**Condensation of Rat Telomere-specific Nucleosomal Arrays Containing Unusually Short DNA Repeats and Histone H1**

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- **Telomeres** are specialized structures found at the termini of eukaryotic chromosomes and have important structural and functional roles in interphase, mitotic, and meiotic chromatin. Vertebrate telomeres consist of the sequence with unusually short and regular repeat lengths (Makarov, V. L., Lejnine, S., Bedoyan, J., and Langmore, J. P. (1993) Cell 73, 775–787; Lejnine, S., Makarov, V., and Langmore, J. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 2393–2397). In order to better define the specific structural features of telomere chromatin, we examined the condensation and H1 content of telomere nucleoproteins from rat liver. Velocity sedimentation analysis shows that telomeric nucleosome arrays condense with increasing ionic strength and molecular weight in a manner comparable with that of bulk chromatin despite the very short repeat length. However, these condensed structures do not exhibit the 100-base pair deoxyribonuclease II repeat characteristic of condensed bulk chromatin. Frictional coefficient calculations suggest that telomere-specific higher order structure is more compact than bulk chromatin. Nucleoprotein gel electrophoresis shows that telomeric dinucleosomes from soluble chromatin contain H1. Finally, direct isolation and analysis of telomere nucleoproteins from formaldehyde-cross-linked nuclei indicate the presence of core histone proteins and H1. These results are consistent with the view that a major fraction of the long telomeres of rat are organized as specialized nucleosome arrays with features similar but not identical to those of bulk chromatin.

- Telomeres are specialized structures found at the termini of eukaryotic chromosomes and have important structural and functional roles in interphase, mitotic, and meiotic chromosomes (1–4). Vertebrate telomeres consist of the sequence (TTAGGG)₉, comprising 0.01–0.2% of the genome (5, 6). The G-rich strand is oriented 5' to 3' toward the chromosome terminus (1–3). The length of telomeres varies from 3 to 150 kb, depending on the species of organism and the state of differentiation of cells (6). For example, telomeres are 2–20 kb in human cells (7, 8), 20–100 kb in rat (6, 9), and 100–150 kb in mouse (10, 11).

The nucleoprotein structure of rat liver telomeres was recently characterized by nuclease and sedimentation analyses (9). Micrococcal nuclease (MNase) digestion of nuclei and soluble chromatin revealed very regular arrays of closely packed telomere-specific nucleosomes with short repeat length (157 ± 2 bp) and unstable mononucleosomes. The internal structure of the telomere nucleosomes was probed with DNase I and found to be indistinguishable from that of bulk chromatin. Sedimentation analysis showed that telomere and bulk mononucleosomes and di- and tri-nucleosomes cosediment at low ionic strength and were both sensitive to BioRex 70 extraction, suggesting that H1 or H1-like protein(s) are present in the telomeric nucleosomes. However, nucleoprotein gels of telomere mononucleosomes did not reveal the presence of histone H1.

Telomere-specific nucleosome arrays are common to many higher eukaryotes. Human, monkey, mouse, chicken, mud puppy, turtle, trout, sea urchin, and plant telomeres were also found to have MNase repeat lengths much shorter than those of bulk chromatin (6, 12, 13). Measurements of the telomere and bulk repeat lengths from animal tissues and cell cultures showed variable telomere spacings from 151–205 bp, systematically about 40 bp shorter than the bulk spacing (6). This difference was also found in plant (13). In contrast, Muyldermans et al. (14) reported that telomere and bulk soluble chicken chromatin have the same spacing and that these telomere nucleosomes with normal repeat length were depleted in histone H5 (relative to histone H1).

Short repeat chromatin is not unique to telomeres. Rabbit and calf cerebral cortex neuron cells show short (162 and 168 bp, respectively) bulk repeat lengths (15, 16). Chromatin of the fungi Aspergillus nidulans also exhibits short repeat lengths of 159 ± 1 and 154 ± 9, respectively (17, 18). Ox neuronal (~162-bp repeat length) and A. nidulans chromatin were shown to contain H1 and an H1-like protein, respectively (19, 20), although neuronal nuclei have a low H1 content of 0.45 molecule of H1/nucleosome on average.

The protein composition of rat telomeres has not yet been directly studied, although the presence of the canonical nucleosome properties of periodic protection from MNase and DNase I and the comigration of telomere and bulk nucleosomes on nucleoprotein gels and sucrose velocity gradients argue strongly for the presence of the four core histones, H2A, H2B, H3, and H4, and suggest the presence of the linker histone, H1. Unlike telomeres of lower eukaryotes, which have nonnucleosomal components (21–24), at least 75% of the long rat telomeres are organized into nucleosomal arrays (9). Recently, a double strand-specific telomere DNA-binding protein, TRF, was cloned and shown by light microscopy to be localized near the ends of human chromosomes (25). A second nonhistone protein, XTEF, has specificity for the ends of single strand telomere DNA (26). The fact that the relatively short metazoan telomeres (from humans and sea urchins) have less distinct MNase ladders than long telomeres from the other species studied, can be interpreted as terminal regions of nonnucleo-

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1 The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); PMSF, phenylmethanesulfonyl fluoride; MNase, micrococcal nuclease; PAGE, polyacrylamide gel electrophoresis; TMV, tobacco mosaic virus.
somal structure or regions of irregular nucleosome arrays (6, 12).

