Destabilization of nucleosomes by an unusual DNA conformation adopted by poly(dA)·poly(dT) tracts *in vivo*

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Poly(dA)·poly(dT) tracts are common and often found upstream of genes in eukaryotes. It has been suggested that poly(dA)·poly(dT) promotes transcription *in vivo* by affecting nucleosome formation. On the other hand, *in vitro* studies show that poly(dA)·poly(dT) can be easily incorporated into nucleosomes. Therefore, the roles of these tracts in nucleosome organization *in vivo* remain to be established. We have developed an assay system that can evaluate nucleosome formation in yeast cells, and demonstrated that relatively longer tracts such as $A_{15}TATA_{16}$ and A_{34} disrupt an array of positioned nucleosomes, whereas a shorter A_5TATA_4 tract is incorporated in positioned nucleosomes of yeast minichromosomes. Thus, nucleosomes are destabilized by poly(dA)·poly(dT) *in vivo* in a length-dependent manner. Furthermore, *in vivo* UV footprinting revealed that the longer tracts adopt an unusual DNA structure in yeast cells that corresponds to the *B′* conformation described *in vitro*. Our results support a mechanism in which a unique poly(dA)·poly(dT) conformation presets chromatin structure to which transcription factors are accessible.

Keywords: chromatin/DNA structure/nucleosome positioning/poly(dA)·poly(dT)/yeast

Introduction

Gene expression in eukaryotic cells involves the interaction of proteins with DNA packaged into nucleosomes; the primary structural units of chromatin are composed of a histone octamer and 145 bp DNA. Since the nucleosome limits the access of *trans*-acting factors to their cognate DNA sites, it is widely accepted that the nucleosome acts as a barrier to transcription. Thus, an alteration of chromatin structure is an essential step for the initiation of transcription. Recent genetic and biochemical studies have shown that chromatin structure is altered by several mechanisms, including chromatin remodeling complexes and the acetylation–deacetylation of histones in living cells (for reviews see Kadonaga, 1998; Kornberg and Lorch, 1999; Travers, 1999; Wolffe and Hayes, 1999).

Nucleosomes are often located at distinct positions in promoter regions and close to *cis*-acting DNA elements. Nucleosome positioning appears to be influenced by a combination of several factors, including histone–DNA interaction, anisotropy of the DNA structure, boundary formation, chromatin folding and interaction of *trans*-acting factors with histones (for reviews see Simpson, 1991; Thoma, 1992). Since DNA structures are considered to act as determinants of nucleosome organization, the effects of DNA sequences on nucleosome formation have been extensively studied using *in vitro* reconstitution from purified histone octamers and DNA such as synthetic polymers, restriction fragments and plasmids. For example, earlier studies found that synthetic homopolymers such as poly(dA)·poly(dT) and poly(dG)·poly(dC) have inherent resistance to nucleosome formation (Rhodes, 1979; Simpson and Kunzler, 1979; Kunkel and Martinson, 1981). Since homopolymeric $A_n·T_n$ sequences are common in most eukaryotes and are often found in promoters (Struhl, 1985; Schlapp and Rodel, 1990; Thiry-Blaise and Loppes, 1990; Karlin *et al*., 1993; Behe, 1995), the influence of A_n tracts on nucleosome organization has been studied in detail by several investigators.

In agreement with the earlier observations, a $T_{14}A_{11}$ tract blocks nucleosome formation over the human NF1-Alu element *in vitro* (Englander and Howard, 1996). On the other hand, Losa *et al*. (1990) found that an $A_n·T_n$ tract, $T_3CCT_6CT_5GCT_5CT_7$, is located in a nucleosome-free region in the yeast *DED1* promoter *in vivo*, whereas it can be incorporated in the nucleosome core *in vitro*. Similarly, human genomic clones containing a longer A_n tract (31, 41 and 69 bp in length) with other mixed sequences readily wrap around histone octamers *in vitro* (Fox, 1992). Other studies also revealed that poly(dA)·poly(dT) sequences can be reconstituted into nucleosomes *in vitro* (Hayes *et al*., 1991; Puhl *et al*., 1991; Schieferstein and Thoma, 1996; Brown and Fox, 1998) and that the free energy of nucleosome formation is virtually identical to that of the nucleosome with a heterologous sequence DNA (Puhl *et al*., 1991). Furthermore, poly(dA)·poly(dT) forms nucleosomes more strongly as the temperature is increased (Puhl and Behe, 1995; Mahloogi and Behe, 1997), suggesting that the DNA conformation adopted at elevated temperatures facilitates nucleosome formation. Thus, the formation and stability of nucleosomes in poly(dA)·poly(dT) tracts remain to be elucidated.

Iyer and Struhl (1995) showed that an A_n tract of 17 bp or more in the yeast *HIS3* promoter stimulates transcription by improving accessibility to the promoters *in vivo*, suggesting that A_n tracts affect local nucleosome formation. Consistent with the above results, Filetici *et al*. (1998) reported that the *HIS3* upstream region containing the A_n tract (T_4CAT_{11}) and a Gcn4p binding site is nucleosome-free. Thiele and his colleagues (Zhu and Thiele, 1996; Koch and Thiele, 1999) showed that a naturally occurring A_{16} element in the *Candida glabrata*

AMT1 gene distorts the nucleosomal DNA within a positioned nucleosome to facilitate transcription factor binding *in vivo*. These studies shed light on the functional importance of poly(dA)·poly(dT) in chromatin structure, but the roles of DNA structures in nucleosome organization *in vivo* are not yet well understood.

