In vitro low propensity to form nucleosomes of four telomeric sequences

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Abstract The structural aspects of nucleosome assembly on telomeres are largely unknown. We analyzed by competitive reconstitution the affinities for the histone octamer of telomeric sequences from four different eukaryotic groups, *Arabidopsis thaliana*, mammals, *Tetrahymena*, and *Saccharomyces cerevisiae*. All telomeres reconstitute in nucleosomes with lower association constants than average nucleosomal DNA. DNase I digestion analysis suggests a multiple translational positioning and the lack of rotational positioning, probably due to telomeric repeats length (in most cases 6–8 bp), out of phase with the DNA helical repeat on the nucleosome (10.2 bp). These results could partly explain the lack of nucleosomes on lower eukaryote telomeres, and suggest a high in vivo mobility of telomeric nucleosomes.

Key words: Telomere; Nucleosome; Competitive reconstitution; Chromatin; DNA repetitive sequences

1. Introduction

Telomeres are specialized nucleoprotein structures at the ends of eukaryotic chromosomes, essential for chromosome stability [1]. In most organisms, telomeric DNA consists of short repeated sequences from 20 bp (*Oxitricha*) to 100–150 kb (mice). The similarity of telomeric sequences (consensus 5′-$C_{1-8}(A/T)_{1-4}$-3′), indicates a common evolutionary origin [2]. The G-rich strand is oriented 5′-3′ towards the end of the chromosome, and often protrudes forming a single-stranded overhang [3]. Whilst the structural features of telomeric DNA have been characterized in vitro, by NMR and X-ray diffraction studies (for a review see [4,5]), less is known about the structural aspects of nucleosome organization on telomeric DNA regions. *Tetrahymena* and yeast telomeres seem to be organized in non-nucleosomal structures. In *Tetrahymena* macronuclei, the telomeric fragments protected from micrococcal nuclease digestion range from 200 to 800 bp, larger than that expected for nucleosomal protection [6]. In *Saccharomyces cerevisiae*, the subtelomeric regions (X and Y′ regions) are packaged in nucleosomes, having features characteristic of transcriptionally inactive chromatin, such as hypoacetylated histones [7]. However, the terminal TG_{1-3} repeats are packaged as non-nucleosomal particles 245–395 bp long, called the telosomes [8].

In contrast to the telomeres of ciliates and yeast, micrococcal nuclease digestion studies indicate that vertebrate and plant telomeres are packaged in nucleosomes [9–12], similarly to the rest of the chromosome. However, the linker region is shorter than in bulk chromatin, and telomeric chromatin seems to lack histone H1, although in chicken erythrocytes telomeric nucleosomes are associated with H1 and H5 [13]. Furthermore, telomeric nucleosomal particles seem to be hypersensitive to micrococcal nuclease [10].

Although some peculiar features of telomeric chromatin seem to depend on the interactions of telomeres with specific proteins, such as RAP1 [8,14,15] or TRF [16], nevertheless it is reasonable to suppose that telomeric nucleosomes could be different from bulk nucleosomes on account of the smaller propensity to curve of telomeric sequences. This feature may be attributed to the sequence repeat length (less than 10 bp) that characterizes telomeric DNA in most biological systems.

In this paper the different propensity of four different telomeric sequences to assemble in mononucleosomes compared to average sequence DNA was evaluated by competitive reconstitution [17–19]; micrococcal nuclease (MNase) and DNase I were used to investigate some structural features of telomeric nucleosomes.

2. Materials and methods

2.1. Synthesis and cloning of telomeric sequences

Oligonucleotides corresponding to a dimer of the telomeric repeat were chemically synthesized and purified on a 20% polyacrylamide/urea gel. After phosphorylation by polynucleotide kinase, complementary strands were annealed by slow cooling from 80°C to 4°C. The strands were designed in order to have two 5′ bp protruding after the annealing. The double stranded monomers were then ligated with T4 DNA ligase; the multimeric ladders obtained were separated on a polyacrylamide gel. Fragments about 160–180 bp long were excised and eluted in TE (10 mM Tris pH 8, 1 mM EDTA). Purified fragments were filled with Klenow enzyme and cloned into the *Sma*I site of pUC18. Clones were screened by the alkaline lysis method and verified by dideoxy sequencing. Telomeric DNAs for the experiments were extracted from the plasmid with *Eco*RI and *Bam*HI, gel purified, and 5′-labelled with polynucleotide kinase.