Understanding how the nucleoprotein structure at the ends of chromosomes differs from the well-characterized nucleoprotein structure found throughout the length of chromosomes may lead to a better understanding of how terminal nucleoproteins might affect 1) the expression of genes adjacent to the telomeres (27, 28), 2) the localization of telomeres within the nucleus, and 3) the accessibility of this region to proteins that could be involved with the regulation of length and stability of chromosome ends and with telomere metabolism including replication, recombination, and repair.

The present study addresses the questions of whether 1) rat telomere-specific nucleosome arrays can condense into higher order structure similar to that of bulk chromatin and 2) rat telomere chromatin is associated with histone H1. The question of condensation was addressed using velocity sedimentation and DNase I cleavage analyses, and that of H1 content using nucleoprotein gels and analysis of purified telomere nucleoprotein from formaldehyde-cross-linked nuclei. Sedimentation analysis shows that telomeric nucleosome arrays condense with increasing ionic strength and molecular weight in a manner comparable with that of bulk chromatin despite the very short repeat length. Fractional coefficient calculations are consistent with greater compaction of the telomere chromatin. The condensed telomere nucleosome arrays do not exhibit the ~100-bp DNase I repeat characteristic of condensed bulk chromatin. Telomeric dinucleosomes contain histone H1 as determined by nucleoprotein gel electrophoresis. Finally, direct isolation and analysis of telomere nucleoproteins from formaldehyde-cross-linked nuclei indicate that in addition to the core histones, H1 is bound to telomeres. These results are consistent with the view that a major fraction of the long telomeres of rat are organized as specialized nucleosome arrays with features similar but not identical to those of bulk chromatin.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin (as hemisulfate), MNase, and N-laurylsarcosine (sarkosyl) were from Sigma. PMSF, EGTA, and proteinase K were from Boehringer Mannheim. Porcine spleen deoxyribonuclease II (DNase II) was from Worthington. Random prime DNA-labeling kit, T4 kinase, 100- and 123-bp DNA ladders, λ-HindIII digest, and λ-DNA were from Life Technologies, Inc. Zeta-Probe nylon membranes, and Bio-Rex 70 (200–400-mesh) ion exchange were from Bio-Rad. Oligonucleotide TEL4G, (TTAGGG)₄, was synthesized on an Applied Biosystems model 391 DNA synthesizer. Rats (Rattus norvegicus, CD VAF or C1 CD BR VAF) were from Charles River Laboratories. 125I was from Amersham Corp. pHur93 plasmid (containing 258 bp of human telomere DNA) was from American Type Culture Collection.

Preparation of Rat Nuclei and Soluble High Molecular Weight Chromatin—Rat liver nuclei from 3-4-month-old male outbred rats were prepared by a modified Hewish and Burgeyne method as described by Makarov et al. (9) and stored at ~70 °C for 1–2 months. The same batch of nuclei was used throughout the sedimentation studies. Three mg of nuclei in 1 ml of buffer A (15 mM Tris-HCl (pH 8.0), 60 mM KC1, 15 mM NaCl, 0.25 mM mercaptoethanol, 0.15 mM spermine, 0.15 mM spermidine, and 0.34 mM sucrose) were preincubated by adding 1 mg CaCl₂ for 5 min at 37 °C, and digested with 0.3 units of MNase for 30 s. After stopping the reaction with EDTA to 10 mM and gentle sedimentation of nuclei, soluble chromatin was released by lysing in 1 ml Tris-HCl (pH 8.0), 0.2 mM EDTA, 6 μM leupeptin, and 0.2 mM PMSF for 1 h on ice.

Preparation of Isokinetic Sucrose Gradients—Preparative sucrose gradients were prepared and centrifuged at low ionic strength as described (9). Briefly, 150–400 μl of soluble chromatin (24–43 μg of DNA) was loaded onto 5–31% isokinetic exponential sucrose gradients. The gradients were prepared at room temperature and placed at 4 °C for at least 30 min before loading the samples and centrifuging at 25,000 rpm for 9 h. Fractions of about 200 μl were collected from the top of the gradients by using a gradient fractionator (ISCO).