We have developed an assay system with which to examine the effects of DNA sequences on the formation and organization of nucleosomes in yeast minichromosomes *in vivo*. We found that longer $A_n \cdot T_n$ sequences disrupt an array of positioned nucleosomes, but shorter sequences are included. Furthermore, *in vivo* UV photo-footprinting revealed that the longer $A_n \cdot T_n$ sequences adopt an unusual DNA conformation in yeast cells, supporting the notion that a unique DNA structure prevents wrapping around histone octamers *in vivo*.

Results

Experimental design

Previous studies indicate that the yeast α2/Mcm1p precisely positions nucleosomes adjacent to the α2 operator in genomic **a**-specific genes as well as in minichromosomes (Roth *et al.*, 1990, 1992; Shimizu *et al.*, 1991; Ganter *et al.*, 1993), together with co-repressors Ssn6p-Tup1p (Cooper *et al.*, 1994). Tup1p directly interacts with deacetylated histones H3 and H4 (Edmondson *et al.*, 1996), indicating that the mechanism of the positioning involves interaction of the multiple repressor complex with histones.

Roth *et al.* (1990) constructed the TALS plasmid by inserting 356 bp of the *STE6* promoter, including the α2 operator, into the *Eco*RI site of the TRP1/ARS1 plasmid. Figures 1 and 2 show the chromatin structure of the TALS minichromosomes and DNA inserts used in this study. The α2 operator is located in the region of 1535–1565 m.u. (map units). Figure 2 indicates that the main location of nucleosome IV is between 1376 and 1522 m.u. and that the pseudo-dyad axis is mapped at 1448 m.u., as determined by high resolution mapping of micrococcal nuclease (MNase) cleavage sites (Shimizu *et al.*, 1991; Kladde and Simpson, 1994). The *Sac*I site is located at 1460 m.u., corresponding to 63 bp from the edge of nucleosome IV at 1522 m.u., whereas the *Dra*III site at 1412 m.u. corresponds to 111 bp from the edge. Thus, Figure 2 shows that when DNA sequences were inserted into the *Sac*I site, the inserts lay around the dyad of nucleosome IV (pOM801, -802, pTS711, -713 and -703). On the other hand, when a sequence was inserted into the *Dra*III, it lay proximal to the linker region between nucleosomes III and IV (pTS704). These TALS derivatives were introduced into yeast α cells to assemble chromatin *in vivo*. The chromatin structure of the minichromosomes was examined by limited digestion of the isolated nuclei with MNase, which preferentially cuts the linker DNA region between neighboring nucleosomes.

High resolution mapping of MNase cleavage sites in the nucleosome IV region

Figure 3 shows high resolution mapping of MNase cleavage sites in the region of nucleosome IV in TALS and their derivative minichromosomes. In the TALS chromatin, a region of ~140 bp adjacent to the α2 operator
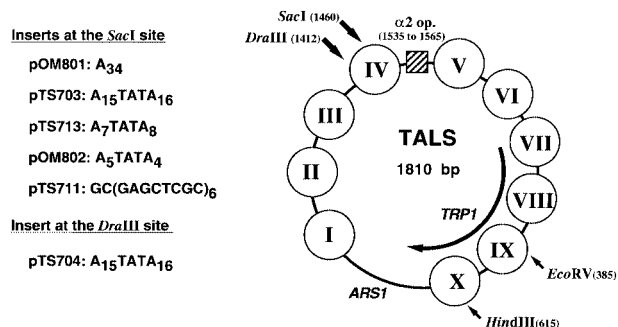

Fig. 1. Chromatin structure of TALS minichromosomes in yeast α cells and DNA inserts used in this study. TALS contains the α2 operator (indicated by the hatched box) from the *STE6* promoter at the *Eco*RI site of TRP1/ARS1 plasmid, and the location of nucleosomes in TALS was determined previously (Roth *et al.*, 1990). DNA inserts were cloned into the *Sac*I site of TALS, corresponding to near the pseudo-dyad axis of nucleosome IV, or into the *Dra*III site corresponding to the linker region between nucleosomes III and IV.
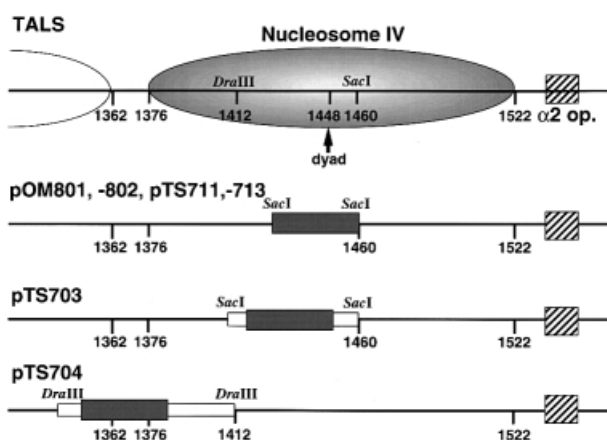

Fig. 2. Locations of the positioned nucleosome IV, α2 operator and poly(dA)·poly(dT) inserts in TALS and its derivative minichromosomes as described (Shimizu *et al.*, 1991; Kladde and Simpson, 1994). The *Sac*I and *Dra*III sites are at 1460 and 1412 m.u. (map units), respectively, in nucleosome IV as shown by the shaded ellipse. The α2 operator and poly(dA)·poly(dT) tracts are shown as hatched and shaded boxes, respectively. The polylinker attached with the $A_{15}TATA_{16}$ insert is indicated by white boxes (see Materials and methods).