2.2. Competitive reconstitution

The procedure used for competitive reconstitution was that of Shrader and Crothers [17,18], with minor modifications. 1 μg of H1- and H5-depleted polynucleosomes, obtained from chicken erythrocytes [20], was mixed with 30 ng of radiolabeled telomeric DNA and various amounts of competitor DNA (calf thymus DNA digested with *Hae*III) in 1 M NaCl, 10 mM Tris-HCl pH 8, 0.1% Nonidet P-40, 100 μg/ml BSA (bovine serum albumin), in a final volume of 20 μl. After incubation at room temperature for 30 min, the salt concentration was lowered to 0.1 M NaCl with three additions of 60 μl 10 mM Tris pH 8, 0.1% Nonidet P40 (30 min apart, room temperature). Chicken erythrocytes mononucleosomal DNA (about 165 bp) was terminally labeled and reconstituted under identical experimental conditions. Samples were resolved on 5% polyacrylamide gels. The relative quantities of complexed and free DNA were assayed either by scanning dried gels with an Instant Imager (Packard) or by excising and quantifying the radioactive bands in a scintillation counter. The free energy for a given telomeric sequence (Tel) was calculated from the equation $E(\mathrm{Tel}) = RT\ln[\alpha(\mathrm{Nuc})] - RT\ln[\alpha(\mathrm{Tel})]$, where α(Nuc) is the ratio of labeled reconstituted nucleosome to labeled free DNA for mononucleosomal DNA, and α(Tel) is the analogous ratio for telomeric sequences [17–19]. Each fragment was reconstituted in three

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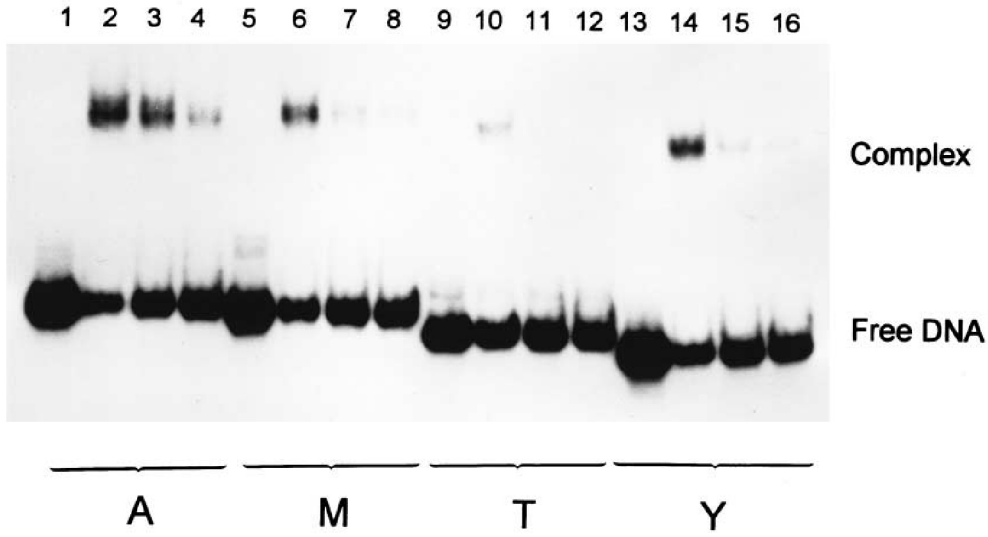

Fig. 1. Competitive reconstitution experiment of the telomeric fragments reported in Table 1. Letters at the bottom of the gel indicate the origin of the DNAs used in the experiment (A, *Arabidopsis*; M, mammals; T, *Tetrahymena*; Y, yeast). Lanes 1, 5, 9, 13, free DNA; lanes 2, 6, 10, 14, reconstituted samples without added competitor DNA; lanes 3, 7, 11, 15, samples reconstituted with 1 μg competitor DNA; lanes 4, 8, 12, 16, samples reconstituted with 2 μg competitor DNA.

separate experiments and the results were averaged. The reproducibility of the experiments was within ~0.1 kcal/mol.

