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Nucleosomal Organization of Telomere-Specific Chromatin in Rat

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Summary

Rat liver interphase chromosomes have telomeres 20–100 kb in length. Micrococcal nuclease digestion of nuclei cleaves telomeres with a uniform 157 bp periodicity, producing soluble particles that sediment in sucrose gradients exactly like oligonucleosomes. The monomeric telomere particles comigrate with nucleosome core particles on nucleoprotein and DNA gels but do not bind H1. DNAase I cleaves telomere nucleoprotein into a series of bands spaced by about 10.4 bp and with the same intensity distribution as bands from bulk nucleosomes. Removal of H1 from chromatin alters the sedimentation properties of telomeres in parallel with bulk chromatin. Thus, telomeres of mammals are constructed of closely spaced nucleosomes, in contrast with the telomeres of lower eukaryotes, which show no evidence of nucleosomal structure.

Introduction

Eukaryotic chromosomes are terminated with specialized structures called telomeres, which have many important structural and functional roles in interphase, mitotic, and meiotic chromosomes (e.g., Blackburn and Szostak, 1984; Zakian, 1989; Blackburn, 1991a, 1991b). Telomeric DNA from most organisms has the consensus sequence $5'\text{-}C_{1\text{-}8}(A/T)_{1\text{-}4}\text{-}3'$ (Blackburn, 1984). In Oxytricha and Euplotes macronuclei, the chromosomes have very short double-stranded telomeres (20 and 18 bp, respectively) with single-stranded G-rich 3′ protrusions (Klobutcher et al., 1981). Tetrahymena and yeast telomeres are about 400–600 bp in length (Blackburn and Gall, 1978; Shampay et al., 1984). Mammalian telomeres consist of the sequence $(TTAGGG)_n$, terminally repeated for 5–20 kb in humans (Moyzis et al. 1988; Allshire et al., 1988) and 100–150 kb in mice (Kipling and Cooke, 1990; Starling et al., 1990).

Very little is known about the nucleoprotein structure of telomeres. Telomerase is an enzyme that specifically adds telomeric repeats to the 3′ ends of telomeric DNA, compensating for incomplete replication at the ends of the chromosomes (Blackburn, 1992). Without telomerase activity chromosomes appear to shorten gradually, which might influence cell senescence (Harley et al., 1990). It was shown that, in the ciliates Oxytricha and Euplotes, specialized proteins bind tenaciously to the G-rich single-strand termini and protect against chemical modification and nucleases (Gottschling and Cech, 1984; Gottschling and Zakian, 1986; Price and Cech, 1987; Price, 1990). Tetrahymena and yeast telomeres were also demonstrated to have nonnucleosomal organization (Blackburn and Chiou, 1981; Budarf and Blackburn, 1986; Wright et al., 1992). Several proteins that bind tightly to $(TTAGGG)_n$ have been identified or isolated from eukaryotes (Gottschling and Zakian, 1986; Berman et al., 1986; Buchman et al., 1988; Price, 1990; Liu and Tye, 1991; Coren et al., 1991; McKay and Cooke, 1992; Zhong et al., 1992). These findings suggest that telomeres are specialized structures, unlike the nucleosomal substructure characteristic of the bulk of the eukaryotic genome. Specifically, there are no reports that telomeres bind histone proteins or have any repeating subunits.

This paper addresses the question of the nucleoprotein structure in mammalian telomeres. Rat was chosen for study, because the properties of rat chromatin have been investigated in detail (reviewed by van Holde, 1989). Using standard electrophoretic and sedimentation analyses of nuclease-treated telomeres, we unexpectedly found that the bulk of the telomeric DNA is organized into nucleoprotein subunits that are nearly indistinguishable from nucleosomes. The subunits were cleaved with a 10.4 bp periodicity by DNAase I, had a sedimentation coefficient of 11.2S, and were digested with micrococcal nuclease (MNase) to form a stable 146 bp core particle that comigrated with nucleosome core particles on nucleoprotein gels. Each of these properties is characteristic of nucleosome core particles. Oligomers of the telomeric subunits sedimented identically to oligomers of bulk chromatin and were less condensed after treatment of the chromatin with Bio-Rex 70, which removes histone H1 from bulk chromatin. The unique features of telomeres in nuclei are an unusually short and regular repeat length of 157 bp, a slightly altered MNase sensitivity in nuclei, and an absence of demonstrable binding of histone H1 to the monomer subunit. We conclude that the telomeres of rat are composed of closely spaced nucleosomes. This result, combined with our finding of very short repeat lengths in telomeres of other organisms (unpublished data), suggests a conserved, unique chromatin structure for telomeres in higher eukaryotes that is probably important for telomere function.

Results

Restriction Enzyme and Bal31 Digestions Show That Rat Telomeres Are Very Long and Are Terminally Located

To confirm that the telomere sequences were primarily at the ends of the chromosomes, intact rat DNA was trimmed with exonuclease Bal31 for increasing times, digested to completion with HaeIII, separated by pulse-field electrophoresis, and stained with ethidium bromide before transfer to filters for hybridization with the telomere-specific probe TEL4. Without Bal31 treatment, the telomere fragments were extremely long, consistent with telomere

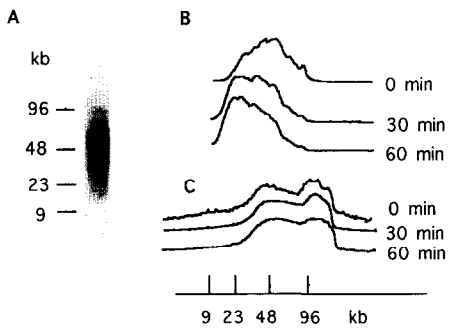

Figure 1. Electrophoretic Analysis of Rat Telomere DNA Size and Location

(A) Agarose-embedded DNA was digested with HaeIII, electrophoresed by pulse-field inversion in 1% agarose, electrotransferred to a filter, and hybridized with TEL4. The average size of bulk DNA was ≤2 kb (data not shown). To test the specific digestion of the telomeres by Bal31, nuclei in agarose blocks were digested with Bal31 for 0, 30, and 60 min and subsequently divided into roughly equal parts, digested with HaeIII or XhoI to achieve an average size of ~50–100 kb for telomere and bulk DNA, respectively, and electrophoresed by pulse-field inversion. Bulk and telomere DNA were detected by ethidium bromide staining and TEL4 filter hybridization.

(B) Electrophoretic pattern of telomeres after cleavage with HaeIII, showing a gradual decrease in the average size of telomere DNA from ~50 kb to ~30 kb.

(C) Electrophoretic pattern of bulk DNA after cleavage with XhoI, showing a constant molecular size of ~100 kb.

tracts that were 20–100 kb long (Figure 1A). This result was also found with nine other frequently cutting enzymes (MboI, MspI, CfoI, RsaI, HpaI, HinfI, DdeI, Sau3AI, and AluI). Bal31 progressively reduced the size (Figure 1B) and intensity of telomere DNA, in agreement with previous results in human and mouse (Allshire et al., 1988; Starling et al., 1990). Control digestions of the same Bal31-digested samples with XhoI showed no change in the length of high molecular weight bulk DNA, indicating that Bal31 was specific to the ends of the chromosomes (Figure 1C). Thus, the TEL4 probe is specific to long tracts of hexameric repeats at the ends of the chromosomes. Cytological studies have also been unable to detect interstitial hexameric repeats in rat (Meyne et al., 1990).