Two Pools of three to five fractions were combined from several gradients, concentrated 2 times by dialysis (29), using 60% sucrose, 10 mM HEPES (pH 7.5), 1 mM EDTA, 3 μM leupeptin, and 0.2 mM PMSF as the first dialysis buffer and 10 mM HEPES (pH 7.5), 1 mM EDTA, 3 μM leupeptin, and 0.2 mM PMSF as the wash buffer. The first dialysis was carried out for 3.5–4.5 h, and washing was carried out for 5–14 h with one change of buffer. Sample aliquots (containing chromatin at 0.14 μg DNA for P1 and 0.06 μg DNA for P2) were mixed with 200 μl of 10% formaldehyde at 4 °C with an equal volume of 10 mM HEPES (pH 7.5), 1 mM EDTA, and 0.2 mM PMSF containing 0, 40, 80, 120, and 160 mM NaCl. About 5 μg of tobacco mosaic virus (TMV) (gift from Dr. G. Stubbis) was added to each sample, and the samples (400 μl containing <30 μg of DNA) were immediately loaded onto isokinetic sucrose gradients at the same salt concentration. Analytical centrifugation was performed at 25,000 rpm for 4 h (P1) or 2.5 h (P2), followed by fractionation.

MNase Digestion of Pooled Fractions—P1 and P2 (150-μl aliquots) were adjusted to 3 mM CaCl₂, incubated for 5 min at 37 °C, and digested with 0.07 units of MNase/μg of DNA. Aliquots of 20 μl were collected at different times, quenched with 12 mM EDTA, and treated overnight at 37 °C with 0.5% (w/v) sarkosyl and 0.5 mM proteinase K.

Polyacrylamide Nucleoprotein Gel Electrophoresis Analysis—Nuclei were washed three times with either buffer A (see above) or the same buffer without polyamines but replaced with 3 mM MgCl₂, CaCl₂ was added to 1 mM, and nuclei were incubated for 5 min at 37 °C before digestion with MNase at 0.06 unit/μl final concentration (about 0.1 unit/μg of DNA) at 37 °C. Aliquots were collected after different incubation times, and the reaction was terminated with formaldehyde. Nuclei were lysed in 1 mM Tris-HCl (pH 8.0), 0.2 mM EDTA, and 0.2 mM PMSF for 1 h on ice. Soluble chromatin was H1-depleted as described using Bio-Rex 70 resins (9). Nucleoprotein gels were prepared by a modification of the methods of Varshavsky et al. (30) and Pennings et al. (31). Polyacrylamide nucleoprotein gels (5%, 30:1 acrylamide: N,N′-methylenebisacrylamide; 1.5-mm thickness) contained 20% (v/v) glycerol and 35 mM Tris-HCl, 5 mM boracic acid, 0.1 mM EDTA (pH 8.3; 0.056 × TBE (1 × TBE: 89 mM Tris borate, 89 mM boric acid, and 2 mM EDTA)). Preelectrophoresis was performed for at least 4.5 h at 4 °C and 2 mA with intensively recirculating buffer (>20 ml/min, 0.056 × TBE). Chromatins were prepared from four different samples (5–7 μg of DNA in 25 μl) were loaded in 20% glycerol, 0.4% bromophenol blue, and 0.05% (w/v) xylene cyanol. The gels were stained with 0.5 μg/ml ethidium bromide in 0.056 × TBE for 15 min, and images were digitized (9). The gels were then incubated in 0.5 × TBE containing 0.5% sarkosyl and 0.5 mM proteinase K for 1 h at 37 °C. The gels were washed twice for 15 min with 0.5 × TBE, and then transferred into 1× TBE or into nylon membranous in the same buffer (see below).

DNase II Digestion Analysis—Soluble high molecular weight chromatin was prepared using MNase (0.08 unit/μg DNA; 0.08 unit/μl; 15 min at 37 °C) as before. The MNase reaction was stopped using 12 mM EDTA; nuclei lysis was performed using 1 mM Tris-HCl (pH 7.0), 0.2 mM EDTA (pH 7.0), and 0.2 mM PMSF, and H1 depletion was carried out as described before (9). Soluble chromatin was digested with DNase II according to conditions established by Horz and Zachau (32) and Horz et al. (33). Briefly, soluble H1-containing or H1-depleted chromatin was digested with DNase II (428 units/ml; 3–5 μg/μl DNA) at 37 °C in 10 mM Tris-HCl (pH 7.0), 0.1 mM EDTA (pH 7.0) with or without 0.6 mM MgCl₂. The reaction was terminated by the addition of 22 mM EDTA, 0.5% sarkosyl, and 0.5 mg/ml proteinase K and overnight incubation at 45 °C. DNA was purified and electrophoresed on 1.5% agarose gels (see below).

Isolation and Analysis of Fixed Telomere Nucleoprotein—A special procedure was devised to isolate telomere proteins after partial cross-linking to telomere DNA in nuclei. Thawed rat liver nuclei were washed twice with 5 ml of buffer A (15 mM Tris-HCl (pH 7.5), 60 mM KC1, 0.25 mM NaCl, 0.25 mM mercaptoethanol, 0.15 mM spermine, 0.15 mM spermidine, and 3.4 mM sucrose) preincubated by adding 1 mg CaCl₂ for 5 min at 37 °C, and digested with 0.3 units of MNase for 30 s. After stopping the reaction with EDTA to 10 mM and gentle sedimentation of nuclei, soluble chromatin was released by lysing in 1 ml Tris-HCl (pH 8.0), 0.2 mM EDTA, 6 μM leupeptin, and 0.2 mM PMSF for 1 h on ice.