2.3. Micrococcal nuclease assay

Telomeric DNAs for the MNase assay were internally labeled by 30-cycle PCR amplification in the presence of [α-^{32}P]ATP (dATP/[α-^{32}P]ATP molar ratio equal to 125). A 19-mer (TTGGGAAACAGCTATGACCAT) and a 21-mer (ACAGGAAACAGCTATGACCAT), which hybridize outside the polylinker region of pUC18, were used as primers. Amplification products were then digested with *Eco*RI and *Bam*HI, and purified on a 5% native polyacrylamide gel. For the reconstitution reaction 1.2 μg of histone octamer was mixed with 50 ng of labeled DNA and 1.3 μg of chicken erythrocyte nucleosomal DNA in 1 M NaCl, 10 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.1% Nonidet P-40, 100 μg/ml BSA, in a final volume of 10 μl. After incubation at room temperature for 30 min, the salt concentration was lowered to 50 mM NaCl with additions of 5, 10, 25, 50, and 100 μl 10 mM Tris pH 8, 0.1 mM EDTA, 0.1% Nonidet P40 (10 min apart, room temperature). $CaCl_2$ was then added to a final concentration of 3 mM. The mixture was preincubated for 5 min at 37°C, and MNase was added to a final concentration of 1 U/ml. Aliquots (50 μl) were removed at various times and stopped with an equal volume of 20 mM EDTA, 0.2% SDS. Samples were phenol extracted and ethanol precipitated. Pellets were resuspended in 5 μl loading buffer and run on a 5% native polyacrylamide gel.

2.4. DNase I assay

To 5′-label only the G-rich strand, the plasmid containing *Arabidopsis* telomeric DNA was digested with *Bam*HI, 5′ end-labeled with polynucleotide kinase, digested again with *Eco*RI, and gel purified. DNA fragments were reconstituted as described in Section 2.3. After reconstitution, $MgCl_2$ was added to a final concentration of 5 mM. Samples were then digested for 1 min at 25°C with 1–4 U/ml (free DNA) or 10–40 U/ml DNase I (reconstituted complex) in a 50 μl total volume. The reactions were stopped by adding 50 μl of 20 mM EDTA, 0.2% SDS. After extracting with phenol/chloroform, DNA was precipitated with ethanol in the presence of 1 μg tRNA. Pellets were washed with 75% ethanol, air dried, resuspended in 4 μl sequencing loading buffer, and run in a 6% polyacrylamide-urea gel. Gels were dried under vacuum and autoradiographed. Autoradiographs were scanned with a BioRad GS-670 Imaging Densitometer. Lanes from the same autoradiograph, equivalent in extent of digestion and band intensity, were used for the calculations of difference probability plots. The same procedure was used for experiments on the C-rich

Table 1
Telomeric sequences used in competitive reconstitution experiments

DNA	Monomer Sequence	Ligated Polymer
Arabidopsis thaliana	5' GGTTTAGGGTTTAG 3' 3' AAATCCCAAATCCC 5'	(GGTTTAGGGTTTAG)$_{13}$
Mammals	5' GGTTAGGGTTAG 3' 3' AATCCCAATCCC 5'	(GGTTAGGGTTAG)$_{15}$
Saccharomyces cerevisiae	5' GGTGTGTGGGTGTGTG 3' 3' ACACACCCACACACCC 5'	(GGTGTGTGGGTGTGTG)$_{10}$
Tetrahymena	5' GGTTGGGGTTGG 3' 3' AACCCCAACCCC 5'	(GGTTGGGGTTGG)$_{14}$

Monomers correspond to dimers of the telomeric repeats. The strands were designed to have 5′ two bp protruding.

strand (not shown), digesting first with *Eco*RI and then with *Bam*HI (after labeling). The experiments were repeated at least three times with identical results.

3. Results

3.1. Competitive nucleosome reconstitution of telomeric sequences

Telomeric sequences from four different eukaryotic groups, *Arabidopsis thaliana* [21], mammals [9], *Tetrahymena* [6], and *Saccharomyces cerevisiae* [14], were synthesized and multimerized by ligation, in order to obtain telomeric DNA fragments about 170 bp long (Table 1). *Arabidopsis* telomeric sequence also represent tobacco telomeric repeats [12], maize [22] and barley [23]. TTAGGG corresponds to all vertebrate telomeres sequenced to date [11] and to some fungi like *Neurospora* [24] and slime molds like *Physarum* [25]. The yeast sequence corresponds to the binding domain of Rap1 protein [14].