MNase Digestion Reveals That Telomeres Have a Periodic Subunit Structure

When digested with MNase, bulk DNA exhibited the expected nucleosome ladder with an average repeat of 197 bp (Figure 2A). Surprisingly, the telomere DNA also had a strong band pattern (Figure 2B). Control digestion of the purified rat DNA with MNase produced a broad molecular weight distribution of telomere and bulk DNA. There are three differences between the behavior of telomeres and bulk chromatin. First, each telomere band migrated faster than the corresponding band on the stained gel, indicating

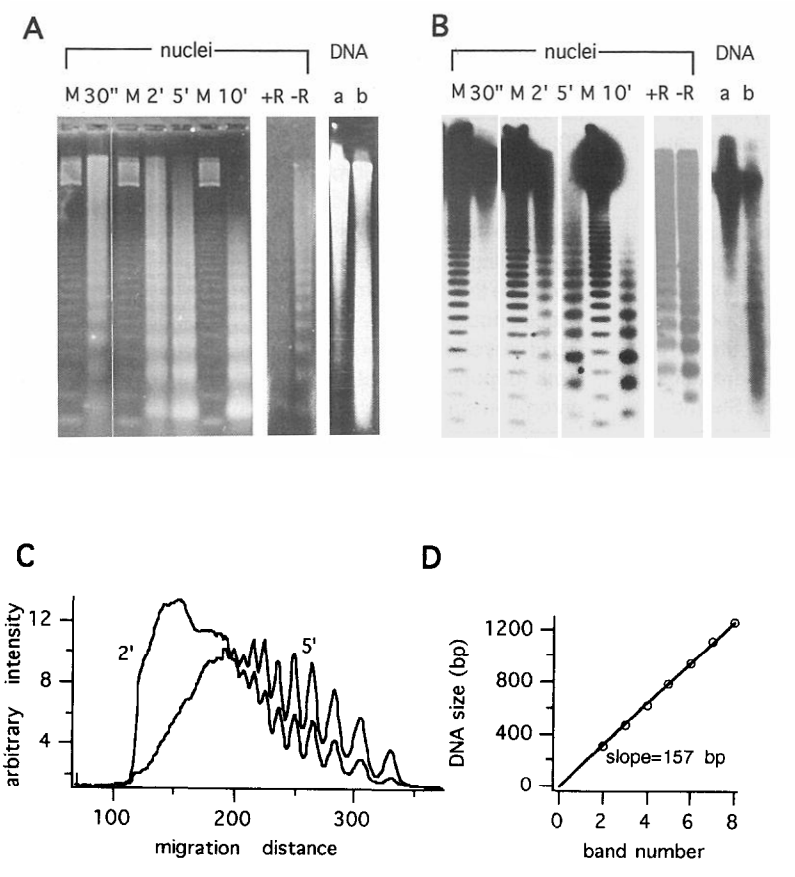

Figure 2. MNase Cleavage Patterns of Rat Liver Nuclei, Assayed by Electrophoresis in 1.5% Agarose

Nuclei were incubated with 0.04 U of MNase per μg of DNA for the indicated times. Control DNA samples were incubated for 1 min with 0.002 U of MNase per μg of DNA (lane a); and for 10 min with 0.01 U of MNase per μg of DNA (lane b). To demonstrate that the telomere ladder was not caused by telomere sequences interspersed with nontelomere sequences, nuclei were digested with 0.04 U of MNase per μg of DNA for 2 min (lane −R), purified, and then digested for 2 hr with 3 U of HaeIII per μg of DNA (lane +R).

(A) Fluorogram of total DNA.

(B) Autoradiogram of telomere DNA and marker DNA hybridized to TEL4 and a probe made from the 123 bp DNA ladder.

(C) Autoradiogram densities from 2 min and 5 min digests in (B).

(D) Linear regression analysis of the size of telomere DNA fragments after 2 min digestion of nuclei with MNase. The slope indicates a telomere repeat length of 157 bp.

that the repeat length of telomere nucleoprotein was significantly shorter. Second, the telomere bands were sharper and could be visualized at higher molecular weights than bulk chromatin, indicating that the telomere nucleoprotein structure was more regular. Third, the cleavage of the telomeres was slightly slower than that of bulk chromatin. For example, after 10 min of digestion, the bulk chromatin distribution peaked at the monomer, whereas the telomere DNA distribution peaked at the tetramer and pentamer.

The molecular weights of telomere bands were determined and plotted as a function of band number. Figure 2C shows the autoradiogram densities for the 2 min and 5 min time points in Figure 2B. Linear regression analysis shows that all bands fit a straight line (Figure 2D). Analysis of the products of 2, 5, and 10 min digestions gave slopes (and intercepts) of 157 (9), 157 (−6), and 157 (−1) bp, respectively. We found this short repeat length from six different preparations of rat nuclei. Two preparations were measured carefully and gave repeats of 157 and 158 bp. Therefore, we estimate that the true telomere repeat is 157 ± 2 bp. This value is much less than the 197 bp average repeat of bulk rat liver chromatin.

Nucleosomes can move along DNA at high temperatures or salt concentrations, particularly if depleted in histone H1 (Spadafora et al., 1979; Watkins and Smerdon, 1985; van Holde, 1989). As a control for subunit sliding, we digested nuclei with MNase at 0°C, 20°C, and 37°C and determined that the telomere repeat lengths were independent of temperature (data not shown). We conclude that the 157 bp ladder reflects the true average periodicity of telomeres.

MNase Digestion Shows That Most of the Telomere Nucleoprotein Is Organized as a Repeating Unit That Is Neither Unusually Accessible nor Protected

Figures 2A and 2B show that telomeres have a nuclease ladder more definite than that of bulk chromatin, which is consistent with less heterogeneity in the length of the DNA in each repeating unit. The bands still are not as sharp as those of restriction fragments, perhaps owing to some heterogeneity in linker length, cutting at a distribution of sites in the linker DNA, enhanced cleavage of core DNA due to reduced accessibility of the linker DNA, or exonucleolytic trimming. To test directly whether those bands were composed primarily of hexameric repeats of telomere DNA, we redigested the MNase ladder with HaeIII (Figures 2A and 2B, lanes −R and +R). HaeIII obliterated the bulk nucleosome repeat but had no effect on the telomere repeat, consistent with the 157 bp ladder consisting of pure telomere DNA sequences. Digestion of naked DNA did not reveal a telomere repeat.

To test whether a substantial fraction of the telomere sequences were exceptionally susceptible or protected from MNase, we blotted the digestion samples shown in Figure 2 onto nylon and quantitated the amount of telomere and bulk DNA during the progressive digestion by probing with TEL4 and RAT (the bulk rat DNA probe). The ratio of telomere DNA to bulk DNA remained constant (± 10%), indicating that telomeres are not rapidly degraded (data not shown). The fact that the 10 min digests in Figure 2B do not have appreciable telomere DNA above 1 kb shows that there are not long tracts of highly protected telomere DNA. After extensive MNase digestion, more than 75% of the telomere sequences were present in the monomer, dimer, trimer, and tetramer bands, with the remaining 25% being in the regions between these bands (data not shown). In comparison, Noll (1974a) used MNase digestion to estimate that at least 85% of the DNA in rat liver was organized into nucleosomes. These data make it likely that the predominant structure of telomeres is similar to that of bulk chromatin and rule out the possibility that telomeres contain large amounts of free DNA or large refractile complexes.

Telomere Nucleoprotein Is Soluble at Low Ionic Strength

The role of attachment to the nuclear envelope is often attributed to telomeres, owing to evidence of telomere localization at the periphery of the nucleus (e.g., Gruenbaum et al., 1984; Foe and Alberts, 1985; van Dekken et al., 1989; Chung et al., 1990; Rawlins et al., 1991) and interaction of telomere DNA with nuclear matrix (de Lange, 1992). Therefore, telomeres might be insoluble, even under conditions that solubilize most chromatin but leave the nuclear matrix and associated DNA intact.

To study telomere solubility, nuclei were mildly digested and lysed to give 30%–50% solubilization of bulk chromatin (measured by A_{260}). Dot blots indicated that the telomere fragments were neither enriched nor depleted during solubilization (data not shown). This shows that the telomere fragments are as soluble as bulk chromatin in low salt without divalent cations. Redigestion of the soluble chromatin (or H1-depleted chromatin, shown below) with MNase gave the same repeating subunit patterns as found in nuclei, indicating that the subunits were stable to solubilization (data not shown).

Sucrose Gradient Centrifugation Shows That Telomere Nucleoprotein Sediments as Independent Oligomers with the Same Sedimentation Coefficients as Mononucleosomes and Oligonucleosomes

Neutral sucrose density gradient sedimentation has been extensively used to separate multimeric nucleoprotein complexes, including chromatin (e.g., Noll and Noll, 1989). The sedimentation of intact chromatin is characterized by a monomer peak with an $S_{20,w}$ of 11.2S and a series of faster bands related to the molecular weight by a power law, which represent oligomers of the nucleosome subunit (Finch et al., 1975; Osipova et al., 1986). We are not aware of any other cellular structures having the same sedimentation coefficients as chromatin fragments.