(shown by a bracket in Figure 3, lanes 1 and 3) is largely protected against MNase digestion, compared with the digestion of naked DNA (lane 2), confirming the precise translational positioning of nucleosome IV (Shimizu *et al.*, 1991).

A mixed sequence, $GC(GAGCTCGC)_6$ (50 bp), was inserted at the *Sac*I site, near the pseudo-dyad axis of nucleosome IV (pTS711) for a control sequence. Figure 3 (lanes 16–18) shows that the nucleosome IV region in pTS711 chromatin was protected from MNase digestion, and that the MNase cleavage profile is very similar to that of vector TALS chromatin (lanes 1–3). We also analyzed the effect of $(CA)_{17} \cdot (TG)_{17}$ (34 bp) insertion at the *Sac*I site and found no alterations in the nucleosome positioning (data not shown). These results indicate that DNA insertions *per se* do not affect nucleosome formation. This is consistent with the fact that the nucleosomes are positioned independently of DNA sequences flanked by
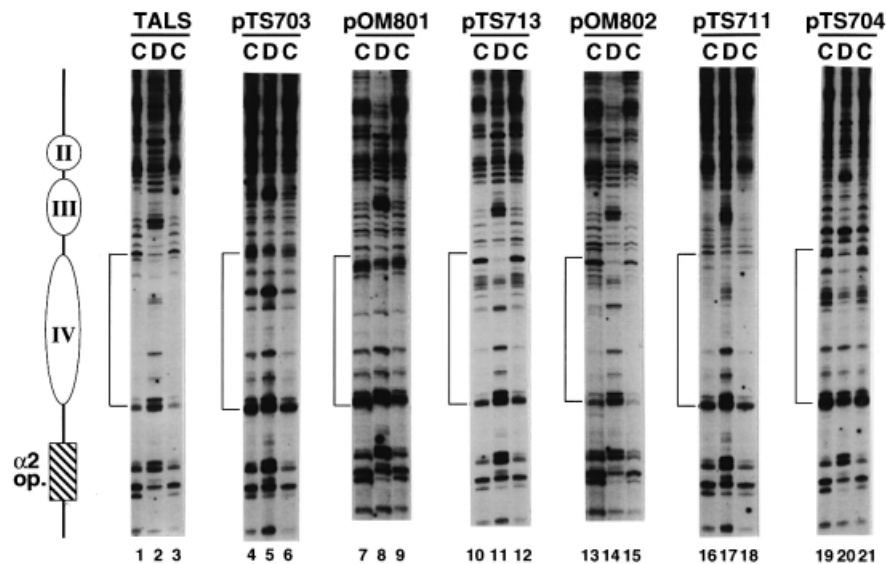
Fig. 3. Primer extension analysis of MNase cleavage sites in the nucleosome IV region of TALS and its derivative minichromosomes. Plasmids pTS703, pOM801, pTS713, pOM802 and pTS711 contain $A_{15}TATA_{16}$, A_{34}, A_7TATA_8, A_5TATA_4 and $GC(GAGCTCGC)_6$, respectively, at the *Sac*I site of TALS. Plasmid pTS704 contains $A_{15}TATA_{16}$ at the *Dra*III site. Chromatin (in isolated nuclei) (lanes labeled C) and naked DNA (lanes labeled D) were digested at 37°C for 10 min with various concentrations of micrococcal nuclease as follows: 2.5 U/ml (lanes 1, 4, 7, 10, 13, 16 and 19), 1.25 U/ml (lanes 3, 6, 9, 12,15,18 and 21) and 0.05 U/ml (lanes 2, 5, 8, 11, 14, 17 and 20), as described (Shimizu *et al.*, 1991). The nucleosome IV regions are shown on the left of each gel by brackets. The locations of nucleosomes II to IV and the α2 operator are schematically shown to the left of the gel.

the α2 operator. At least seven such examples of precise positioning of nucleosomes adjacent to the α2 operator have been reported: three **a**-cell-specific genes and four minichromosomes (Roth *et al.*, 1990; Simpson, 1990; Shimizu *et al.*, 1991; Ganter *et al.*, 1993; Simpson *et al.*, 1993; Patterton and Simpson, 1994).

In contrast to the control insert, relatively longer tracts such as $A_{15}TATA_{16}$ and A_{34} (pTS703 and pOM801, respectively) remarkably affected nucleosome formation. That is, the nucleosome IV region became hypersensitive to MNase and the cleavage profile of chromatin was the same as that of naked DNA (Figure 3, lanes 4–6 and 7–9, respectively). This demonstrated that the insertion of longer $A_n \cdot T_n$ tracts disrupts nucleosome positioning by the α2 repressor *in vivo*. The MNase cleavage sites in the nucleosome IV region of pTS713 chromatin containing the A_7TATA_8 insert were partially protected, and the region near the tract became more accessible to MNase (Figure 3, lanes 10–12), compared with the vector TALS or the control pTS711. On the other hand, a shorter A_5TATA_4 sequence that matched the ARS (autonomously replicating sequence) consensus sequence (ACS) in *Saccharomyces cerevisiae* was incorporated into nucleosome IV (pOM802; Figure 3, lanes 13–15). These results demonstrate that $A_n \cdot T_n$ tracts disrupt or distort the nearby nucleosome structures and that a certain length is required to disrupt nucleosome positioning.

