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Unusual Chromatin in Human Telomeres

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We report that human telomeres have an unusual chromatin structure characterized by diffuse micrococcal nuclease patterns. The altered chromatin manifested itself only in human telomeres that are relatively short (2 to 7 kb). In contrast, human and mouse telomeres with telomeric repeat arrays of 14 to 150 kb displayed a more canonical chromatin structure with extensive arrays of tightly packed nucleosomes. All telomeric nucleosomes showed a shorter repeat size than bulk nucleosomes, and telomeric mononucleosomal particles were found to be hypersensitive to micrococcal nuclease. However, telomeric nucleosomes were similar to bulk nucleosomes in the rate at which they sedimented through sucrose gradients. We speculate that mammalian telomeres have a bipartite structure with unusual chromatin near the telomere terminus and a more canonical nucleosomal organization in the proximal part of the telomere.

Most eukaryotic telomeres are specialized nucleoprotein complexes with at least two functions (7, 67). First, these chromosomal elements can ensure the complete replication of chromosomal DNA by recruiting a telomere-specific DNA polymerase to chromosome ends (5, 28). This enzyme, telomerase, is thought to elongate telomeres and balance the gradual loss of terminal DNA that accompanies the replication of linear DNA by conventional DNA polymerases (29). The second known telomeric function is to provide a protective cap to the chromosome end. Nontelomeric DNA ends, such as DNA ends created by chromosome breakage or enzymatic cleavage, are unstable (40, 47, 55). In part, the instability of broken ends is due to DNA damage control factors that monitor and repair lesions in the genome (55). Natural chromosome ends apparently go undetected by this system, most likely because they are effectively concealed by the telomeric nucleoprotein complex. Telomeres may have additional roles in mitotic and meiotic chromosomes, but these functions are still poorly defined.

While the telomeric nucleotide sequences are known in representatives of most eukaryotic phyla, telomeric proteins have been identified in only a few species. The telomeres of *Saccharomyces cerevisiae* contain the double-stranded DNA-binding protein RAP1 (2, 10, 15, 34, 38). A different telomeric factor is found in several hypotrichous ciliates, whose telomere termini are encapsulated by a protein that recognizes the telomeric 3′ overhang (26, 27, 50; for a review, see reference 51). Although recent experiments suggest a similar telomere terminus binding activity in *Xenopus* eggs (12), it remains to be determined whether telomeric proteins are evolutionarily conserved.

Compelling evidence for stably bound telomeric proteins comes from the inspection of the overall chromatin structure of chromosome ends. In *Tetrahymena thermophila* macronuclear chromosomes, the terminal 400 bp are packaged in a large complex that does not resemble the nucleosomal chromatin (8, 11). The small DNA molecules in macronuclei of *Oxytricha nova* also end in a nonnucleosomal DNA-protein complex (26), and yeast chromosomes terminate with a large nuclease-resistant chromatin domain covering the 250- to 400-bp telomeric repeat region (66).

The idea that interactions at the telomere profoundly alter the chromatin structure of chromosome ends is consistent with the influence of telomeres on neighboring genetic elements (for a review, see reference 56). In *S. cerevisiae*, subtelomeric genes and replication start sites are down-modulated apparently by long range effects of the telomeric nucleoprotein complex (22, 25, 36). Possibly, telomeric silencing involves spreading of an altered chromatin state into subtelomeric domains (24, 53).

The nucleoprotein complex of vertebrate telomeres is still poorly defined. All vertebrate chromosomes carry a terminal array of TTAGGG repeats that ranges from ~1.5 kb in severely shortened human telomeres to 150 kb in the longest repeat regions of mouse telomeres (9, 19, 33, 43, 46, 60; for reviews, see references 18 and 28). To be fully replicated, this DNA presumably interacts with its cognate telomerase, which adds TTAGGG repeats to the 3′ end of the chromosome (44). We have recently identified two vertebrate DNA-binding activities that have properties expected for telomere factors. TRF is a double-stranded DNA-binding protein which binds the sequence $(TTAGGG)_{n \geq 6}$ (68). This factor is ubiquitously expressed in primate, rodent, and bird cell nuclei and could potentially bind along the length of the telomere (21, 68). A second factor (XTEF) recognizes the presumed structure of vertebrate telomere termini, a 3′ overhang of TTAGGG repeats (12). However, despite the specificity of TRF and XTEF for vertebrate telomeric DNA, direct evidence for their role at telomeres is not available. Indirect evidence for the association of telomeric DNA with a nuclear matrix protein was previously inferred from the attachment of human telomeres in lithium-diiodosalicylate-extracted nuclei (17).

This report describes the chromatin structure of telomeric DNA in cell lines of human and mouse origin. A recent analysis of rat telomeres has suggested that mammalian telomeres are packaged in normal nucleosomal arrays (39). However, our results indicate the presence of a specialized chromatin at the termini of human chromosomes.

MATERIALS AND METHODS

Cell lines. Suspension cultures of HeLa-S (ATCC CCL2.2; HeLa-II in reference 54) human cervical carcinoma and mouse J558 (ATCC TIB6) plasmacytoma cells were grown in roller

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bottles in Joklik's medium with 10% iron-supplemented bovine serum to 5×10^5 cells per ml. HeLa-L cells (described as HeLa and HeLa-I in references 19 and 54, respectively) and RD human rhabdomyosarcoma cells (ATCC CCL136) were grown in Dulbecco modified Eagle medium–10% iron-supplemented bovine serum to 10^6 cells per 10-cm-diameter dish. HL-60 human promyelocytic leukemia cells (ATCC CCL240) were grown in roller bottles in RPMI 1640–10% fetal calf serum to 5×10^5 cells per ml.