To compare telomere to bulk nucleoproteins, we analyzed low ionic strength isokinetic sucrose gradients of soluble chromatin (Figure 3). Electrophoresis of the collected fractions showed the expected bands of bulk nucleosome oligomers. Figure 3A shows the ethidium bromide–stained gel of the fractions containing mononucleosomes and some of the dinucleosomes. Figure 3B shows the autoradiogram of the same gel after transfer and hy-

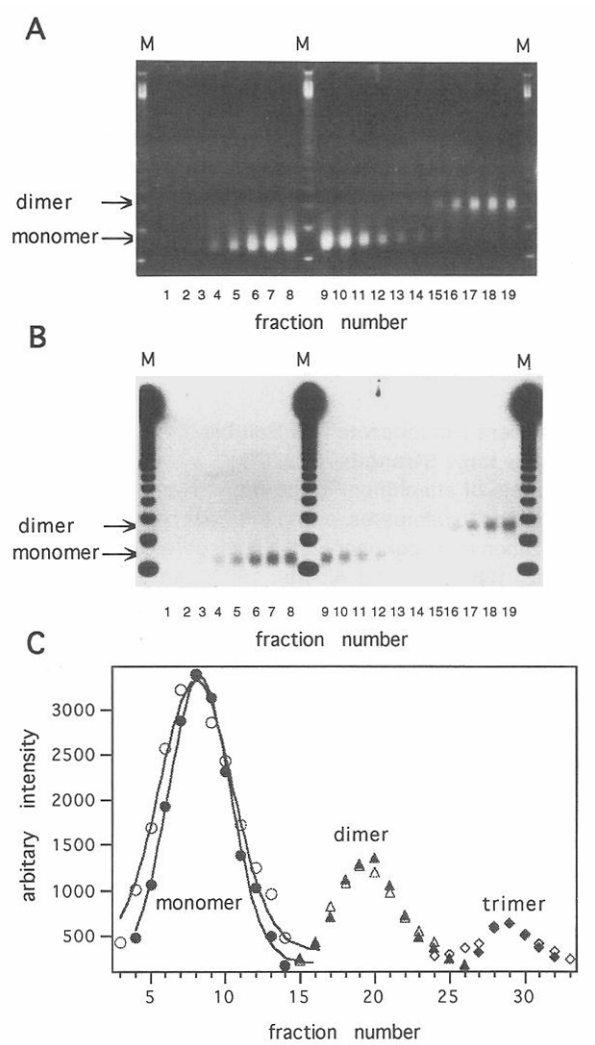

Figure 3. Sedimentation Analysis of Bulk and Telomeric Chromatin Using Isokinetic Sucrose Gradients

Gradient fractions were analyzed by proteolysis, electrophoresis, ethidium bromide staining of total DNA, transfer, and hybridization to TEL4 and M (123 bp marker).

(A) Fluorogram of the first 19 fractions.

(B) Autoradiogram of hybridization to the DNA in (A). The positions of bulk mono- and dinucleosomes are shown.

(C) Graphical analysis. Integrated fluorescence of each fraction (closed circle, triangle, and diamond). Integrated autoradiogram density of each fraction (open circle, triangle, and diamond). The intensity of each telomere band has been multiplied by an arbitrary constant to allow direct comparison of the sedimentation of the bulk and telomere multimers. Lines are Gaussian fits.

bridization to TEL4. The monomer peak on the gradient contained virtually pure monomer-length bulk and telomere DNA, and the same purity was found across the multimer peaks. Quantitation of each of the lanes from fractions containing monomers, dimers, and trimers showed that the profiles of the bulk and telomere bands were indistinguishable (Figure 3C). Table 1 compares the sedimentation coefficients of the telomere and bulk bands with previous chromatin studies. We conclude that the hydrodynamic properties of the telomere nucleoprotein fragments are virtually identical to those of bulk mono- and oligonucleosomes at low ionic strength.

H1 Depletion Results in Parallel Changes in Sedimentation of Telomeres and Bulk Chromatin

Removal of histone H1 from chromatin dramatically decreases the sedimentation coefficients of the oligonucleosomes, owing to decondensation of the fibers (Noll and Noll, 1989). To determine whether telomeres underwent the same structural change, we compared sedimentation of intact and H1-depleted chromatin. Bio-Rex 70 was used to remove histone H1 selectively from bulk chromatin at low ionic strength without dissociation of other major proteins (Marekov and Beltchev, 1981). SDS–polyacrylamide gel elctrophoresis analysis showed ⩽3% differential loss of core histones or HMG14/17 and ⩾85% removal of H1 (data not shown). Intact and Bio-Rex 70–treated oligonucleosomes from the same preparation of soluble chromatin were centrifuged and analyzed as above (Table 1), giving the same quality of results as shown in Figure 3. Bio-Rex 70 treatment reduced the sedimentation coefficients of both bulk and telomere oligomers, although the telomere fragments sedimented more rapidly than bulk chromatin. We attribute this to the 4-fold shorter linker DNA. These results indicate that telomere multimers are normally condensed but decondense subsequent to treatment with Bio-Rex 70, possibly owing to removal of a nonhistone protein or histone H1.

Extensive MNase Digestion of Telomeres in Nuclei and H1-Depleted Chromatin Produces Stable Nucleosome Core Particles

The studies above show that telomeres are organized into repeating subunits that could have the same molecular weight, density, and shape as nucleosomes, but they do not address the issue of internal structure. Extensive MNase digestion and DNAase I digestion produce nucleosome-specific patterns of cleavage within the nucleosome, reflective of the protein–DNA contacts as well as the deformation of the DNA on the surface of the nucleosome (van Holde, 1989).

As MNase progressively cleaves chromatin, there is a brief pause at 166 bp, characteristic of the chromatosome, which consists of the histone octamer and one copy of histone H1 (Simpson, 1978), and a stronger pause at 146 bp, characteristic of the nucleosome core particle, which does not have H1 (van Holde, 1989). These particles can be unambiguously identified by electrophoresis of the nucleoproteins or DNA. Nucleoprotein electrophoresis has proven very sensitive to the protein composition of mononucleosomes and is able to detect the presence of histone H1, ubiquitinated H2A, and HMG14/17 (Varshavsky et al., 1976; Levinger and Varshavsky, 1982; Barsoum and Varshavsky, 1985). If telomeres are organized as nucleosomes, it is expected that nuclease digestion would produce stable telomere core particles that could be resolved on nucleoprotein and DNA gels.

As the most sensitive test for the presence of nucleosome core particles in telomeres, we digested solublized rat liver chromatin with MNase and performed nucleopro-

Table 1. Hydrodynamic Properties of Bulk and Telomere-Specific Chromatin

Samples	Source	Sedimentation Coefficients ($S_{20,w}$) of Chromatin			
		Monomer	Dimer	Trimer	Tetramer
Bulk	Noll and Noll (1989)	11.2 (10.9)	16.0 (14.3)	19.7 (16.5)	22.7 (18.2)
Bulk	Osipova et al. (1980)[a]	10.7 (10.6)	15.6 (14.1)	19.4 (16.7)	22.7 (18.8)
Bulk	Present study[b]	11.2 (10.9)	15.5 (14.0)	19.4 (16.2)	22.8[c] (17.8)
Telomere	Present study[b]	11.1 (10.9)	15.5 (14.2)	19.4 (17.0)	22.6[c] (18.7)

Data in parentheses represent those for H1-depleted chromatin. We estimate the accruacy of our calculated sedimentation values to be ±0.2S.
[a] Calculated using the equations for oligonucleosomes with and without H1 from Osipova et al. (1980).
[b] Calculated from the sedimentation velocity of the peaks relative to that of the monomer particle of H1-containing bulk chromatin, assuming a value of 11.2S for the mononucleosome.
[c] Data from a different gradient of H1-containing chromatin.

tein gel electrophoresis under the conditions most sensitive to charge, mass, and conformation of the nucleosome core particles (Huang and Garrard, 1989; Pennings et al., 1992). Bulk chromatin showed two clearly resolved monomer bands, previously identified by Varshavsky et al. (1976) as a nucleosome core particle (MN1) and a chromatosome (MN2) (Figure 4A). Transfer of the DNA and filter hybridization to TEL4 showed a single band that comigrated with MN1 (Figure 4B). Excision of the MN1 band and electrophoresis of the naked DNA showed that it contained 146 bp of DNA for both bulk and telomere sequences (Figures 4C and 4D). We conclude that the monomer subunit of telomeres is a stable nucleosome core particle that does not bind histone H1.

To probe the resistance of telomere core particle DNA to MNase, rat liver nuclei were digested with MNase and analyzed using high resolution DNA gels (Figure 5). Bulk chromatin (Figure 5A, lanes 1 and 2) showed overlapping bands at 166 bp and 146 bp, indicative of the presence of the chromatosome digestion intermediate and core particle. In contrast, the telomere fragments showed no evidence of the chromatosome (Figure 5A, lanes 3 and 4), in agreement with the nucleoprotein gels in Figure 4. Better-resolved 166 and 146 bp bands in bulk DNA can be obtained by redigestion of soluble chromatin (data not shown) or nuclei of certain tissues such as chicken erythrocytes (Bavykin et al., 1990), perhaps because more uniform MNase exonucleolytic trimming is achieved when the linker DNA is more accessible. Figure 5 (lanes 3 and 4) also shows that the telomere core particles are digested to subnucleosomal particles more rapidly than the core particles of bulk chromatin. This result warrants more careful analysis of the MNase digestion of telomeres.