Furthermore, we examined the effect of the location of the destabilizing sequence ($A_{15}TATA_{16}$) on nucleosome formation by inserting the sequence into the *Dra*III site (pTS704; Figure 2) to cover the region corresponding to the linker between nucleosomes III and IV. High resolution mapping of pTS704 (Figure 3, lanes 19–21) revealed similar cleavage profiles between naked DNA and chromatin samples in the region of nucleosomes III

and IV, indicating that nucleosome positioning is disrupted by the insert. This result implies that longer A_n tracts inserted in the linker region as well as in the center of the nucleosome prevent wrapping of histone octamers.

Nucleosome positioning in the minichromosomes analyzed by indirect end-labeling

The chromatin structures of minichromosomes were also analyzed by indirect end-labeling and the results are shown in Figure 4. For the vector TALS, the four MNase cleavage sites (indicated by *a, *b, *c and *d) in the naked DNA (Figure 4, lane 2) were protected in the chromatin sample (lane 1). Also, the cleavage sites with equal spacing (140–150 bp) in TALS chromatin were enhanced (Figure 4, lane 1). This result is in good agreement with published reports (Roth *et al.*, 1990, 1992), confirming that the TALS chromatin is assembled with precisely and stably positioned nucleosomes. The same profile of MNase cleavage sites was obtained for the pTS711 minichromosomes containing the control sequence $GC(GAGCTCGC)_6$ (Figure 4, lane 11), indicating that DNA insertion *per se* does not affect nucleosome positioning in the minichromosomes.

In contrast, the two bands (*a and *d) that were protected in TALS chromatin were evident in the pTS703 chromatin ($A_{15}TATA_{16}$) (Figure 4, compare lanes 1 and 2 with lanes 3 and 4). Furthermore, the discrete bands in TALS chromatin that corresponded to the linker regions between nucleosomes II and IV became broader or disappeared in pTS703 chromatin (Figure 4, lane 3). Similarly, the band *a was present in pOM801 chromatin (A_{34}; Figure 4, lane 5) and the bands corresponding to the linker regions between nucleosomes II and IV were broader or lost. These results indicate that nucleosome positioning is disrupted by the longer inserts, not only in
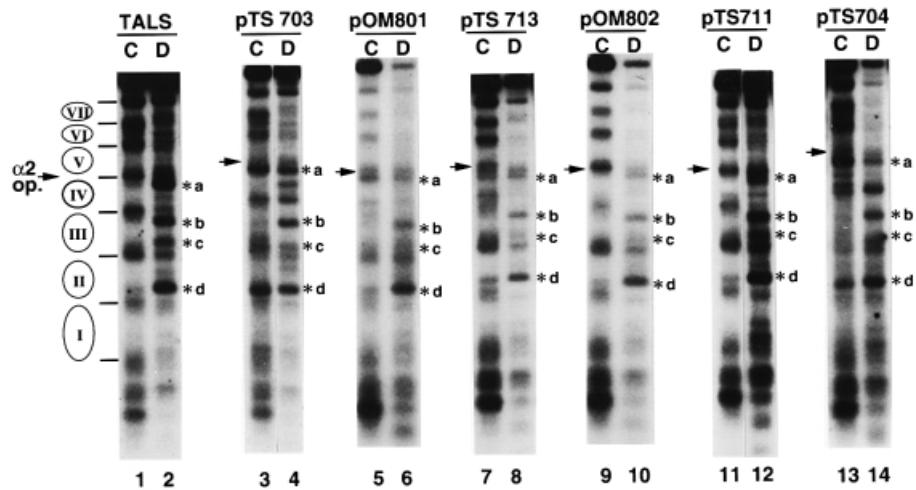
Fig. 4. Chromatin structure of TALS and its derivatives analyzed by indirect end-labeling. Plasmids pTS703, pOM801, pTS713, pOM802 and pTS711 contain $A_{15}TATA_{16}$, A_{34}, A_7TATA_8, A_5TATA_4 and $GC(GAGCTCGC)_6$, respectively, at the *Sac*I site of TALS. Plasmid pTS704 contains $A_{15}TATA_{16}$ at the *Dra*III site. Chromatin (in isolated nuclei) (lanes labeled C) and naked DNA (lanes labeled D) were digested at 37°C for 10 min with micrococcal nuclease as follows: 2.5 U/ml (odd-numbered lanes) and 0.05 U/ml (even-numbered lanes). The samples were digested with *Eco*RV and resolved by electrophoresis on 1.2% agarose gel, then the MNase cleavage sites were detected by indirect end-labeling using the *Eco*RV–*Hin*dIII fragment (385–615 m.u. in TALS; see Figure 1) as a probe (Roth *et al.*, 1990). The locations of nucleosomes I to VII and the α2 operator are shown to the left of the gel. The four cleavage sites of TALS in naked DNA, but not in chromatin, are indicated by *a, *b, *c and *d.

nucleosome IV but also in nucleosomes II and III. For pTS713 containing an A_7TATA_8 insert (Figure 4, lanes 7 and 8), the MNase cleavage pattern of chromatin sample was similar to that of TALS chromatin, although the characteristic band *a in naked DNA was detected to some extent in the chromatin sample, and the band corresponding to the linker regions between positioned nucleosomes III and IV was broader than that in TALS chromatin. This implies that nucleosome positioning in pTS713 chromatin is less stable than that in TALS chromatin.

The MNase cleavage profile of the minichromosomes containing A_5TATA_4 (pOM802; Figure 4, lane 9) was similar to that of vector TALS (lane 1) and the control pTS711 (lane 11), indicating that the shorter tract did not significantly affect the formation of positioned nucleosomes.

