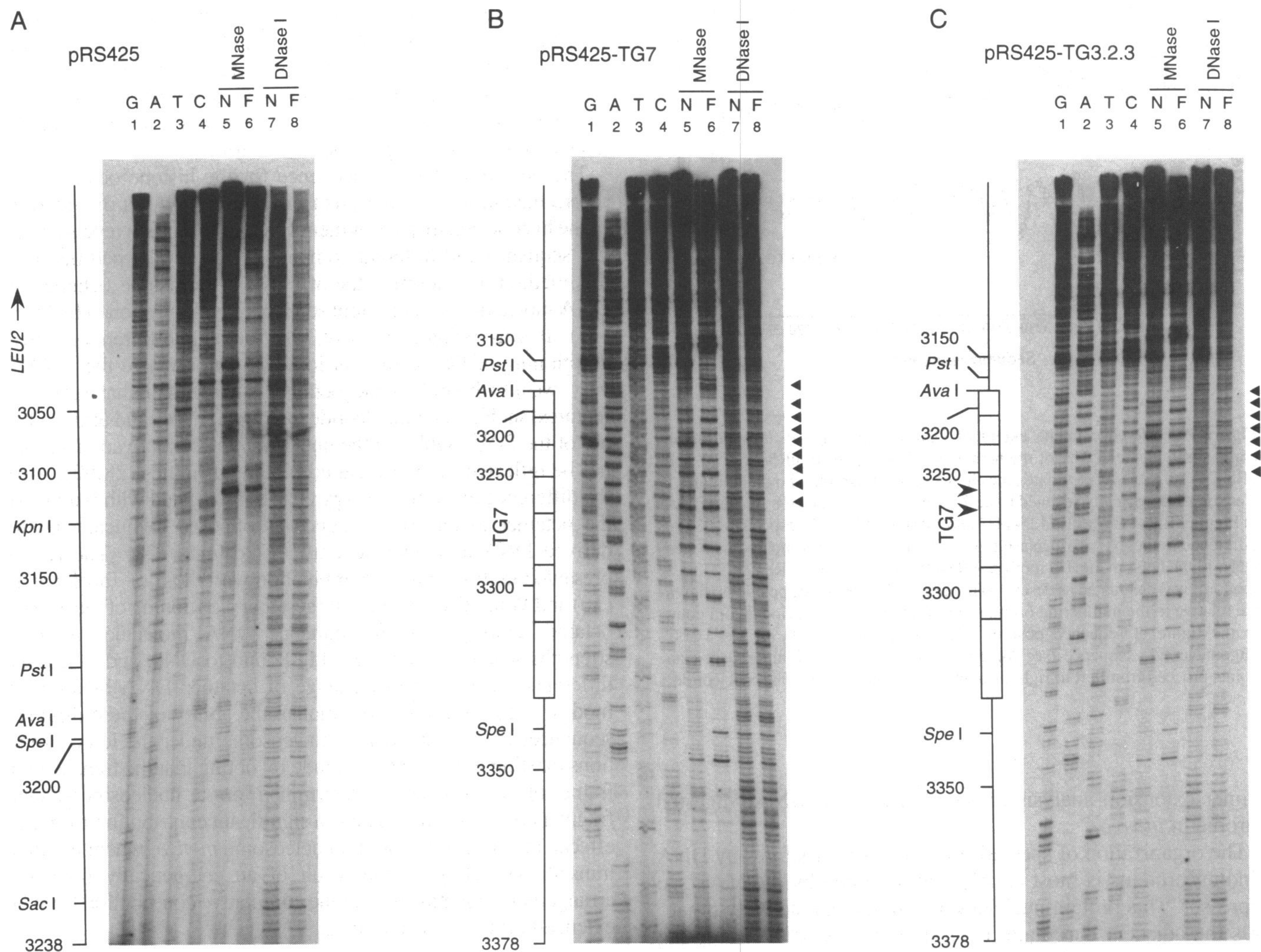

Figure 6. The chromatin structure of the native and modified TG repeats determined by primer extension. Nuclei were prepared from yeast strains containing pRS425 (**A**), pRS425-TG7 (**B**) or pRS425-TG3.2.3 (**C**). Chromatin in isolated nuclei (N, lanes 5 and 7) and purified free DNA (F, lanes 6 and 8) were subject to MNase (lanes 5 and 6) or DNase I (lanes 7 and 8) digestion, followed by extension of a radiolabeled primer complementary to a sequence in each plasmid. An autoradiograph of the primer extension products separated on a 6% polyacrylamide–8 M urea gel is shown. Sequencing standards are shown in lanes 1–4 for the appropriate plasmid in each panel. The sequence positions and the location of relevant restriction endonuclease sites are shown to the left of each panel. The location of the tandemly repeated TG sequence is indicated by the series of rectangles in (B) and (C) and the locations of the two inserted base pairs in the central TG monomer of the heptameric TG repeat are shown by the arrowheads in (C).

spatial organization of the large DNA molecule *in vivo*. Assuming that the binding affinity of the octamer to the TG sequence measured *in vitro* gives an accurate indication of the inherent ability of this sequence to accommodate nucleosomes, we may consider the possible involvement of each of these factors in turn in the absence of stably positioned nucleosomes on the TG sequences *in vivo*.

Considering the transcriptional activity first, we note that we have investigated the nucleosomal structure of the native and modified TG heptamer both upstream of the *LEU2* selectable marker in the pRS425 experiments reported above and downstream of the *TRP1* selectable marker in the pRS424ΔZ plasmid (data not shown). Identical results were obtained in the two cases, suggesting that the absence of a stably positioned nucleosome on the TG sequence is not due to the frequent passage of RNA polymerase II.