The high resolution MNase digestion patterns of the telomeres are more complex than those of bulk chromatin because of the sequence specificity of the enzyme. Rather than a simple distribution of lengths representing the protection of the DNA from nuclease, the telomere pattern of protein protection was superimposed on a strong 6 bp repeat, owing to the sequence specificity of MNase (e.g., Figure 5C, lanes 1–3). Quantitative analysis of the high resolution gels showed that the length of the DNA in the bands, L, is described by the expression $L = 6n - 3$ bp, where n is an integer. Digestion of naked DNA gave the same 6 bp ladder, owing to the specificity of MNase (Figure 5C, lane D), which is known to cleave A–T 30 times faster than C–G (Drew, 1984). A pause at $(C–G)_3$ during trimming of both telomere strands would account for the band positions.

Telomere DNA consistently failed to show a pause at 166 bp (Figure 5A, lanes 3 and 4), in agreement with the short repeat of 157 bp and the absence of an H1-containing telomere band on the nucleoprotein gel (Figures 4A and 4B). Telomere DNA consistently showed a

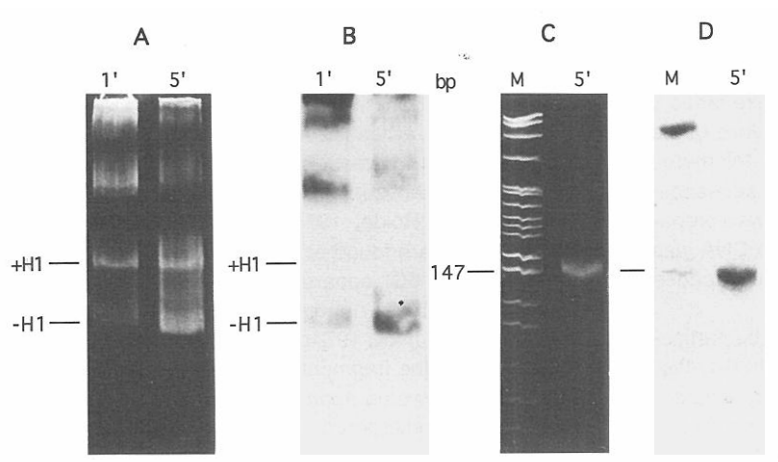

Figure 4. Nucleoprotein Gel Analysis of Material Solubilized from Rat Liver Nuclei after Digestion with 0.3 U of MNase per μg of DNA for 1 or 5 min

(A) and (B) show soluble nucleoprotein electrophoresed on 6% polyacrylamide; (C) and (D) show DNA from the MN1 bands cut from lanes duplicate to the 5 min digestion in (A) and electrophoresed on 8% agarose to confirm that the putative MN1 bands contained 146 bp of DNA.
(A) Fluorogram of the nucleoprotein gel.
(B) TEL4 hybridization to DNA electrotransferred from the gel in (A).
(C) Fluorogram of DNA cut from the MN1 bands.
(D) TEL4 hybridization to DNA electrotransferred from the gel in (C). The telomere bands comigrated with bulk MN1 bands and contained 146 bp DNA.

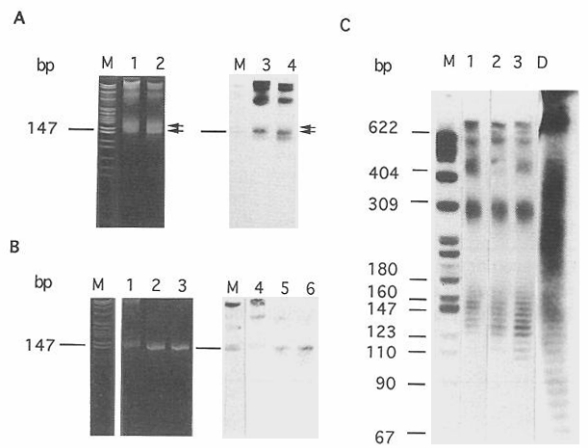

Figure 5. Polyacrylamide Gel Electrophoresis Analysis of Extensive MNase Cleavage of Intact Nuclei and H1-Depleted Chromatin

Samples were digested at 37°C, the DNA was purified, electrophoresed on polyacrylamide, stained with ethidium bromide, transferred to nylon, and hybridized to TEL4, and a probe was made from the MspI-digested pBR322 DNA. The MspI-digested pBR322 DNA was used as a size marker (lane M).

(A) Digestion of nuclei. Lanes 1 and 2, fluorogram, showing resistance of bulk nucleosomes to digestion below ~146 bp after digestion of nuclei with 0.4 U of MNase per μg of DNA for 1 and 2 min; lanes 3 and 4, autoradiogram of TEL4 hybridization after electrotransfer of lanes 1 and 2, showing a pause at ~146 bp. Lines show the position of the 147 bp restriction fragment; arrows show the expected positions of DNA fragments with the lengths of chromatosomes (166 bp) and core particles (146 bp). At this level of digestion, the chromatosomes and core particles are clearly present (but not well resolved) in bulk chromatin, but no evidence of the chromatosome is seen in telomeres.

(B) Digestion of soluble chromatin depleted of H1 by washing in 0.5 M NaCl. Lanes 1, 2, and 3, fluorogram showing resistance of bulk DNA below ~146 bp after digestion with 0.1 U of MNase per μg of DNA for 0.5, 1, and 2 min; lanes 4, 5, and 6, autoradiogram of TEL4 filter hybridization of DNA in lanes 1, 2, and 3, showing resistance of telomere core particles to digestion below ~146 bp.

(C) MNase digestion of a different nuclear preparation, which shows rapid digestion of telomeres to subnucleosomal particles. Lanes 1, 2, and 3, TEL4 hybridization to nuclei digested with 1 U of MNase per μg of DNA for 0.5, 2, and 10 min. Control DNA was digested with 0.004 U of MNase per μg of DNA for 10 min (lane D).

pause at about 146 bp. The 146 bp peak was usually stronger after redigestion of soluble chromatin or H1-depleted chromatin (Figure 5B). During digestion the 146 bp core particle is digested further to form subnucleosomal fragments (Figure 5A, lane 4; Figure 5C, lanes 1–3). The telomeres were usually cut into subtelomere particles faster than bulk chromatin (Figure 5A; compare lanes 2 and 4). We speculate that the short-lived presence of the 146 band might be due to association of the telomeres with nuclear matrix or nonhistone proteins or to sequence-specificity of MNase. Figure 5C (lanes 1–3) shows a preparation of rat liver nuclei in which the telomere DNA was quickly cut from ~150 bp to ~120 bp, without a pause at ~146 bp.

To test whether the telomere subunits might be particularly unstable, rat liver nuclei were treated with 0.1, 0.6, 0.8, 1.0, and 2.0 M NaCl, digested with MNase, and assayed by DNA electrophoresis (data not shown). Treatments with less than 0.8 M NaCl gave bulk and telomere patterns that were identical to those of untreated nuclei. Above 0.8 M NaCl the MNase ladders were dramatically and comparably attenuated for both bulk and telomere sequences, presumably owing to removal of histones H2A and H2B (Burton et al, 1978). These salt experiments were not able to detect a difference in the salt stability of the bulk and telomere nucleosomes.

DNAase I Digestion of Telomeres Demonstrates a Nucleosome-like Protection of One Side of the DNA Molecules

The internal structure of the telomere subunit was also probed with DNAase I (Figure 6). Rat liver nuclei and DNA were digested with DNAase I, electrophoresed in a denaturing polyacrylamide gel, transferred to nylon, and hybridized to TEL4 and the marker probe, M. Figure 6A shows that bulk chromatin produced a ladder with ~10 bp spacing extending from 30 bp to 160 bp, with strong bands at ~80 bp and ~90 bp and a weak band at ~60 bp, in agreement with previous findings (Noll, 1974b; Prunell et al., 1979). Figure 6B and the densitometer tracing of the autoradiogram (Figure 6C) show that telomere nucleoprotein has the same type of repeat pattern, with a very similar distribution of weak and strong bands. A control digestion of purified rat DNA with DNAase I gave a featureless smear for bulk and telomere DNA. Although a 10 bp DNAase I ladder has been observed for DNA bound to inorganic or protein surfaces (e.g., Liu and Wang, 1978; Rhodes and Klug, 1980; Price and Cech, 1987; Price, 1990), strong peaks at ~80 bp and ~90 bp and a very weak peak at ~60 bp have never been observed for nonnucleosomal DNA. The extents of DNAase I digestion of bulk and telomere chromatin seem to be identical. We conclude that the telomere 10 bp periodicity is specific to a telomere nucleoprotein with the properties of a bulk (i.e., transcriptionally inactive) nucleosome.