To evaluate the propensity of different sequences to form nucleosomes, a competition for a limited number of histone octamers is established between radiolabeled test DNA and unlabeled, heterogeneous-sequence DNA. The fraction of labeled DNA present in the nucleosomal band depends on the intrinsic ability of that sequence to reconstitute in nucleosomes compared to bulk DNA (Fig. 1). To be sure that equilibrium is reached under the assay conditions, control reconstitutions were carried out in which a radioactive fragment was initially added either as free DNA or in nucleosomal complex. The final nucleosome/free DNA ratio was the same in the two cases, showing that equilibrium was reached (data not shown). Increasing incubation times to 1 h did not affect the percentage of DNA incorporated in nucleosomes (not shown). Free DNA and nucleosomal complex were separated on a polyacrylamide gel (Fig. 1). The relative fractions of labeled DNA in the nucleosomal and free DNA bands were used to calculate the free energy difference of nucleosome formation on telomeric sequences relative to average nucleosomal DNA. The results are shown in Table 2. All telomeric sequences tested show positive free energy values compared to average nucleosomal DNA, corresponding to lower tendencies to reconstitute. The free energy differences range from 1.10 kcal/mol for *A. thaliana* telomeric DNA to 2.00 kcal/mol for *Tetrahymena*. In the literature there are no reported sequences, either natural or synthetic, with such a low propensity to form nucleosomes. In the case of *Tetrahymena* telomeric sequence, the difference with a strong nucleosome binder such as the *Xenopus borealis* somatic 5S rRNA gene is 3.75 kcal/mol [26], and in the case of the synthetic sequence named TG from Shrader and Crothers [17] the difference is 4.85 kcal/mol. These results can be related to the nucleosomal

Table 2
Comparative reconstitution free energies

DNA	ΔΔG (kcal/mol)
Arabidopsis thaliana $(GGGTTTA)_{26}$	1.10
Mammals $(GGGTTA)_{30}$	1.35
Saccharomyces cerevisiae $(GGTGTGTG)_{20}$	1.60
Tetrahymena $(GGGGTT)_{28}$	2.00
Mononucleosomal DNA	~0

Reconstitution free energies measured for the four telomeric sequences examined. All energies are relative to the fraction of mononucleosomal DNA that is reconstituted into nucleosomes under identical conditions.

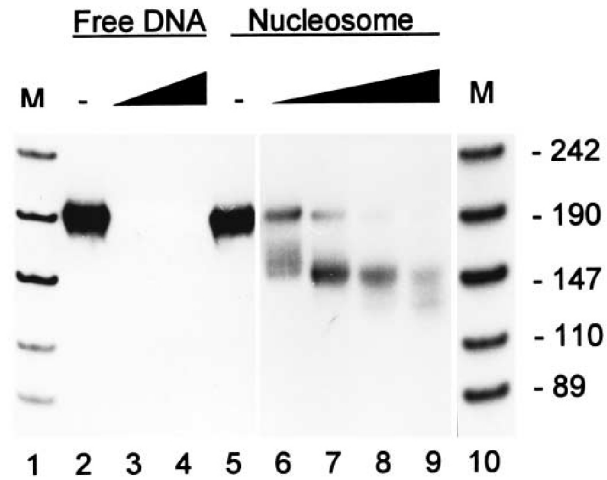

Fig. 2. Micrococcal nuclease digestion analysis of nucleosomes reconstituted on telomeric DNA. Naked *A. thaliana* telomeric DNA (lanes 2–4) and reconstituted cores (lanes 5–9) were digested with 1 U/ml of micrococcal nuclease for 0, 1, 3 min (naked DNA, lanes 2–4), and 0, 1, 3, 6, 10 min (reconstituted samples, lanes 5–9). M (lanes 1, 10) is a molecular weight marker (pUC18 cut with *Hpa*II).