The effect of inserting $A_{15}TATA_{16}$ into the linker DNA region (pTS704; Figure 4, lanes 13 and 14) was also analyzed by indirect end-labeling. The cleavage profile of pTS704 chromatin was quite different from that of TALS chromatin (Figure 4, compare lanes 1 and 13). Two characteristic bands, *a and *d, were clear, while no obvious MNase cut sites were observed in the region containing bands *b and *c in pTS704 chromatin (Figure 4, lane 13). Thus, an insertion into the linker region also disrupted the positioning of nucleosomes II to IV in the minichromosomes. These results are consistent with those of the high resolution mapping described above.

Nucleosome organization in minichromosomes analyzed by nucleosome repeat assay

We further examined whether or not the regularity of the nucleosome array is disrupted by the A_n·T_n tracts using the nucleosome repeat assay (Southern blots of nucleosome ladders produced by MNase digestion followed by hybridization with a unique oligonucleotide probe).

Figure 5A shows the results of nucleosome repeat analysis detected using a proximal probe with a complementary sequence (1502–1473 m.u.) flanked by the inserts in the nucleosome IV region. We examined MNase digests corresponding to the mono-nucleosome up to the hexanucleosomes, as observed for TALS chromatin (Figure 5A, lanes 1 and 2). The nucleosome ladders of pTS711 and pOM802, which contain the control $GC(GAGCTCGC)_6$ insert and the shorter A_5TATA_4 tract, respectively (Figure 5A, lanes 9–12), were similar to that of the vector TALS (Figure 5A, lanes 1 and 2). Thus, this result indicates that these inserts were incorporated into the nucleosome array with regular spacing.

Nucleosome ladders for more than tri-nucleosomes were not clear for the pTS713 (A_7TATA_8) chromatin (Figure 5A, lanes 7 and 8), indicating less periodicity of nucleosome spacing compared with the vector TALS. Furthermore, the ladders became much more smeared for pTS703 ($A_{15}TATA_{16}$) and pOM801 (A_{34}) (Figure 5A, lanes 3–6), indicating that the nucleosome IV regions near the inserts completely lost spacing periodicity. This implies that the regularity of the nucleosome array is disrupted by the longer A_n inserts.

After stripping the proximal probe off, the blots were rehybridized with a distal probe that has a sequence complementary to a region within nucleosome IX (409–445 m.u.) (Figure 5B). Figure 5B shows that ladders from the minichromosomes appeared nearly identical to the canonical nucleosome ladders, demonstrating that the perturbation of nucleosome organization does not propagate into the distal region.

Unusual conformation of A_n·T_n tracts detected by in vivo UV photofootprinting

Poly(dA)·poly(dT) adopts at least two double-helical conformations (e.g. Premilat and Albiser, 1997), depending on conditions such as temperature and relative
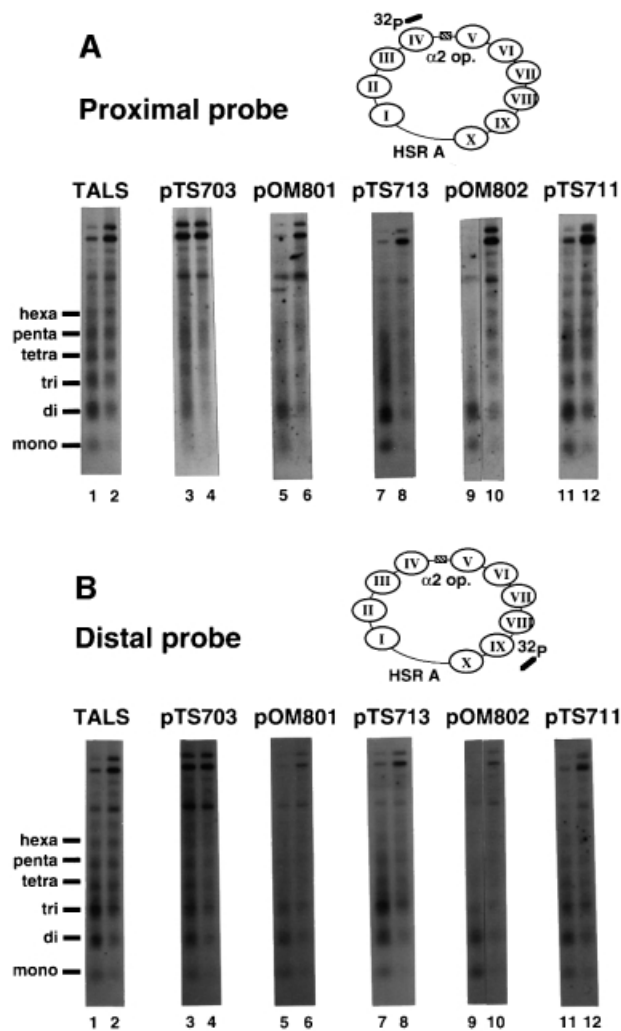
Fig. 5. Nucleosome repeat analysis of TALS and its derivative minichromosomes. Plasmids pTS703, pOM801, pTS713, pOM802 and pTS711 contain $A_{15}TATA_{16}$, A_{34}, A_7TATA_8, A_5TATA_4 and $GC(GAGCTCGC)_6$, respectively, at the *Sac*I site of TALS. Chromatin (in isolated nuclei) was digested at 37°C for 10 min with micrococcal nuclease: 2.5 U/ml (odd-numbered lanes) and 1.25 U/ml (even-numbered lanes). The MNase digest samples were resolved by 1.3% agarose gel electrophoresis. (**A**) Southern blots of MNase-digested chromatin were probed using a ^{32}P-labeled oligonucleotide with a complementary sequence adjacent to the *Sac*I site in nucleosome IV (proximal probe, top of panel; see Materials and methods). (**B**) After stripping the proximal probe off, the blots were re-probed using a ^{32}P-labeled oligonucleotide with a sequence complementary to the nucleosome IX region (distal probe, top of panel; see Materials and methods).