Actually, the strong peaks in bulk DNAase I patterns in Figure 6A have lengths of 83 bp and 94 bp, which has been interpreted as revealing a 10.4 bp repeat of the DNA on the surface of the nucleosome core (e.g., Prunell et al., 1979). A graph of the telomere fragment lengths versus band number (Figure 6D) has a slope of 10.4 bp, indicating an average periodicity close to that of bulk chromatin. The strongest telomere bands are centered at 83 bp (Figure 6C) and 94 bp.

There are three additional interesting features of the telomere DNAase I sensitivity seen in the densitometer trace in Figure 6C. First, the 10 bp ladder continues beyond 160 bp to at least 240 bp. The same observation has been made for yeast chromatin and interpreted as indicating a quantized spacing between nucleosomes (Lohr and van Holde, 1979). Second, clear telomere-specific bands are found at ~160, ~320, and ~480 bp (Figures 6B and 6C), apparently representing monomers, dimers, and trimers, noticed previously in bulk rat and yeast chromatin (Noll, 1974b; Lohr and van Holde, 1979). Third, a plot of the fragment length versus band number (Figure 6D) shows an apparently overlapping pattern of telomere bands staggered by 5 ± 1 bp. This pattern has been reported for yeast and chicken chromatin, presum-

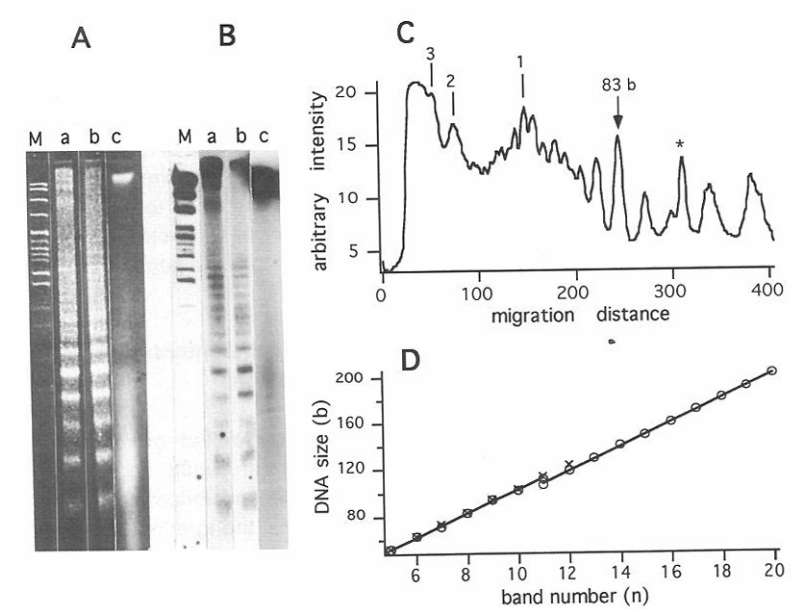

Figure 6. Denaturing Polyacrylamide Gel Electrophoresis Analysis of DNAase I Cleavage of Telomeres and Bulk Chromatin

Rat livers were digested at 37°C, and the DNA was purified, electrophoresed in 8% polyacrylamide–7 M urea, stained with ethidium bromide, transferred to nylon, and hybridized to TEL4 and calibration marker DNA. Lane M, MspI-digested pBR322 DNA calibration.

(A) Fluorogram.

(B) Autoradiogram.

Lanes a and b, nuclei digested for 10 min with 2 U of DNAase I per μg of DNA and for 5 min with 10 U of DNAase I per μg of DNA; lane c, purified DNA digested for 10 min with 0.1 U of DNAase I per μg of DNA.

(C) Densitometer tracing of lane a of the autoradiogram. The asterisk represents an artifact, adjacent to band 60 bp (see [B], lane a) on the autoradiogram. Numbers 1, 2, and 3 represent telomere-specific bands 160, 320, and 480 bp, respectively.

(D) Graph of band size versus apparent band number, according to the method of Lohr and van Holde (1979). The graphed data (open circles) fit two straight lines: bands 5–11 have a slope of 10.0 bp; bands 11–20 have a slope of 10.4 bp. The accuracy of the results is insufficient to suggest that the difference between the two slopes is meaningful. The data of Prunell et al. (1979) (crosses) are also shown.

ably owing to an overlap between the intracore and intercore cleavage patterns, and interpreted as showing that adjacent nucleosomes form on opposite sides of the double helix (Lohr and van Holde, 1979; van Holde, 1989).

The DNAase I data strongly indicate that the telomere subunits have internal structure indistinguishable from that of nucleosomes.

Discussion

We have studied the nucleoprotein structure of rat telomeres, using classical nuclease, electrophoretic, and hydrodynamic techniques, and found that they are assembled into chromatin with the same repeating core and linker structure as bulk chromatin. By all tested criteria, telomeres are composed of nucleosome core particles. The very short linker is unique to the telomere structure, as is the fact that the monomer does not appear to possess H1 on nucleoprotein gels. Because the majority of the telomere nucleoprotein properties are identical to those of bulk chromatin, but several are unique, we refer to the telomere nucleoprotein as telomere-specific chromatin. This discussion compares our results with studies of bulk nucleosomes and studies of telomeres from lower eukaryotes.

Rat Chromosomes Have Long Terminal Tracts of Telomere DNA

We have found that the bulk of the telomere sequences were in continuous 20–100 kb tracts of the 6 bp repeat, similar to the case in mouse (Kipling and Cooke, 1990; Starling et al., 1990). Assuming an average length of 50 kb, telomere DNA would compose ~0.1% of the rat genome. Bal31 digestions showed that these tracts were located at the ends of rat chromosomes, in agreement with in situ hybridization studies (Meyne et al., 1990). Thus, there is nothing extraordinary about the size, sequence purity, or location of rat liver telomere DNA.

Rat Telomeres Are Organized as Regularly Repeating Subunits Having the Same Properties as Chromatin

MNase has been used to study the repeating nucleosomal substructure of bulk chromatin from many sources, as well as to probe subtle differences between active and inactive genes. There are no known nonnucleosomal structures in prokaryotes or eukaryotes that have the same MNase digestion characteristics as nucleosomes. A MNase digestion ladder is commonly taken as strong evidence that a particular sequence of DNA is organized into nucleosomes.

MNase cleaved the major fraction of rat telomeres at periodic sites that seem to have the characteristics of eukaryotic chromatin. The telomeres were progressively cleaved into lower oligomers of the basic repeat of 157 bp. The length of each oligomer was decreased slightly during extended digestions, as in the case of bulk chromatin during exonucleolytic trimming by MNase. The susceptibility of the telomeres to MNase was slightly less than that of the bulk, which could be the result of the very short repeat length, the constitutive transcriptional inactivity, an interaction with nuclear matrix, or some unknown attribute. The lack of preferential insolubility of the telomere fragments indicates that they are not strongly attached to the nuclear matrix or aggregated into refractile bodies.

The sedimentation coefficient of chromatin is very sensitive to H1 content and salt conditions. Thus, it is very significant that the telomere monomers and multimers had the same sedimentation profiles as H1-containing bulk chromatin. H1 depletion resulted in significant decreases of sedimentation velocities of both bulk and telomere oligonucleosomes, presumably owing to chromatin decondensation (Noll and Kornberg, 1977; Butler and Thomas, 1980). It is likely that H1 or other proteins affecting condensation are bound to telomeres.