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1 mM PMSF for 30 min at 4 °C before adding restriction enzyme at 5 units/µg DNA and incubating 3 h at the recommended temperature. The film was transferred to elution buffer (0.5 × TBE, 0.1% sarcosyl, and 1 mM PMSF) for 5–10 min before electrosieving small, mainly nontelomeric nucleoprotein fragments, at 10 V/cm for 30 min at room temperature. Restriction digestion and electrosieving were repeated 3 times (once with MspI and twice with HinfI) to remove 99.6% of the nontelomere nucleoproteins, leaving 0.10% as telomere and 0.27% as nontelomere material trapped in the agarose. The film was melted in about 1 ml of agarase buffer (Boehringer Mannheim) at 68 °C for 10 min and then digested with 50 units of agarase at 45 °C for 30 min. To further enrich the telomere nucleoprotein, the digested mixture was concentrated to 1.5 ml using a SpeedVac concentrator (Savant), loaded onto a 35-ml Sephacryl S-1000 column equilibrated with 1% SDS, 1% sarcosyl, and 50 µg of carrier RNA, and eluted with 0.5 mM NaCl. The proteins were released from the DNA by overnight decross-linking at 65 °C (34). 125I labeling was performed as described by Biró and Reeder (35) with some modification. Briefly, 20 ng of decross-linked nucleoprotein sample was mixed with about 65 µCi of 125I in 90 mM boric acid, pH 7.6, 30 µM chlorine T, and 1% hexadecytrimethylammonium bromide and incubated at room temperature for 15 min. The reaction was terminated by adding dithiothreitol to 70 mM. Bulk histones (15 µg, as carrier protein) and NaI (1 mM) were added, and the mixture was precipitated with acetone overnight at −20 °C. Finally, labeled proteins from 0.1–0.5 ng of nucleoprotein were resolved on 15% SDS-PAGE as described (36).

The purity of the telomere nucleoprotein at different stages of purification were determined by electrophoresis of the DNA after protein decross-linking (see above), and quantification of the ethidium bromide fluorogram and autoradiogram after hybridization to TELG4 (see Ref. 37). Calibration of the fluorescence and hybridization signals was done using pHuR93 on the same gel. Before column chromatography telomere DNA only represented about 30% of the DNA in the lane. The high molecular weight component of the DNA was >90% telomere DNA (see "Results"). Size fractionation removed 90% of the low molecular weight component, as determined using kinase-labeled fixed nucleoprotein (data not shown). Thus, the final purity of the telomers in the excluded volume of the column was about 80%.

Electrophoresis and Southern Blotting—DNA samples were mixed with loading buffer (2% Ficoll, 0.02% SDS, 0.09% bromphenol blue) and electrophoresed in 1 × TBE at 4 °C and 2 V/cm in 1.8% agarose or 2.5–4.5 V/cm in 0.8–1.0% agarose. Electrophoresis, stringent hybridization, and detection, and nucleosome repeat length determination were carried out as in Makarov et al. (9) using kinase-labeled oligonucleotide TELG4.

Calculation of Sedimentation and Frictional Coefficients—The sedimentation velocity data were analyzed using TMV as an absolute sedimentation coefficient standard, because it has a well established molecular composition and sedimentation coefficient (38, 39) independent of ionic strength from 5 to 85 mM (data not shown). The standard sedimentation coefficients (s20,w) were calculated using the method described by Noll (40) and McCarty et al. (41) with modification. To calculate the standard sedimentation coefficient of the oligonucleosomes in a particular gradient fraction we used the equation,

\[ s_{20,w} = s_{20,w,TMV} (R/C) \]

(Eq. 1)

where \( s_{20,w,TMV} \) is the absolute standard sedimentation coefficient for TMV (192 S) (38); R is the ratio of the sedimentation distances of the oligonucleosomes to that of TMV in the same centrifugation tube; and C is a factor to compensate for the difference in density between chromatin and TMV, which affects the sedimentation velocity and prevents the gradients from being strictly isokinetic (41). The sucrose densities measured by refractometry were in agreement with those calculated with equations by McCarty et al. (41, 42). We measured \( s_{20,w,TMV} \) to be 163.4 S (S.D. = 4.1 S; n = 11).

The weight-average sizes of DNA in the gradient fractions were measured from agarose gels using a calibration graph generated from the molecular weight markers. When necessary, data were fit to Gaussian curves. The weight-average number of nucleosomes was calculated from the molecular weights using the measured nucleosome sizes (195 and 154 bp for bulk and telomere-specific nucleosomes, respectively).