Fig. 6. *In vivo* UV photofootprinting of the T_{34} and $T_{16}ATAT_{15}$ strands in pOM801 and pTS703 minichromosomes. Intact cells (lanes labeled C) and naked DNA (lanes labeled D) were irradiated at 254 nm with different UV dosages as follows: 500 mJ (lanes 1, 4, 7 and 10), 750 mJ (lanes 3, 6, 9 and 12) and 60 mJ (lanes 2, 5, 8 and 11), as described (Shimizu *et al.*, 1997, 1998). The nucleosome IV region and the α2 operator are shown by brackets on the left of the gel. The sequences of T_{34} and $T_{16}ATAT_{15}$ tracts and the α2 operator are shown to the side of gel. + indicates the sites protected from UV irradiation in cells.

humidity. Lyamichev (1991) reported that the structural transition of $A_n \cdot T_n$ tracts from an unusual structure to a structure close to *B*-DNA can be detected *in vitro* by UV photofootprinting as a function of temperature. Thus, we used *in vivo* UV footprinting to determine whether or not the $A_n \cdot T_n$ tracts in minichromosomes adopt the same structure as that *in vitro*. Intact yeast cells harboring the TALS derivatives were irradiated with UV light at 254 nm, and thymine dimerization at $A_n \cdot T_n$ tracts was analyzed by primer extension mapping as described (Axelrod and Majors, 1989; Murphy *et al.*, 1993; Shimizu *et al.*, 1997, 1998).

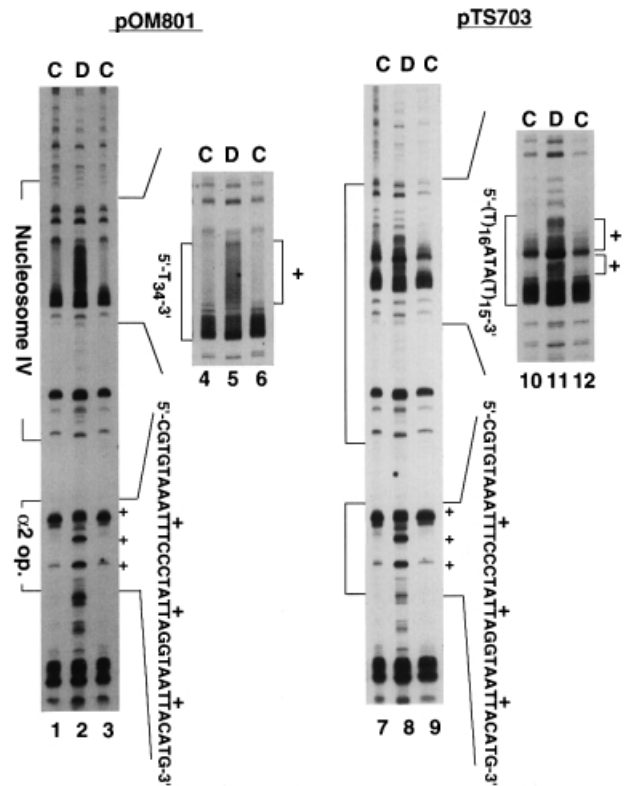Figure 6 shows the *in vivo* UV photofootprints of inserts A_{34} (pOM801) and $A_{15}TATA_{16}$ (pTS703). Eight to ten thymine residues from the 3′-end exhibited high photo-reactivity in the T_{34} tract, whereas thymine bases in the other 2/3 region of the tract were protected in pOM801 chromatin (Figure 6, lanes 1, 3, 4 and 6). Similarly, the photoreactivity of naked DNA decreased gradually from the middle of the tract to the 5′-end, but the degree of protection was much weaker (Figure 6, lanes 2 and 5) compared with samples of intact cells (lanes 1, 3, 4 and 6). The footprints were also similar for the $A_{15}TATA_{16}$ insert, as seen in lanes 7–12 of Figure 6. Yields of thymine dimer formation were maximal at regions near the 3′-end of both T_{16} and T_{15} tracts in the $T_{16}ATAT_{15}$ insert, whereas the 5′-half of each of the T_{16} and T_{15} tracts was protected. Thus, the ATA sequence at the center interrupted the protection associated with the T_n stretch. The UV foot-prints in the yeast cells were in good agreement with the findings of Lyamichev (1991): the yield of thymine dimer formation is maximal at the 3′-ends of the T_n tracts, whereas the photoreactivity of thymine residues is repressed at the 5′-sides. Thus, A_{34} and $A_{15}TATA_{16}$ tracts predominantly form an unusual conformation in yeast cells. The unique protection at the 5′-side was not observed *in vivo* for a shorter insert, T_4ATAT_5 in pOM802 (data not shown), indicating that a certain length of $A_n \cdot T_n$ tract is required to stabilize the unusual conformation.

It should be noted that the pyrimidine dimer formation was also protected in the $\alpha2$ operator in intact cells, and its profile was identical to the *in vivo* UV footprints of $\alpha2$/Mcm1p binding (Murphy *et al*., 1993), indicating that $\alpha2$/Mcm1p proteins are present in these minichromosomes. Thus, destablization of nucleosomes by A_n tracts is not due to the loss of $\alpha2$/Mcm1p binding. In other words, nucleosomes cannot be positioned near A_n tracts, even in the presence of $\alpha2$/Mcm1p.