The Telomere Nucleoprotein Subunits Appear to Have the Same Internal Structure as Nucleosomes

The nucleosome can be defined as a nucleoprotein particle having a core particle, with about 146 bp of DNA and a histone-containing protein core, and a variable amount of DNA linking it to the adjacent core particles. The lengths of the core and linker DNA can be measured directly, as we have done for telomeres in this paper. Although the structure of the globular domain of the histone core has been solved to low resolution by X-ray crystallography (Richmond et al., 1984), the presence of a nucleosome at particular sites in the genome is usually inferred from MNase and DNAase I studies, as described above. In fact, nucleosomes consist of a class of structures, some of them distinguishable by the indirect methods but as yet not characterized by crystallography or microscopy. For example, the class of subunits called nucleosomes includes particles having the following: different DNA sequences; different thermodynamic stabilities; different amounts of linker DNA, from 0 to 100 bp; different overall levels of nuclease sensitivity; different solubilities; different levels of histone posttranslational modifications, such as ubiquitination, acetylation, phosphorylation, and poly ADP-ribosylation; different stoichiometries of histone subtypes; different core histone and H1 stoichiometries; and different nonhistone protein components, including HMG14/17 and satellite-specific proteins (van Holde, 1989 and references therein). The nuclease sensitivities and physical properties of most of those nucleosome subtypes have not yet been studied in detail; however, many of the subtypes are known or suspected to have altered thermodynamic stabilities, sedimentation coefficients, and mobilities on nucleoprotein gels.

Nucleoprotein gel electrophoresis showed that the telomere monomer comigrated with the nucleosome core particle and had a DNA length of about 146 bp. This is a very good indication that the telomere subunit has the same protein composition as a nucleosome core particle, because small changes in protein composition (e.g., binding of HMG14/17, monoubiquitination of H2A, or binding of H1) or in DNA length are known to affect the migration of nucleosomes significantly.

In addition, DNAase I digested telomeres and bulk chromatin at the same rates and gave the 10.4 bp periodicity and specific pattern of strong and weak bands that is a unique signature of protection of the core DNA by the histone octamer core.

Although the MNase and DNAase I data are strong enough to conclude that telomeres are composed of nucleosomes, they do not prove that the telomere core particle has exactly the same structure as the core particle studied by X-ray crystallography, nor that the telomere subunits are organized into the same type of higher order structure as bulk chromatin. Nevertheless, the properties of the rat telomere nucleosomes are close to the center of the spectrum of properties known for nucleosomes at other transcriptionally inactive sites of the genome and in other organisms.

Rat Telomeres Are Organized Differently from Telomere and Subtelomere Regions of Lower Eukaryotes

Rat telomeres are primarily constructed of repeating nucleosomal subunits, although our results do not preclude a small fraction of the telomere nucleoprotein having alternative structure. For example, the terminal 100–500 bp of the chromosomes could have structure similar to the telomeres of lower eukaryotes, but they only represent <1% of the rat telomere DNA. The MNase digestions were unable to detect any highly susceptible telomere material (such as free DNA) or highly refractile telomere material. More than 75% of the soluble telomere nucleoprotein was organized as nucleosomes.

Nucleosomal organization of telomeres has not been reported for any of the other organisms studied previously, which have been limited to lower eukaryotes. In Saccharomyces cerevisiae, a nonnucleosomal nucleoprotein structure has been reported for the terminal tracts of $C_{1-3}A$ repeats (Wright et al., 1992). The yeast telomere nucleoprotein complex, called the telosome, seems to protect the entire ~350 bp terminal tract and contains a number of proteins, including the regulatory protein RAP1, which does not bind to $(TTAGGG)_n$ (e.g., Berman et al., 1986; Buchman et al., 1988; Conrad et al., 1990; Liu and Tye, 1991; Wright et al., 1992). Other lower eukaryotes also show no evidence of a nucleosomal structure on the telomere repeat (e.g., Gottschling and Cech, 1984; Budarf and Blackburn, 1986; Price and Cech, 1987; Price, 1990; Edwards and Firtel, 1984). This difference between the major telomere nucleoproteins of higher and lower eukaryotes might be correlated with differences in the availability of nonhistone proteins that are able to bind telomere DNA or differences in the physical structure of the telomere DNA or might simply be due to differences in length of the terminal tracts, which are 10–100 times larger in higher eukaryotes.

In contrast, the subtelomere regions of lower eukaryotes such as S. cerevisiae, Oxytricha, Tetrahymena, and Dictyostelium discoideum can form nucleosomes (Gottschling and Cech, 1984; Wright et al., 1992; Budarf and Blackburn, 1986; Edwards and Firtel, 1984). However, these subtelomere nucleosomes are unlike the telomere nucleosomes of rat, because those subtelomere nucleosomes are not regularly spaced and have the same average repeat length as bulk chromatin (e.g., 160 bp in yeast and 200 bp in Oxytricha).

Thus, telomere-specific chromatin seems to be specific to higher eukaryotes.

Telomere Chromatin Has an Unusually Short Repeat Length

The repeat length of 157 ± 2 bp was determined at early stages of digestion process, using linear regression analysis, and was reproducibly measured with six different nuclear preparations. Very similar results were obtained after extensive digestions of nuclei, soluble chromatin, and H1-depleted chromatin. This is the smallest repeat ever measured for a higher eukaryote and the largest (40 bp) deviation from an average repeat reported for any eukaryote. It has been hypothesized that the nucleosome repeat of higher eukaryotes is always greater than 165 bp, because the basic subunit might be the chromatosome, consisting of the nucleosome core plus an additional 20 bp of DNA and one molecule of H1. Because the telomere repeat is less than 165 bp, rat telomeres cannot be constructed of chromatosomes, consistent with our nucleoprotein gel results.

We do not understand the origin of unusually short repeat length of rat telomere chromatin. Our controls have ruled out the possibility that the short repeat is due to sliding of nucleosomes during enzymatic digestion. MNase digestions of other tissues and organisms show that the short repeat is probably conserved in all vertebrates and even some invertebrates (unpublished data). Therefore, we suggest that the $(TTAGGG)_n$ motif itself might establish the short repeat. Natural sequence-specific nucleosome spacing for arrays of nucleosomes has not been reported, except in the case of rat satellite chromatin, which has a 185 bp repeat on a 370 bp sequence repeat (Omori et al., 1980). In the case of telomeres, it is not obvious how the 6 bp sequence repeat might be related to the 157 bp nucleosome repeat. Perhaps this average repeat is due to a distribution of nucleosome phases at different multiples of the hexameric repeat, e.g., 156 and 162 bp. Alternatively, the short repeat might be due to strong influences from subtelomere chromatin or nonhistone proteins during assembly or in association with nuclear matrix. For example, strongly cooperative polymerization of nonhistone proteins at the very ends of chromosomes might cause in vivo sliding of nucleosomes into compressed arrays in the proximal end of the telomere and perhaps subtelomere regions.

The only other chromatin molecules with very short repeats are found in certain lower eukaryotes, mammalian neurons, and active genes. In fungi the repeats are ~170 bp in Neurospora crassa, 165 bp in S. cerevisiae, 158 bp in Achlya ambisexualis and 154 bp in Aspergillus nidulans (van Holde, 1989 and references therein). Several of the fungi seem to have complete complements of all five histones, although they often display significant molecular weight and amino acid composition differences from the more highly conserved histones of higher eukaryotes. However, in many cases nucleosome sliding has not been ruled out. A recent careful study of Schizosaccharomyces pombe showed that the repeat length was about 156 bp but was reduced dramatically, owing to sliding, during digestion and resulted in an abnormally large monomer of 160–180 bp (Godde and Widom, 1992). In contrast, the telomere-specific repeat of rat was not sensitive to the extent or temperature of digestion and gave a monomer DNA size less than the repeat length. Each of these properties is consistent with trimming of the telomere fragments rather than sliding of the nucleosomes. The shortest repeat found previously in mammals was ~160–165 bp, in nuclei isolated from neurons in rabbit and rat (van Holde, 1989). It has been proposed that short nucleosome repeats are correlated with transcriptional activity (e.g., Villeponteau et al., 1992), but there is no evidence of telomere transcription in higher eukaryotes.

Telomere-Specific Nucleosomes Are Unusually Uniformly Spaced

In rat liver, the MNase ladders of telomeres seem to be more distinct than those of bulk or even satellite chromatin (see Omori et al., 1980). About 14 telomere bands can be detected, indicating a very regular repeat. The DNAase I ladder of telomeres extends beyond 240 bp, indicating that a substantial portion of nucleosomes have a quantized spacing. Both yeast bulk chromatin and rat telomere chromatin have overlapping patterns staggered by 5 bp, implying that the linker is quantized to lengths of (10n + 5) bp and that adjacent nucleosomes are on opposite sides of the DNA helix. The apparent regularity of the telomere repeat might be the result of one of the following: the short repeat length, which would restrict the number of possible positions of the cores and might interfere with the binding of nonhistone proteins; phased positioning of the cores on the $(TTAGGG)_n$ repeat; increased interactions between adjacent nucleosome cores; or specific interactions between the telomeres and soluble nonhistone proteins or nuclear matrix.