Fractional coefficients, f, were calculated with the equation,

\[ f = \frac{m \cdot N \cdot (1 - v_{60,0})}{s_{20,w}} \]

(Eq. 2)

where m is the mass per nucleosome, N is the number of nucleosomes, and \( v_{60,0} \) is the partial specific volume, and \( p_{20,w} \) is the density of water. m is approximated as \( (M_{H1} + b M_{H1} + M_{DNA}/N_{A}) \), where \( M_{H1}, b M_{H1}, \) and \( M_{DNA} \) are the molecular weights of the nucleosome core proteins, H1, and nucleosome DNA, respectively. \( N_{A} \) is Avogadro’s number. The standard sedimentation coefficient for H1, \( s_{20,w} \), was assumed to be 1 for bulk nucleosomes and a variable for telomeres. The partial specific volume is as follows,

\[ v = \frac{m \cdot N_{A}}{M_{H1} + b M_{H1} + M_{DNA}/p_{DNA}} \]

where \( p_{prot} \) and \( p_{DNA} \) are the densities of protein (1.3 g/ml) and DNA (1.7 g/ml). The molecular masses of histones H2A, H2B, H3, H4, and H1 were assumed to be 13,960, 13,774, 15,273, 11,236, and 22,500 Da, respectively (43). Nucleosome cores were assumed to have two copies of each of the core histones.

RESULTS

DNase I Cleavage Pattern of Telomere Chromatin Is Unlike That of Bulk—DNase I has been shown to cleave nucleosome arrays at either 100- or 200-bp intervals, depending on whether the chromatin is condensed or extended (32, 33, 44). Therefore, we used DNase I to probe for differences between bulk and telomere-specific nucleosome arrays. In the presence of 0.6 mM Mg2+, a condition promoting condensation of H1-containing chromatin (43), DNase I cleavage of soluble long H1-containing and H1-depleted rat liver bulk chromatin was found to occur at about 200- and 100-bp intervals, respectively, (Fig. 1A), in agreement with previous results (32, 33, 44). However, when the same DNA was transferred and probed with TELG4, we found a 158-bp repeat in both H1-containing and H1-de-
completed telomere chromatin (Fig. 1B). The same repeat was obtained for H1-containing or H1-depleted soluble telomere chromatin when Mg²⁺ was absent during the digestion (data not shown).

Saccharomyces cerevisiae chromatin, which has a 165 ± 5-bp MNase repeat (45, 46), undergoes a cation-dependent condensation despite the fact that protein homologous to histone H1 has yet to be definitively identified in yeast (46). Treatment of S. cerevisiae nuclei with DNase II results in an 85 ± 5-bp repeat interval (32), showing that DNase II is capable of discriminating between condensed and uncondensed short repeat chromatin. Therefore, our DNase II results with rat telomeres might indicate the presence of an unusual structure.

Telomere-specific Chromatin Condensation as Analyzed by Velocity Sedimentation Is Comparable with That of Bulk Chromatin—Another approach for studying chromatin condensation into higher order structure has been velocity sedimentation analysis. Therefore, the sedimentation properties of long telomere nucleosome oligomers were determined as a function of ionic strength and compared with the well-known sedimentation properties of bulk chromatin. Soluble chromatin from nuclei mildly treated with MNase was fractionated according to size on a preparative low ionic strength sucrose gradient (Fig. 2A). Fractions were pooled, concentrated, adjusted to the appropriate ionic strength, and analyzed on sucrose gradients of equivalent ionic strength. SDS-PAGE ruled out the possibility of proteolytic digestion of chromatin or loss of histones (data not shown).

Telomere and bulk chromatin maintained the same repeat length during size fractionation, pooling, and dialysis (Fig. 2B). The bulk nucleosomal repeat length of 195 ± 2 bp is in agreement with reported values of 197 ± 2 and 198 ± 6 for rat liver (9, 43). The soluble telomere-specific chromatin repeat length is 154 ± 2 bp in agreement with the value of 157 ± 2 bp reported earlier (9) for total and soluble telomere chromatin. The average size for bulk and telomere DNA in P1 was 5.0 and 4.4 kb, respectively, and in P2 was 10.6 and 9.1 kb, respectively (data not shown). This implies a weight-average number of bulk and telomere-specific nucleosomes in P1 of about 26 and 28 nucleosomes, respectively, and in P2 of about 54 and 59 nucleosomes, respectively.