Discussion

We described an assay system that can evaluate nucleosome formation *in vivo* using well defined yeast minichromosomes (Roth *et al*., 1990; Shimizu *et al*., 1991). Similar approaches have examined the effects of transcription factor binding on nucleosomal DNA (Morse *et al*., 1992; Morse, 1993). The key advantages in this system are as follows. The effect of inserts at specific locations, either at the center or at linker regions in nucleosomes can be examined, and the constructed plasmids are physiologically and naturally assembled into chromatin structures in living yeast cells. Thus, potential artifacts of chromatin assembly arising from reconstitution conditions such as high concentrations of salts and/or urea etc. can be eliminated. We used this system to show the effects of poly(dA)·poly(dT) sequences on the formation of positioned nucleosome *in vivo*.

We examined the chromatin structures of minichromosomes using three different assays: (i) primer extension mapping of positioned nucleosome IV at base-pair resolution; (ii) nucleosome locations in minichromosomes by indirect end-labeling; and (iii) nucleosome spacing by nucleosome repeat analysis. These results were all consistent in terms of the effects of $A_n \cdot T_n$ tracts on nucleosome organization, and led to the following conclusions. Relatively longer tracts such as $A_{15}TATA_{16}$ and A_{34} excluded the positioned nucleosome and a moderate length of tract, A_7TATA_8, was incorporated into a positioned nucleosome, but the nucleosomal DNA near the insert appeared to become more accessible to MNase compared with the control minichromosomes. Finally, a shorter tract (A_5TATA_4) as well as a mixed control sequence [GC(GAGCTCGC)$_6$] did not affect the formation of positioned nucleosomes. Because *in vivo* UV footprints indicated occupancy of the $\alpha2$ operator, the changes of chromatin structure are not due to the loss of $\alpha2$/Mcm1p binding in these minichromosomes.

Nucleosome exclusion by longer $A_n \cdot T_n$ tracts would be accounted for by occupancy of the tracts by a specific DNA binding protein, or by a non-canonical DNA structure that inhibits histone binding. In *S.cerevisiae*, ARS1 lies on the nucleosome-free region between two positioned nucleosomes in yeast chromosome IV (Diffley and Cocker, 1992) as well as in TRP1/ARS1 minichromosomes (Thoma *et al*., 1984; Thoma, 1986; see also Figure 1). The ACS is a 12 bp poly(dA)·poly(dT)-rich sequence [5'-(T/A)TTTA(C/T)(A/G)TTT(T/A)(T/C/G)-3'] and the ORC (origin recognition complex) binds to the ACS (for review see Rowley *et al*., 1994). However, we showed that the inserted ACS is incorporated into the positioned nucleosome in pOM802 chromatin. Thus, ACS alone does not establish a nucleosome-free region. Another

candidate is Dat1p (or Datin protein), the only known yeast poly(dA)·poly(dT) binding protein, which recognizes at least consecutive $A_n \cdot T_n$ tracts of 10 bp and functions as a repressor (Winter and Varshavsky, 1989; Reardon *et al*., 1995). However, our *in vivo* UV footprints argue against this notion. The footprints for $A_{15}TATA_{16}$ and A_{34} indicated enhanced thymine dimer formation in the region near the 3'-end of the T_n tracts and protection in the other 5'-half. This asymmetry is the same as that of an unusual conformation of T_n tract at lower temperatures *in vitro*, as shown by Lyamichev (1991). Also, Iyer and Struhl (1995) reported that dA·dT tracts stimulate transcription from the yeast *HIS3* promoter, and that several lines of evidence argue against the idea that specific proteins binding to poly(dA)·poly(dT) play a positive role in transcription due to increasing accessibility to promoters. Taken together, we conclude that the unusual conformation of $A_n \cdot T_n$ tracts excludes histones from binding, rather than the occupancy of specific binding proteins.

The transition of poly(dA)·poly(dT) DNA fiber proceeds from the B' to the B^* structure (two defined conformations of the double helix) at temperatures >30°C and a relative humidity of ~80% (e.g. Premilat and Albiser, 1997). The B' conformation is rigid and heteronomous with two sugar–phosphate geometries and has a narrow minor groove (Alexeev *et al*., 1987; Nelson *et al*., 1987; Park *et al*., 1987). On the other hand, the B^* structure is a member of the B-DNA family with single sugar–phosphate geometry (Premilat and Albiser, 1997). Thus, the unusual conformation of the T_n tract detected by UV photofootprinting at lower temperatures (Lyamichev, 1991) is most likely to be the B' conformation. The strong protections in the 5'-half may be attributed to the fact that the minor groove of $A_n \cdot T_n$ tracts narrows from the 3' towards the 5'-end of T_n stretches (Alexeev *et al*., 1987; Nelson *et al*., 1987; Park *et al*., 1987). Schieferstein and Thoma (1996) analyzed cyclobutane pyrimidine dimer formation in a positioned nucleosome containing a T_n-rich sequence ($T_3CCT_6CT_5GCT_5CT_7$) from the yeast *DED1* promoter. They showed that most of the T_n stretches formed an unusual conformation in the DNA solution that disappeared upon folding in nucleosomes at 37°C *in vitro*. Thus, our results indicate that the predominant formation of the B' conformation in living yeast cells prevents DNA wrapping around histone octamers. This is also consistent with the observation that poly(dA)·poly(dT) forms nucleosomes *in vitro* much more effectively at higher temperatures (Puhl and Behe, 1995), which then stabilize the B-DNA family conformation.