The Telomere Mononucleosome Often Appears to Be Less Stable to MNase Digestion Than Bulk Nucleosomes

Telomere sequences in rat nuclei, soluble chromatin, and H1-depleted chromatin can be digested into core particles having about 146 bp of DNA, which has been regarded as characteristic of all cellular, viral, and synthetic nucleosomes, even for the short repeat length chromatins of fungi, neurons, and active genes. In most nuclear preparations, however, the discrete telomere band at 146 bp was more quickly degraded into subnucleosomal fragments than were bulk nucleosomes. This unusual feature is perhaps due to a difference in packing of telomere nucleosomes in nuclei, interactions of telomeres with nuclear matrix, or perhaps a special combination of MNase sequence specificity and placement of the nucleosomes on the hexameric repeat. Another possibility is that histone H1 or nonhistone binding to the very short linker DNA alters the stability or accessibility of the ends of the core DNA. Unfortunately, few sequence-specific nucleosome cores have been studied carefully, and no studies have been done to quantitate the relative stabilities of different core particles to MNase. Most studies of nucleosomes are limited to analysis of low resolution gels, which cannot resolve the observed instability of the telomere subunits in nuclei. Therefore, we do not know whether telomere

cores exhibit special instability or merely fall in the range of stabilities that would be found at other sites in the genome.

The Existence of Soluble Telomere-Specific Chromatin Has Implications for the Study of Telomeres

We conclude that the telomeres of rat consist largely of a regular array of very closely spaced nucleosomes. This unique chromatin structure is possibly important for telomere function.

It was unexpected that telomeres were as soluble as bulk chromatin, because cytological and biochemical data indicated that telomeres of higher eukaryotes are closely associated with the periphery of the nucleus or nuclear matrix (e.g., Gruenbaum et al., 1984; Foe and Alberts, 1985; van Dekken et al., 1989; Chung et al., 1990; Rawlins et al., 1991). For example, in HeLa cells, telomere DNA cannot be solubilized from nuclei treated to remove histones and digested with restriction enzymes (de Lange, 1992). We conclude that either the attachments to matrix are sensitive to low salt and EDTA or the attachments are few in number. Possibly the telomere termini, which might have single-stranded G-rich protrusions tenaciously bound to specialized proteins (as found in ciliates), could play a role in attachment. Alternatively, the subtelomere regions could be attached to matrix.

Telomere-specific chromatin might influence the attachments of the ends of the chromosomes to nuclei, the resistance of telomeres to degradation, the expression of adjacent genes, regulation of the length of telomeres, the timing of telomere replication, the binding and activity of telomerase, or the roles of telomeres in recombination. It is not known whether the short, regular repeat length of the telomeres influences any of these possible roles for telomeres. We can speculate, however, that the unique, regular nucleosome repeat could increase the cooperativity of packing of the nucleosomes, perhaps influencing the accessibility of telomeres to nonhistone proteins, or could contribute to interactions between telomeres on different chromosomes, perhaps influencing homologous pairing and recombination. Hypothetically, a highly cooperative interaction of the telomere nucleosomes could propagate into the subtelomere regions and influence the expression of adjacent genes (Wright and Shay, 1992), although nucleosome-mediated effects do not seem to play a part in yeast telomere positional effects (Gottschling et al., 1990).

Experimental Procedures

Materials

Leupeptin (as hemisulfate) and N-lauroylsarcosine (Sarkosyl) were obtained from Sigma. MNase, DNAase I, RNAase A, and proteinase K were obtained from Boehringer Mannheim. Random prime DNA-labeling kit, Bal31, T4 kinase, 123 bp DNA ladder, HindIII-digested λ DNA, λ DNA, and restriction endonucleases AluI, DdeI, HaeIII, HpaII, MboI, and Sau3AI were obtained from Bethesda Research Laboratories. Restriction endonucleases CfoI, HinfI, MspI, and RsaI were obtained from Promega. Msp-digested pBR322 DNA and λ pulse-field gel marker DNA were obtained from New England Biolabs. Zeta-probe nylon membranes were obtained from Bio-Rad. γ-[^{32}P]ATP (3000–6000 Ci/mmol) and γ-[^{32}P]dCTP (3000 Ci/mmol) were obtained from Amersham. Oligonucleotide TEL4, $(TTAGGG)_4$, was synthesized on an Applied Biosystems Model 391 DNA synthesizer. Rats (Rattus norvegicus, Sprague Dawley, CD) were obtained from Charles River Laboratories.

Preparation of Nuclei, Soluble Chromatin, and Purified DNA

Nuclei from 3- to 9-month-old male and female outbred rats were prepared by a modified Hewish and Burgoyne method as described by Kornberg et al. (1989), except all buffers contained 1 mM EDTA, 0.5 mM EGTA and included protease inhibitors 1 mM phenylmethylsulfonyl fluoride (PMSF) and 6 μM leupeptin. Iodoacetate was also present during homogenization (5 mM) and first centrifugation (1 mM). All procedures in this manuscript were carried out at 4°C, except as noted. Nuclei were suspended in buffer A (15 mM Tris–HCl [pH 7.5] 60 mM KCl, 15 mM NaCl, 0.15 mM 2-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 0.5 mM EGTA, 0.34 M sucrose) supplemented with 50% glycerol, frozen in liquid nitrogen as 0.1 mg aliquots, and stored at −70°C for 1–2 months. Concentrations are always stated in terms of DNA concentration, estimated by A_{260}, assuming that one absorbance unit is equivalent to 50 μg/ml. Before use, each aliquot of nuclei was thawed on ice and washed 2 times with 1 ml of buffer C (buffer A without EDTA and EGTA) by centrifugation for 15 min at 180 × g.

To produce immobilized nuclei and DNA, nuclei were washed and resuspended to 2 mg/ml in buffer A, mixed gently with an equal volume of 2% low melting agarose at 37°C, solidified as 10 × 5 × 2 mm plugs, and divided into ~2 μg pieces. To produce salt-treated nuclei, the pieces were washed in 1 ml of buffer C with 0.1–2.0 M NaCl for 3 hr, with hourly buffer replacement to remove soluble protein, and subsequently washed 3 times for 20 min with 1 ml of buffer C.

Chromatin was solubilized by mild MNase digestion of nuclei and low ionic strength lysis (Kornberg et al., 1989). Briefly, ~350 μg of nuclei were suspended in 200 μl of MNase digestion buffer (buffer C with 1 mM $CaCl_2$), incubated for 5 min at 37°C, and digested with 0.23 U/μg for different times at 37°C. Digestion was stopped with 10 mM EDTA, and the nuclei were gently sedimented and resuspended for 1 hr at 4°C either in 1 mM EDTA, 8 mM Na_2HPO_4 (pH 7.0), and 0.1 mM PMSF or in 1 mM Tris–HCl (pH 8.0) and 0.2 mM EDTA. Soluble chromatin was recovered by microcentrifugation for 5 min.

High molecular weight DNA in agarose was prepared by incubating 100 μl of immobilized nuclei with 0.5 ml of solution of 200 μg/ml proteinase K, 0.5% SDS, and 100 mM EDTA (pH 8.0) for 12 hr at 50°C, followed by washing with ~10 ml of TE (10 mM Tris–HCl [pH 8.0], 1 mM EDTA). Soluble DNA was prepared by standard phenol–chloroform extraction, ethanol precipitation, washing with 70% ethanol, and resuspension in TE at 1 mg/ml. It was incubated with 50 μg/ml RNAase (DNAase-free) for 1 hr at 37°C and purified again, as described above.

MNase and DNAase I Digestion

Washed nuclei were suspended at a concentration of 1 mg/ml in MNase digestion buffer or DNAase I buffer (buffer C with 3 mM $MgCl_2$, 0.1 mM $CaCl_2$). Aliquots (100 μl) were heated at 37°C for 1 min, added to nuclease, and incubated at 37°C, unless stated otherwise (time and enzyme concentrations in figure legends). Digestion was stopped by adding EDTA to 20 mM, SDS to 0.5%, and proteinase K to 200 μg/ml, followed by incubation at 37°C or 50°C for 12 hr. DNA was extracted twice with phenol–chloroform, ethanol precipitated, washed with 70% ethanol, and dissolved in TE (after MNase) or deionized formamide with 1 mM EDTA (after DNAase I). After DNAase I digestion, the samples were incubated with RNAase and repurified.