A typical molecular weight distribution after analytical velocity sedimentation of P1 is shown in Fig. 3A (bulk nucleoprotein) and Fig. 3B (telomere nucleoprotein). Fig. 3C shows a typical sedimentation profile after Gaussian fits for bulk and telomere oligonucleosomes from which the average sedimentation...
Figure 4: Ionic strength and size dependence of the sedimentation coefficients of bulk and telomeric nucleosome oligomers from P1 and P2. A, sedimentation coefficients of bulk (open symbols) and telomere-specific (closed symbols) oligomers from P1 (circles) and P2 (triangles) as a function of ionic strength. All sedimentation coefficients for bulk and telomere oligonucleosomes were calculated from the Gaussian average of the respective fraction profiles as shown in Fig. 3C. The exponent n in the simple power-law equation \( S_{20,w} \propto I^n \) is 0.14 for bulk 26-mer and 0.14 for telomere 28-mer with standard deviations of 0.01 for both. Butler and Thomas (47) found the exponent to be 0.16 ± 0.01. B, sedimentation behavior of bulk (open symbols) and telomere (closed symbols) oligonucleosomes with different average DNA size from P1 (circles) and P2 (triangles) at an ionic strength of 85 mM. The data for bulk and telomere-specific chromatin were fit to a straight line by a linear regression. C, estimation, using the linear fit in B, of the frictional coefficients of bulk (dashed line) and telomeric (solid lines) chromatin as a function of number of nucleosomes. Calculations for bulk assumed one H1 per nucleosome, whereas calculations for telomere chromatin were done for zero and one H1 per nucleosome. Closed circle shows the frictional coefficient for bulk rat liver chromatin containing an average of 76 nucleosomes, as calculated by Clark and Kimura (51).
linker length in agreement with the x-ray and electron microscopic data (52, 53).

SolubleTelomereChromatinContainsHistoneH1—Because histone H1 has been implicated and correlated with chromatin higher order structure, solubility, and function (43) and the calculation of the frictional coefficient depends upon the amount of H1 present, it is important to determine whether H1 is associated with telomere-specific nucleosome arrays. Low ionic strength polyacrylamide nucleoprotein gel electrophoresis has been used to address questions of nucleosome heterogeneity, protein composition, structure, and function (30, 54–57). Consequently, we used this technique to address the question of H1 content in soluble rat telomere chromatin.

H1 depletion increased the mobility of bulk mono- and dinucleosomes as expected, showing the presence of H1 on bulk chromatin (Fig. 5). Similar differences were observed for telomere nucleosomes, also indicating the presence of H1. (The bulk and telomeric bands were confirmed to be mononucleosomes, dinucleosomes and higher oligomers by stripping the proteins and separating the DNA in a second dimension (data not shown).) The similar effects of H1 stripping of bulk and telomere nucleoproteins on electrophoretic gels and sucrose gradients (9) suggests that H1 was present in telomeric chromatin, although the substitution of an H1-like protein sensitive to BioRex 70 treatment cannot be ruled out. Although the predominant form of the undepleted bulk mononucleosome contains H1, only about 20% of the undepleted telomere mononucleosome comigrated with the form containing H1. This could be directly related to the fact that the telomeric mononucleosomes do not form chromatosomes and are unusually sensitive to degradation into subnucleosomal particles (9). The spectrum of telomere electrophoretic forms of dinucleosomes and higher oligonucleosomes for depleted and undepleted samples is similar to that of bulk samples (Fig. 5C). Our results are consistent with those described by Varshavsky et al. (30), who find three discrete bands corresponding to fast (minor), intermediate, and slow migrating bulk dinucleosomes containing zero, one, and two molecules of H1, respectively. Telomere dinucleosomes exhibit the same three bands including a fast component that comigrates with the H1-depleted band. All three telomere bands have the same DNA size of about 310 bp on second dimension DNA gels (data not shown). Thus, in our preparations telomere and bulk dinucleosomes can be separated into three distinguishable forms that seem to have zero, one, and two molecules of H1, with comparable stoichiometry.

The nucleoprotein gels show some subtle differences between

\[3\] Soluble undepleted chromatin from nuclei washed with polyamine-containing buffer instead of Mg\(^{2+}\) showed more H1-lacking bulk and telomere dinucleosomes (data not shown), perhaps due to the polyamines displacing some H1.
telomere and bulk chromatin structures. 1) Telomere multimers (with and without H1) migrated faster than bulk multimers (Fig. 5C). This could be due to the shorter DNA sizes of rat telomere oligonucleosomes or to differences in the protein composition and/or charge/mass ratio. 2) In general, telomere bands had less of a slowly migrating "tail" than bulk (most obvious when comparing dinucleosomes: Fig. 5C). This could be the result of greater homogeneity in protein composition of the telomere chromatin, consistent with the very regular rat telomere nucleosome repeat. 3) Although H1-stripped telomere trimers migrated slightly faster than unstripped trimers, as expected, bulk trimers were anomalously retarded after depletion (Fig. 5C). Perhaps the H1-depleted bulk trimers exceeded a critical size for normal migration in 5% acrylamide gel that was not yet attained by the shorter linker telomere fragments. 4) Finally, in some undepleted telomere samples, we observed a weak, slowly migrating band between the dimers and trimers (Fig. 5B; asterisk). Whether this band is caused by binding of nonhistone proteins such as TRF (25) remains to be determined.

Histone H1 Is Associated with Rat Telomere in Nuclei—We also wanted to address the question of H1 binding to telomeres in nuclei, to avoid the potential effects of H1 exchange during solubilization of chromatin. Formaldehyde cross-linking has been extensively used to examine the composition and rearrangement of chromatin structure in vitro and in vivo (34, 58–60). Consequently, we applied this approach to partially cross-link protein to DNA in nuclei and then purified cross-linked-telomere nucleoproteins and assayed for the presence of H1 on protein gels. This method has the potential of positively identifying the species of H1 and other proteins bound to telomeres in nuclei.