When the size of the A_n tract is moderate, the region near the A_7TATA_8 insert is more accessible to MNase within the positioned nucleosome. This result may correspond to the observations of Thiele and colleagues (Zhu and Thiele, 1996; Koch and Thiele, 1999). They showed that an A_{16} element distorted the DNA structure in a positioned nucleosome, facilitating nucleosomal access to transcription factors in the *C.glabrata* metal response promoter. We consider that the dynamic changes of the $A_n \cdot T_n$ structure between the unusual conformation and the B-form family may distort the local nucleosome structure.

Regarding the biological implications of the $A_n \cdot T_n$ tracts, analyses of *S.cerevisiae* chromosome III have shown that ~25% of yeast genes contain $A_n \cdot T_n$ tracts

$n \geqslant 10$ bp (Karlin *et al.*, 1993; Behe, 1995; Iyer and Struhl, 1995; Reardon *et al.*, 1995). We extended this type of analysis, since a database of the completed yeast genome sequence is now available. Consistent with earlier reports, $A_n \cdot T_n$ tracts $n \geqslant 10$ bp are found in 2495 sites in the yeast genome, among which 1760 are located within 500 bp of the untranslated region of the open reading frame (ORF) (6183 ORFs >100 amino acids long were reported). Longer $A_n \cdot T_n$ tracts ($n \geqslant 20$ bp) are found in 130 sites in the genome, and 98 of these are located within 500 bp upstream of the ORF. Although many of the loci mapped are hypothetical ORFs, the longer A_n tracts have been mapped in many housekeeping genes such as metabolic enzymes and ribosomal proteins. This supports the notion that poly(dA)·poly(dT) modulates chromatin structures to make transcription factors accessible to the promoters of constitutively expressed genes.

In summary, we showed that yeast minichromosomes provide a unique system with which to examine the effect of specific DNA sequences and hence their intrinsic DNA structures on nucleosome organization *in vivo*. We found that poly(dA)·poly(dT) tracts disrupt or at least modulate nucleosome organization in yeast. Using this assay, we are currently extending these studies to other specific DNA sequences associated with non-*B*-DNA structures such as Z-DNA, cruciforms and triplet repeat sequences. The effects of these DNA structures on chromatin organization *in vivo* will be published elsewhere.

Materials and methods

Plasmids and strains

The TALS-pUC19 plasmid was provided by Drs S.Y.Roth and R.T.Simpson. To manipulate plasmid construction, TALS linearized by *Hin*dIII digestion was inserted into the *Hin*dIII site of pBR322ΔRI. Plasmid pBR322ΔRI was constructed as follows: pBR322 was digested with *Eco*RI, then the site was filled-in with Klenow fragment and dNTPs, and ligated. A set of oligonucleotides synthesized chemically was annealed and ligated with the vector TALS-pBR322ΔRI digested with *Sac*I to construct pTS711, -713, pOM801 and -802. To construct pTS703, oligonucleotides $A_{15}TATA_{16}$ and $T_{16}ATAT_{15}$ were annealed and cloned into the *Sma*I site of a pRS424 derivative, forming pKB103, and then pKB103 was digested with *Bam*HI and *Eco*RI to obtain the fragment containing $A_{15}TATA_{16}$. The 5′-overhang of the $A_{15}TATA_{16}$ fragment was filled-in, ligated with the *Sac*I linker, digested with *Sac*I and cloned into the *Sac*I site of the TALS-pBR322ΔRI, forming pTS703. To construct pTS704, pKB103 was digested with *Eco*RI and *Not*I, ligated with a *Dra*III adapter and cloned into the *Dra*III site of TALS-pBR322ΔRI, forming pTS704. All inserts were verified by DNA sequencing. The location of the inserts is schematically shown in Figure 2.

TALS-pBR322ΔRI and its derivatives containing test DNA inserts were digested with *Hin*dIII to eliminate the pBR322 backbone. TALS or TALS derivative plasmids were then isolated, ligated and introduced into the yeast strain FY24 (*MAT*α *ura3-52 trp1Δ63 leuΔ21*).

Analysis of chromatin structure

Yeast cells harboring plasmids were cultured in SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose) supplemented with 0.02% uracil and 1% leucine until the OD_{600} reached ~0.7. Nuclei were isolated and MNase digestion proceeded as described (Shimizu *et al.*, 1991; see legends to Figures 3–5). Cleavage sites for MNase were analyzed by primer extension mapping using the primer MS-2 (1735–1701 m.u. in TALS) (Shimizu *et al.*, 1991) and indirect end-labeling (Thoma *et al.*, 1984; Thoma, 1986; Roth *et al.*, 1990). Nucleosome spacing was analyzed using the nucleosome repeat assay (Cavalli and Thoma, 1993; Becker *et al.*, 1994). Oligonucleotides 5′-GAAAAC-TGTTGCATTATTGCCGTTCATCATTTTCGA-3′ (1502–1473 m.u.) and 5′-GAGTCGTGGCAAGAATACCAAGAGTTCCTCGGTTTGC-3′ (409–445 m.u.) were used as proximal and distal probes, respectively.

In vivo UV photofootprinting

In vivo UV photofootprinting was performed as described (Axelrod and Majors, 1989; Shimizu *et al.*, 1997, 1998). The sites of UV photoproducts were analyzed by primer extension using the primer MS-2 (Murphy *et al.*, 1993).

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