Agarose-embedded and salt-treated nuclei (2 μg of DNA) were preincubated in 50 μl of MNase buffer for 30 min and then with 0.4 U of MNase for 15 min to let the enzyme diffuse into the gel. Digestion was started by heating to 37°C and stopped by chilling to 4°C, and the products were incubated overnight with 10 μl of 20 mM EDTA, 0.5% Sarkosyl, and 200 μg/ml proteinase K at 50°C, removed from the gel at 68°C, and extracted with phenol–chloroform.

Purified DNA (200 μg/ml) was digested as above with 0.002–0.01 U of MNase or 0.1 U of DNAase I per of μg DNA, treated with SDS and proteinase K, and extracted with phenol–chloroform.

DNA Restriction and Bal31 Digestion

Pieces (2 μg) of immobilized DNA were washed twice for 30 min in TE with 1 mM PMSF, washed 3 times in TE, incubated for 30 min in 100 μl of appropriate restriction buffer at room temperature, and then

restricted. Digestion with 5 U of enzymes per μg of DNA was done in 100 μl of recommended buffer for 16 hr at 37°C. Bal31 digestion of immobilized intact DNA was performed in 100 μl of buffer, containing 20 mM Tris–HCl (pH 7.5), 600 mM NaCl, 12 mM $MgCl_2$, 12 mM $CaCl_2$, and 3 U of Bal31. Samples were incubated with enzyme at 4°C for 30 min and then at 37°C for 30, 90, and 120 min. Reactions were stopped by 100 mM EDTA, 0.5% SDS, and 200 μg of proteinase K, and the DNA was digested overnight at 37°C, washed as above, digested with 5 U of HaeIII per μg of DNA, and deproteinized as above. Samples were stored in TE buffer before electrophoresis.

DNA Electrophoresis, Southern Blot, and Dot Blot Analyses

Agarose, polyacrylamide, and denaturing polyacrylamide–urea gel electrophoresis was performed as described elsewhere (Maniatis et al., 1982). DNA samples were mixed with 5 × loading buffer containing 10% (w/v) Ficoll, 0.1% SDS, 0.35% bromophenol blue, and 5 × TBE, and loaded onto 0.5 × 19.6 × 25.3 cm or 0.5 × 19.6 × 15.4 cm agarose, or 0.1 × 16 × 16 cm acrylamide gels in TBE. Agarose gels (0.8%–2%) were run at 4°C at ~4 V/cm. Nondenaturing (6%–8%) acrylamide gels were prepared from 19:1 acrylamide:bisacrylamide solution and run at 4°C at 5 V/cm. Denaturing gel samples were diluted in 90% formamide containing 1 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, heated to 95°C for 10 min, loaded onto 8% acrylamide–7 M urea gel, and electrophoresed at room temperature at 30 V/cm. Field reversal pulse electrophoresis was done using a programmable power inverter PPI-200 (MJ Research, Inc.) at 8 V/cm. During electrophoresis the forward and reverse pulses were varied from 0.15 to 4.8 s and 0.05 to 1.6 s, respectively. The gels were stained with 0.5 μg/ml ethidium bromide and photographed as described elsewhere (Vincenz et al., 1991). DNA was transferred to nylon for 12 hr at 0.5 A, using 0.5 × TBE using a Hoefer Transphor unit. The DNA was fixed and denatured using NaOH (Vincenz et al., 1991). Prehybridization was done in 1.5 × SSPE, 0.1 mg/ml Escherichia coli DNA, 1% SDS, and 0.5% Carnation nonfat milk at 50°C overnight, using a Hoefer hybridization oven. Hybridizations were done at the same conditions for 16 hr using one or more of four probes: TEL4, kinase-labeled synthetic oligonucleotide $(TTAGGG)_4$; RAT, random primer–labeled bulk rat DNA; and M, random primer–labeled 123 bp marker DNA or MspI-digested pBR322 DNA. Membranes were washed at 25°C for 10 min with 2 × SSC, 1 × SSC, 0.5 × SSC, 0.1 × SSC, and then for 30 min at 50°C with 0.1 × SSC, 0.1% SDS. If the last washing step was at 55°C, the telomere hybridization decreased more than 10-fold, indicating a high stringency at 50°C. If salmon sperm DNA was substituted for E. coli, the telomere hybridization significantly decreased, as expected. These precautions are important for eliminating nonstringent hybridization to nontelomere sequences that are found near the centromeres of certain cells such as CHO (D'Anna and Tobey, 1989). Dot blot analyses were performed as described by Vincenz et al. (1991), using the same prehybridization and hybridization conditions. After hybridization, the telomere probe was stripped twice with 0.1 × SSC, 0.5% SDS at 95°C for 15 min, and the membrane was hybridized with random primer–labeled bulk rat DNA. Membranes were exposed to preflashed X-OMAT film (Kodak) for 6–60 hr at −70°C. Autoradiograms and images of ethidium bromide–stained gels were digitized using a cooled CCD camera (Vincenz et al., 1991). The data were analyzed by using IGOR (Wave Metrics).

Nucleoprotein Gel Electrophoresis

Solubilized chromatin was digested at 37°C for different times with 0.3 U of MNase per μg of DNA in 10 mM HEPES (pH 7.5), 1 mM $CaCl_2$ mixed with an equal volume of glycerol and loaded onto a 6% acrylamide gel, prepared as described (Varshavsky et al., 1976) except using 10 mM Tris–acetate (pH 8.5). The gel was preelectrophoresed for 3 hr at 50 V and 2 mA, 5 μg of nucleoprotein was loaded, and the gel was electrophoresed at 150 V and 9 mA for 2 hr with intensively recirculating buffer (10 ml/min). The gel was stained with ethidium bromide, photographed, and incubated for 1 hr at 37°C with 500 ml of 1% Sarkosyl solution containing 10 mg of proteinase K, and then the DNA was transferred to the nylon membrane.

Preparation of H1-Depleted Chromatin and Protein Analysis

H1 depletion for sedimentation analyses was by the method of Marekov and Beltchev (1981). Rat nuclei (350 μg) in 200 μl of buffer C were incubated with 1 mM $CaCl_2$ for 5 min at 37°C and digested with 80 U of MNase for 3.5 min. The reaction was stopped with 10 mM EDTA, the nuclei were sedimented gently and lysed for 1 hr on ice in 280 μl Bio-Rex 70 buffer (1 mM EDTA, 8 mM Na_2HPO_4 [pH 7.0], and 0.1 mM PMSF), and the soluble chromatin was collected from the supernatant after microcentrifugation for 5 min. To remove H1 selectively, the chromatin was mixed with an equal volume of Bio-Rex 70 (Bio-Rad, 200–400 mesh), equilibrated with Bio-Rex 70 buffer, gently shaken for 2 hr, and then sedimented at 300 × g for 5 min.

H1 depletion for MNase studies of the nucleosome core particles was performed by conventional treatment with 0.5 M NaCl (Butler and Thomas, 1980). Chromatin in Bio-Rex 70 buffer (500 μg/ml, 0.5 ml) was mixed with an equal volume of 1 M NaCl, placed into a Centricon 100 centrifugal microconcentrator (Amicon), and spun at 1000 × g for 25 min. To remove salt, centrifugation was repeated after addition of 1 ml of TE buffer.

Electrophoresis in 15% polyacrylamide–SDS gels confirmed lack of protein degradation of nuclei, soluble chromatin, H1-depleted chromatin, and oligonucleosomes after sucrose gradient centrifugation and also confirmed complete removal of H1 after both depletion procedures. Quantitation of Coomassie staining was done using the CCD camera.

Analysis of Sedimentation Coefficients

Soluble chromatin (30–35 μg) in 250 μl was loaded onto cold 5%–31% isokinetic exponential sucrose gradients (9.3 ml mixing volume) containing 10 mM HEPES (pH 7.5), 1 mM EDTA, and 0.2 mM PMSF. Centrifugation was done at 4°C with an SW-40 rotor in a Beckman L5-65 at 29,000 rpm for 12 hr. Fractions of 100 μl were collected, incubated overnight at 37°C in 0.5% SDS, 0.5 mg/ml proteinase K, and then mixed with loading buffer. Electrophoresis, staining, electrotransfer, hybridization, and quantitation were carried out as above. Integrated fluorescence and densities of oligonucleosomes with the same molecular weights were analyzed using IGOR. The centers of the sedimentation profiles of bulk or telomere DNA were determined by Gaussian fit and agreed with centers chosen by eye from the fluorograms and autoradiograms. The profiles of fluorescence agreed quantitatively with the A_{260} recorded during fraction collection. The gradients were confirmed to be isokinetic and exponential by centrifugation of tobacco mosaic virus for different times and measurement of refractive index.

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