Our method of isolating telomere nucleoprotein employs sequential enrichment by electroelution and size exclusion chromatography. After embedding nuclei in a thin agarose film and treating with formaldehyde, the DNA is subjected to repeated steps of restriction and electroelution. Telomeres, which are devoid of sites for frequently cutting restriction enzymes, remain as high molecular weight DNA in contrast to bulk DNA, which is reduced to lower molecular weight fragments. During electroelution, telomere nucleoproteins remain trapped inside the agarose (for unknown reasons), whereas most bulk fragments are removed. After melting the agarose, the nucleoprotein is subjected to size-exclusion chromatography to further enrich telomeres to about 80% purity (Fig. 6A; see "Experimental Procedures"). Decross-linking and iodination were followed by SDS-PAGE to resolve the protein components. Although iodination does not seem to affect the relative migration of the histones on SDS or charge-sensitive gels (61), we have found it difficult to quantify the amounts of protein present from the autoradiograms. We attribute this fact to uncertainties in the efficiencies of 1) the initial cross-linking, 2) inadvertent decross-linking during electroelution, melting of agarose, and other steps, and 3) handling and iodinating nanogram amounts of nucleoprotein. Therefore, although we feel confident about our identification of proteins that appear on the autoradiograms, we cannot quantify the abundance of those proteins or draw conclusions about missing protein bands(s).

Typical SDS-PAGE results from an iodination are shown in Fig. 6B, for the uncross-linked bulk, cross-linked and chromatographically purified nontelomere nucleoprotein, and electroelution and chromatographically purified telomere nucleoprotein. All three samples showed similar bands. Focusing on the telomere nucleoprotein, we observed two H1-like bands that comigrate with H1-1 and H1-2 from the bulk, although H1-2 is only weakly labeled in both fixed samples. In other experiments, H1-2 was more strongly labeled than H1-1 (data not shown). The results suggest that telomeres have the same two H1 variants as bulk chromatin but do not rule out a preference for one variant over another. It is noteworthy that rat has at least five H1 variants (62); thus, a more definitive assignment of the type of H1 variant observed here will require two-dimensional gels (i.e. SDS/acid-urea gels). Similarly, it is difficult to place importance on the absence of a strong H2A band in the telomere sample or the exceptionally strong intensity in the H2B region of the fixed nontelomere sample, especially because the DNase I and nucleoprotein gel experiments clearly indicate normal structure to the telomere nucleosome core. More definitive analysis of the protein content of telomeres will require two-dimensional electrophoresis and better methods of handling and labeling small amounts of nucleoprotein.

**DISCUSSION**

Vertebrate telomeres exhibit a number of specialized structural and functional features: 1) they define the ends of chromosomes and protect them against fusion and degradation (44); 2) they have highly conserved sequences and consistently exhibit an abnormally short nucleosomal spacing (6, 9, 12); 3) they are associated with the periphery of the nucleus or nuclear matrix (63–65); and 4) they might not participate in looped domains capable of supercoiling. Consequently, the organization of telomere-specific nucleosomal arrays into a higher order structure could potentially be different from that of bulk nucleosomes. In this study we have focused our attention to questions of structural condensation and histone H1 composition of telomere chromatin because of the unique organization of rat telomeres into closely packed nucleosomal arrays (6, 9, 12) and the importance of H1 in chromatin higher order structure (43).