Sequence-specific DNA binding proteins can affect nucleosomal structure by either sterically excluding nucleosomes from the bound DNA sequence or by participating in the active organization of the chromatin in the surrounding region. Steric exclusion of nucleosomes by bound sequence-specific proteins, irrespective of the molecular path resulting in such a final state, is shown by the upstream regions of several transcriptionally active genes (45). However, it appears unlikely that a nucleosome is sterically excluded from both the native and modified TG sequences due to a bound sequence-specific protein, since a search of the TFD database (release 6.3; 46) did not reveal the presence of a single DNA recognition site within either sequence. Furthermore, no clear footprint or hypersensitive site consistent with the stable presence of a sequence-specific DNA binding protein on the native or modified TG sequences was evident in the

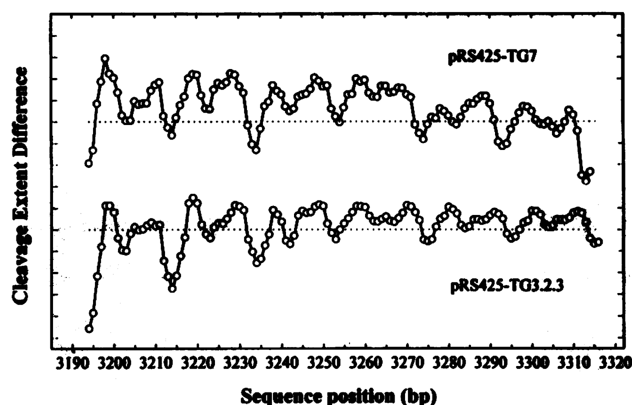

Figure 7. Difference in the extent of DNase I cleavage in the chromatin compared with free DNA of the heptameric TG regions in pRS425-TG7 and pRS425-TG3.2.3. Appropriate lanes in the autoradiographs shown in Figure 5 were densitometrically scanned (lane 7 for chromatin and lane 8 for free DNA in Figure 5B and C for pRS425-TG7 and pRS425-TG3.2.3, respectively). The difference in the extent of DNase I cleavage in chromatin compared with free DNA in the heptameric TG motif of pRS425-TG7 (top) and pRS425-TG3.2.3 (bottom) was calculated as described in the text. The sequence positions shown are relative to the numbering origin of plasmid pRS425. The first datum point in the figure at sequence position 3194 corresponds to T_7 in the TG0C oligonucleotide sequence (see Materials and Methods) and subsequent data points proceed from left to right through the sequence of the oligonucleotide.

primer extension analysis of the DNase I- or MNase-digested chromatin *in vivo*.

The organization of specialized chromatin structures by DNA binding proteins is most clearly demonstrated by the MATα2p repressor. This protein nucleates a complex that actively organizes the adjacent chromatin (47), irrespective of the bordering DNA sequence (33). In the experiments reported above, however, there was no evidence of any coherent nucleosomal organization enclosing the native or modified TG sequences nor in the corresponding region of the parental plasmid. This suggests that the absence of nucleosomes on the TG sequences *in vivo* is not due to active organization of the chromatin in the immediate surroundings of the TG sequence. The absence of stably positioned nucleosomes partially overlapping the edges of the TG sequence further suggests that nucleosomes are not sterically excluded from this sequence due to the tight placement of bordering nucleosomes.

It was previously shown *in vivo* that insertions in one region of a yeast plasmid influenced the positioning of nucleosomes in a distal region by a mechanism that was suggested to involve chromatin folding (48). However, this phenomenon was confined to only one region of the investigated plasmid molecule (48). Since the TG heptamer is not incorporated into a nucleosome at either of two different positions in pRS425 and pRS424ΔZ, we suggest that the exclusion of a nucleosome from the TG sequence by a similar mechanism is unlikely. The presence of the TG sequence on a circular DNA molecule and the accompanying topological constraint is similarly not expected to influence the positioning properties of the TG sequence, since it was previously shown that unrestrained negative superhelical stress equivalent to a specific linking difference (see 12) of –0.04 did not influence the placement of a positioned nucleosome *in vitro*.

It therefore appears that careful consideration of factors known to influence nucleosome placement does not provide a reasonable explanation for the absence of stably positioned nucleosomes on the TG heptamer *in vivo*. An alternative that must be considered involves identification of the TG heptamer as a favorable nucleosome positioning sequence *in vitro*.

The affinity of the TG sequence for the histone octamer was determined *in vitro* by dialysis from high to low salt of a mixture of the histone octamer, a fragment containing tandem repeats of the TG sequence and different competitor DNAs. The pertinence of this method to determine the affinity of the histone octamer for DNA molecules with different sequences was questioned by Drew (49). It was pointed out (49) that binding differences reflect partitioning of the octamer at ionic strengths where the DNA is very weakly bound to the octamer surface and that structural features, such as the dinucleotide distribution (14) and underwinding of the DNA duplex at the nucleosomal pseudodyad (10), may not be reflected in the measured binding affinities (28,50). Also, the difference in the free energy change associated with binding of the octamer to the TG sequence as opposed to nucleosomally derived DNA amounted to only ~3 kcal/mol (28). The results presented in this study, in combination with those of Tanaka *et al.* (30) and Wallrath *et al.* (31), suggest that partitioning of the histone octamer among different sequence DNA molecules at ionic strengths where it is only weakly bound to DNA and where the differences in free energy change of octamer–DNA binding are modest does not accurately emulate the ability of these different sequences to accommodate a nucleosome under the ionic conditions existing *in vivo*. We conclude by noting that the translational frame of a nucleosome determined on a nucleosome-length fragment *in vitro* will also have a significant contribution from end effects, in contrast to that of a nucleosome *in vivo*, where the final translational setting is the result of an energetically favorable arrangement that may involve the cumulative contribution from an extended array of nucleosomes.

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