DNA pathway around the histone octamer. Satchwell et al. [27] showed that different dinucleotides occupy preferentially different locations in the DNA wrapped around the histone octamer, and exhibit a periodic modulation which corresponds to the DNA period on the nucleosome, which is, on average, 10.2 [28]; A/T-rich sequences preferentially occur at sites where the minor groove faces toward the protein, whereas G/C-rich sequences preferentially occupy positions where the minor groove points outward. The sequence repeat length of telomeres is in most cases 6–8 bp; therefore, telomeric sequence repeats are out of phase with nucleosomal helical repeats. As a consequence, at some points sequences that would be energetically favored in occupying inward facing minor groove positions will be forced to point outward, resulting in a increase of nucleosome formation free energy.

3.2. Micrococcal nuclease digestion reveals a canonical nucleosome length

Naked and reconstituted *A. thaliana* telomeric DNA were treated with MNase for increasing times. The results are shown in Fig. 2. Naked DNA was rapidly degraded by MNase (lanes 2–4), whereas reconstituted DNA was trimmed to a fragment of about 147 bp (lanes 5–9). A small degradation is visible only after prolonged digestion (lane 9). This result indicates that telomeric nucleosomes are similar to bulk nucleosomes, showing the canonical protection of about 146 bp (Fig. 2). In vivo experiments [9,10] show an increased MNase sensitivity of telomeric regions compared to bulk nucleosomes. A similar hypersensitivity does not emerge from our experiments, suggesting that the enhanced degradation found in vivo could not depend on an increased sequence-dependent accessibility to MNase. However, a more extensive analysis is necessary to conclusively check this point.

3.3. Multiple nucleosome translational positioning without rotational phasing

A predictable consequence of telomeric sequence repeat length is the lack of rotational positioning. We studied this feature by DNase I digestion analysis, since this enzyme is