Condensation—The direct measurement of chromatin condensation using velocity sedimentation shows that telomeric nucleosomes condense with increasing ionic strength in a manner comparable with that of bulk chromatin. However, we found three subtle differences between the structure of tel-
omere and bulk chromatin. 1) Telomere chromatin exhibits no significant change in the DNase II repeat pattern upon addition of magnesium or depletion of H1, unlike the behavior of bulk nucleoprotein. 2) The frictional coefficient calculated for large telomere oligonucleosomes is smaller than that of bulk with the same number of nucleosomes irrespective of the assumed amount of H1 per nucleosome. 3) The migration difference between the H1-containing and H1-depleted telomere multimers on nucleoprotein gels (a method also sensitive to conformation) is less than that for the corresponding bulk multimers. These three differences might be attributable to an increase in compaction of rat telomere chromatin relative to bulk and might be explained in different ways. 1) A more compact structure for telomere might prevent DNase II from cleaving at the second site within nucleosomes. 2) A smaller diameter and/or shorter length for telomere chromatin fiber could account for the reduction in frictional coefficient. This observation is also consistent with earlier observations that chromatin fiber diameter decreases with decreasing length of linker DNA (52, 53, 66). 3) The ~40-bp shorter linker length of rat telomere chromatin might cause less shape change upon H1 depletion than a similar depletion for bulk, causing the observed anomalies in migration on nucleoprotein gels. Although the differences between the behavior of telomere and bulk nucleosome arrays can be qualitatively rationalized in terms of these proposed subtle differences in structure, we cannot exclude the possibility of other structural differences between telomere and bulk chromatin. Because other vertebrate and invertebrate telomeres have different linker lengths, it should be possible to use them to test specific hypotheses about telomere structure. For instance, sea urchin sperm telomere nucleosomes, which have a 405-bp repeat, might have properties similar to those of bulk chicken erythrocyte nucleosomes, which have a 204-bp repeat. Histone H1 Composition—In contrast to sedimentation, low ionic strength polyacrylamide nucleoprotein gel electrophoresis fractionates macromolecules according to charge, size, and conformation. Using this method, we find that soluble telomeric dinucleosomes contain H1. From the number of different electrophoretic forms and their profiles, telomere and bulk dimers appear to have comparable H1 stoichiometries. Thus, short linker chromatin is not necessarily correlated with a deficiency in histone H1, as found in ox neurons (19). The clarity of telomere electrophoretic patterns suggests a more homogeneous protein composition for telomere chromatin. Analysis of the protein composition of isolated formaldehyde-fixed telomeres showed the presence of the core histone proteins and H1. This confirms that the nucleoprotein gel results were not artifacts due to exchange during handling of the soluble chromatin. Differential association of linker histones with telomeric nucleosomes was recently reported for chicken erythrocytes (14); however, those results are limited to a fraction of the soluble telomeres that apparently have the same repeat length as bulk chromatin (210 bp) and therefore might not be representative of the majority of the telomere nucleosomes, which have a nucleosome-specific repeat of 167 bp (6). It is important to estimate how much H1 exchange might affect our sedimentation and nucleoprotein gel results. Histone H1 and H2A/H2B are known to exchange in and out of chromatin in vitro at physiological concentration of monovalent cations. For example, in similar experiments by others at 4 °C there is <10% of H1 redistribution at about 8 m ionic strength over several hours (60, 67). Although H1 exchange during nuclear isolation cannot be ruled out and cannot be accounted for here, we attempted to limit H1 redistribution by preparing the nuclei in the presence of divalent cations (or polyamines) and performing the lysis, preparative sedimentation, and nucleoprotein gel electrophoresis at <12 m ionic strength at 4 °C as commonly done by others (30, 31, 47, 48, 60, 67). In all of the sedimentation experiments performed by us and others, extensive H1 exchange in chromatin can be expected at elevated ionic strength.

Similarly, it is important to consider the level of H1 exchange during the formaldehyde cross-linking used for immobilizing H1 in nuclei. Our fixation conditions were milder than those employed by J. Jackson and Chalkley (68), who find that formaldehyde did not induce redistribution of histones at pH 7.4 and that formaldehyde-treated free histones cannot rebind and fix to DNA or chromatin. Consequently, we believe that H1 exchange during and after formaldehyde cross-linking was unlikely.

The finding of histone H1 in very compact telomere nucleosome arrays raises questions about the role of H1 in chromatin with very short linker DNA, nominally 8 bp long. Histone H1 contains 65–67 lysine/arginine residues (depending on the H1 subtype (69)), of which ~60 residues are bound to bulk linker DNA, neutralizing ~60% of the linker DNA charges (51). The remaining positively charged residues of H1 are thought to be exposed to solvent (70) or bound to the folded globular domain of H1 or to core DNA of the same or neighboring nucleosome (71, 72). H1 in rat liver telomeres probably binds less strongly to linker DNA than in bulk chromatin due to the shorter linker, yet the nucleoprotein gels show about the same amount (or slightly more) H1 bound to the telomere dinucleosomes. The release of at least 45 basic residues of H1 from linker DNA in telomeres might promote greater internucleosomal bonding, perhaps stabilizing higher order structures. Maximally, only about 16 positive residues of H1 could be bound to the linker DNA of telomere nucleosome arrays, leaving at least 49 basic residues for binding to core DNA or water. Polyelectrolyte effects might also contribute to increased condensation of the telomere nucleosome arrays, because the net formal charge on each nucleosome is only ~110 in telomeres, rather than the ~192 in bulk chromatin. The greater neutralization on telomeres might also reduce the capacity of the nucleosomes to bind other basic proteins or might encourage the binding of acidic telomere-specific proteins such as TRF.

Our data are in agreement with the view that a major fraction of the long telomeres of rat are organized as specialized nucleosome arrays with features similar but not identical to those of bulk chromatin. It is impossible to predict, however, whether telomere-specific nucleosome arrays have functionally distinguishable structure in vivo. The repetitious DNA sequence and ability to form regular nucleosome arrays suggest that in vitro reconstituted telomere nucleoproteins might be good models for higher order structure amenable to study by biophysical techniques. Unfortunately, the current studies were unable to detect the nonnucleosomal components of telomeres, which should have important roles in telomere function, but possibly constitute only minor components of the long telomeres of rat.

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