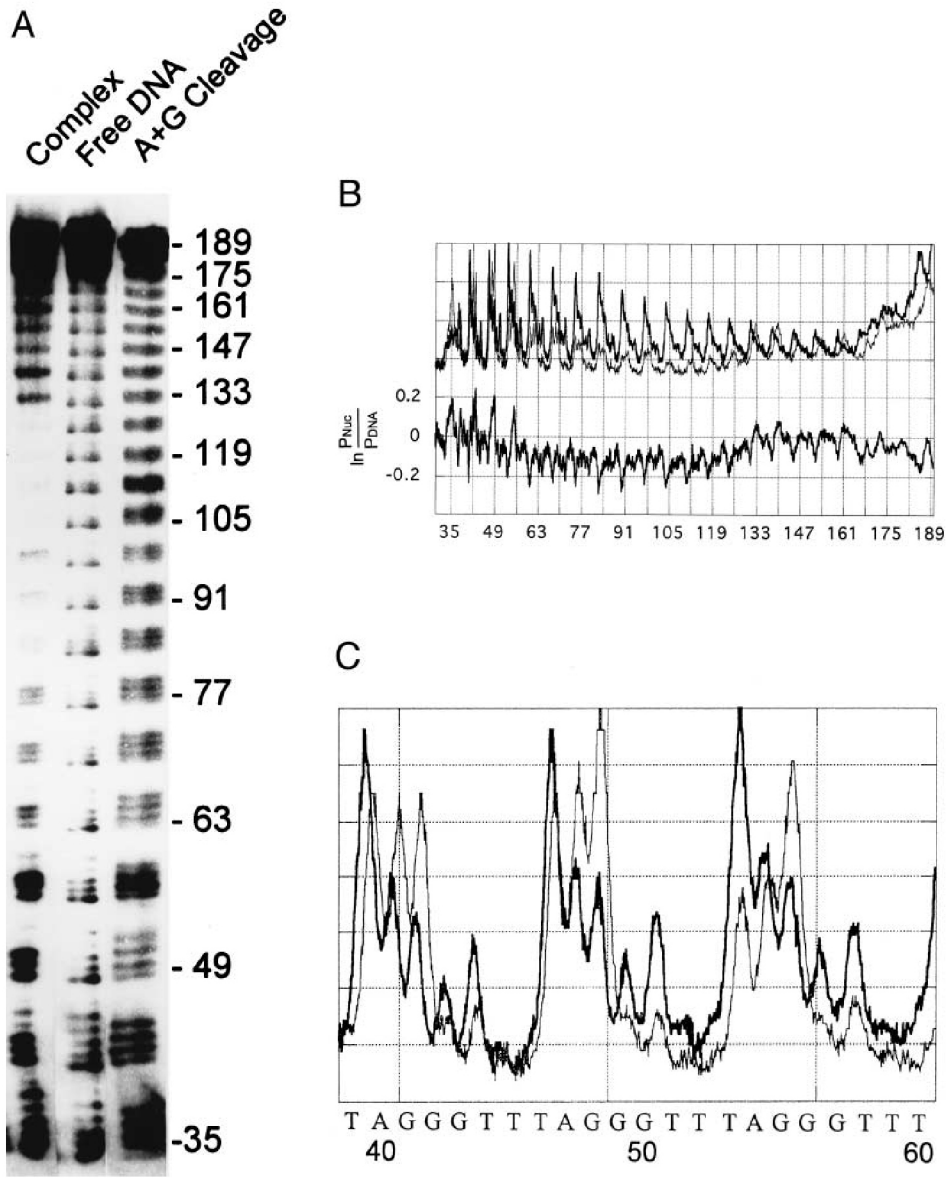

Fig. 3. DNase I digestion analysis. A: Autoradiograph of the DNase I cutting on the G-rich strand of *Arabidopsis* telomeric DNA. Free DNA was digested with 2 U/ml DNase I and the reconstituted complex with 20 U/ml for 60 s. A+G cleavage was carried out according to Maxam and Gilbert [30]. B: Densitometric profiles of the DNase I cleavage sites in free (thick line) and complexed DNA (thin line). At the bottom of the figure, the difference probability plot is reported. The plot was calculated by subtracting the ln (probability of cutting, *P*) at each individual bond in free DNA from those in the reconstituted complex. Therefore, negative values represent protected positions. The scale on the left is in units of ln(probability). C: Enlarged section of the densitometric profiles from 39 to 60 nt.

well known to provide information on helical periodicity of DNA-protein interactions. In Fig. 3A, the cleavage patterns of naked and reconstituted *A. thaliana* G-rich strand telomeric DNA are shown. Both in the free DNA and in the complex pattern a cutting periodicity of 7 bp was found, reasonably depending on DNase I sequence specificity. An underlying periodicity of about 10 bp is not detectable. This suggests that nucleosomes are distributed all along the sequence without particular preferred positions. Similar indications emerge from the C-rich strand DNase I footprinting and from exonuclease III digestion (data not shown). When complexed with the histone octamer, the bands corresponding to the central region of the fragment are depressed compared to the free DNA pattern (Fig. 3A,B). If the logarithm of the DNA versus nucleosome cleavage frequency is considered (Fig. 3B, bottom), a bell-shaped curve emerges, having negative values in a 70 bp central tract; this region should be occupied by every nucleosome formed on the telomeric sequence, causing the DNase I protection observed. As it is possible to observe in the enlarged section (Fig. 3C), bases are not equally cleaved by DNase I in naked DNA and nucleosome, suggesting the interesting presence of different local geometrical parameters in naked as well as in reconstituted telomeric DNA. To obtain further information on this point the DNase I cutting patterns of different telomeric sequences and their nucleosome complexes are under investigation.

4. Discussion

We find that the telomeric sequences examined, which rep-

resent a great number of organisms, reconstitute in nucleosomes with a remarkably low affinity; moreover, at least in the case of *A. thaliana,* telomeric nucleosomes occupy multiple positions without rotational phasing. The telomeric sequences from yeast and *Tetrahymena*, which are relatively short and do not form nucleosomes in vivo, show the highest positive values of free energy differences compared to average nucleosomal DNA. The lack of nucleosomes from yeast and *Tetrahymena* chromosome ends could be a consequence of the shortness (300–400 bp) of the telomeric sequences; this makes it unlikely that specific nucleoprotein structures and nucleosomes could occur contemporaneously. Anyway, the formation of specific nucleoprotein complexes instead of nucleosomes could be favored by the remarkably low affinity for the histone octamer of their telomeric sequences. By contrast, in multicellular eukaryotes telomere lengths range from a few [21] to 150 kb [11]; it is likely that only the very ends of multicellular eukaryote telomeres may be organized in a non-nucleosomal nucleoprotein structure. Nucleosomes and specific end-capping nucleoprotein structures could form contemporaneously on higher eukaryote telomeres, with nucleosomes extended over the proximal part of telomeres. In addition, nucleosome formation on *A. thaliana* and mammals telomeres could benefit from higher sequence-dependent affinities for the histone octamer compared to yeast and *Tetrahymena.*

Our data suggest the possibility of an easier nucleosome sliding over telomeric sequences than bulk DNA. As shown by Meersseman et al. [29], even strongly positioned nucleosomes are characterized by histone octamer mobility among multiple positions having the same rotational settings. The low affinity for the histone octamer and the lack of rotational positioning of telomeric nucleosomes should imply a high nucleosome mobility, at least in the case of higher eukaryotes.

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References

[1] Rhodes, D. and Giraldo, R. (1995) Curr. Opin. Struct. Biol. 5, 311–322.
[2] Blackburn, E.H. (1994) Cell 77, 621–623.
[3] Henderson, E. and Blackburn, E.H. (1989) Mol. Cell. Biol. 9, 345–348.
[4] Williamson, J.R. (1994) Annu. Rev. Biophys. Biomol. Struct. 23, 703–730.
[5] Henderson, E. (1995) in: Telomeres (Blackburn, E.H. and Greider, C.W., Eds.), pp. 11–34, CSHL Press, New York.
[6] Budarf, M.L. and Blackburn, E.H. (1986) J. Biol. Chem. 261, 363–369.
[7] Brauenstein, M., Sobel, R.E., Allis, C.D., Turner, B.M. and Broach, J.R. (1996) Mol. Cell. Biol. 16, 4349–4356.
[8] Wright, J.H., Gottschling, D.E. and Zakian, V.A. (1992) Genes Dev. 6, 197–210.
[9] Makarov, V.L., Lejnine, S., Bedoyan, J. and Langmore, J.P. (1993) Cell 73, 775–787.
[10] Tommerup, H., Dousmanis, A. and De Lange, T. (1994) Mol. Cell. Biol. 14, 5777–5785.
[11] Lejnine, S., Makarov, V.L. and Langmore, J.P. (1995) Proc. Natl. Acad. Sci. USA 92, 2393–2397.
[12] Fajkus, J., Kovarik, J., Kralovics, R. and Bezdek, M. (1995) Mol. Gen. Genet. 247, 633–638.
[13] Muyldermans, S., De Jonge, J., Wyns, L. and Travers, A. A. (1994) Nucleic Acids Res. 22, 5635–5639.
[14] Gilson, E., Roberge, M., Giraldo, R., Rhodes, D. and Gasser, S.M. (1993) J. Mol. Biol. 231, 293–310.
[15] Konig, P., Giraldo, R., Chapman, L. and Rhodes, D. (1996) Cell 85, 125–136.
[16] Chong, L., van Steensel, B., Broccoli, D., Erdjument-Bromage, H., Hanish, J., Tempst, P. and de Lange, T. (1995) Science 270, 1663–1667.
[17] Shrader, T.E. and Crothers, D.M. (1989) Proc. Natl. Acad. Sci. USA 86, 7418–7422.
[18] Shrader, T.E. and Crothers, D.M. (1990) J. Mol. Biol. 216, 69–84.
[19] Jayasena, S.D. and Behe, M.J. (1989) J. Mol. Biol. 208, 297–306.
[20] Cacchione, S., Cerone, M.A., De Santis, P. and Savino, M. (1995) Biophys. Chem. 53, 267–281.
[21] Richards, E.J. and Ausubel, F.M. (1988) Cell 53, 127–136.
[22] Burr, B., Burr, F.A., Matz, E.C. and Romero-Stevenson, J. (1992) Plant Cell 4, 953–960.
[23] Kilian, A. and Kleinhofs, A. (1992) Mol. Gen. Genet. 235, 153–156.
[24] Schechtman, M.G. (1990) Gene 88, 159–165.
[25] Forney, J., Henderson, E.R. and Blackburn, E.H. (1987) Nucleic Acids Res. 14, 9143–9151.
[26] Schild, C., Claret, F.-X., Wahli, W. and Wolffe, A.P. (1993) EMBO J. 12, 423–433.
[27] Satchwell, S.C., Drew, H.R. and Travers, A.A. (1986) J. Mol. Biol. 191, 659–675.
[28] Drew, H.R. and Calladine, C.R. (1987) J. Mol. Biol. 195, 143–173.
[29] Meersseman, G., Pennings, S. and Bradbury, E.M. (1992) EMBO J. 11, 2951–2959.
[30] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.