MNase digestion of chromatin. All manipulations were done at 4°C unless otherwise indicated. Suspension cells were harvested by centrifugation at 2,000 rpm for 10 min in a Sorvall RT6000 centrifuge and suspended in buffer A (100 mM KCl, 10 mM Tris-HCl [pH 7.5], 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM phenylmethylsulfonyl fluoride) at approximately 2×10^6 cells per ml. Adherent cells were trypsinized, suspended in growth medium, centrifuged as described above, and suspended in buffer A. The cells were subsequently washed twice with the same volume of buffer A, suspended in buffer A at 5×10^6 cells per ml, and lysed by the addition of Nonidet P-40 to 0.3% for J558 cells and 0.5% for HeLa cells. After addition of detergent, the cells were gently mixed and incubated for 15 min on ice. Nuclei were harvested at 1,000 rpm for 10 min, washed four times with buffer A, and suspended at 10^8 nuclei per ml of buffer A. Aliquots of 200 μl were digested with a range of micrococcal nuclease (MNase) concentrations (~2 to 6,000 U/ml; Boehringer Mannheim) at 30°C for 5 min. Nuclease reactions were terminated by addition of 1 volume of TES-protK (10 mM Tris-HCl [pH 7.5], 10 mM EDTA, 0.1% sodium dodecyl sulfate [SDS], proteinase K at 50 μg/ml) and incubated 2 h at 37°C. The DNA was isolated by extraction with phenol-chloroform and precipitation with isopropanol in the presence of 0.2 M sodium acetate (pH 5.5). The DNA was dissolved in 200 μl of 10 mM Tris-HCl (pH 7.5).

Formaldehyde-fixed chromatin. The method used is essentially as described in reference 58. Briefly, J558 and HeLa-S cells were stirred slowly in medium supplemented with 1% formaldehyde at $\sim 2 \times 10^5$ cells per ml for 16 h at 4°C. Cells were harvested by centrifugation and washed by stirring in Dulbecco modified Eagle medium for 16 h to remove all traces of formaldehyde. Subsequent processing of the cells and MNase digestion were as described above with the difference that formaldehyde-fixed cells were permeabilized by addition of 1% Nonidet P-40 and 1% Triton X-100. This step also served to monitor the effectiveness of the formaldehyde fixation, since cross-linked cells remain morphologically intact during the detergent treatment. After MNase digestion, the material was incubated with 1% SDS and 500 μg of proteinase K per ml at 37°C for 1.5 days to remove protein-DNA cross-links. Additional proteinase K was added after 1 day of incubation. Further processing of the DNA was as described above. Control samples that were not digested with MNase yielded high-molecular-weight DNA, indicating that no significant DNA degradation had occurred during the procedure.

Fractionation of MNase products. Electrophoresis and blotting procedures of agarose gels were described previously (19). Denaturing gel electrophoresis was carried out in 5% (19:1 acrylamide/bisacrylamide ratio) polyacrylamide gels containing 7 M urea. DNA samples (2 μg of mouse DNA and 6 μg of human DNA) were loaded in 95% formamide with 10 mM EDTA and 0.05% both xylene cyanol and bromophenol blue after denaturation for 5 min at 95°C. Electrophoresis was performed at 25 V/cm in 0.5× Tris-borate-EDTA. The gels were soaked in transfer buffer for 15 min (10 mM Tris-HCl [pH 7.5], 5 mM sodium acetate [pH 5.5], 0.5 mM EDTA [35]) and electroblotted onto Hybond-N (Amersham).

DNA size markers. For denaturing gels, two molecular weight markers were used. *Msp*I-cleaved pBR322 DNA was dephosphorylated and 5′ end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The $(TTAGGG)_{6n}$ ladder marker was prepared from the phosphorylated oligonucleotides $(TTAGGG)_6$ and $(CCTAAC)_6$, annealed at 0.1 μg/μl, and ligated with T4 ligase at 37°C for 12 h. Ligation products were extracted with phenol-chloroform, precipitated with ethanol, and dissolved in formamide loading dye for analysis on denaturing acrylamide gels (~10 ng per lane) (see above). This marker DNA was visualized with a labeled telomeric RNA probe (see below).

DNA and RNA probes and annealing conditions. A riboprobe was synthesized with SP6 polymerase in the presence of [α-^{32}P]CTP (3,000 Ci/mmol) on *Bam*HI-linearized pSP73.Sty11, which contains ~800-bp TTAGGG repeats (54). The resulting RNA is composed of the sequence $(CCCUAA)_{135}$ and a short polylinker segment. DNA probes were prepared by primed Klenow labeling using [α-^{32}P]dCTP (3,000 Ci/mmol). Klenow labeling of the 800-bp $(TTAGGG)_{135}$ *Eco*RI fragment from plasmid pSP73.Sty11 was primed with $(CCCTAA)_3$. Klenow labeling of a fragment containing two human *Alu* repeats (a gift from Catrin Pritchard, University of California, San Francisco) and a 800-bp *Pst*I fragment from the mouse *Bam*HI repeat (20) was primed with random hexamers. Labeled nucleic acids were isolated by Sephadex G-50 chromatography. The telomeric riboprobe was supplemented with 20 μg of tRNA for hybridization; *Alu* and *Bam*HI repeat probes were supplemented with 2 μg of sheared genomic DNA from human and mouse cells, respectively. Hybridizations were performed at 65°C for at least 6 h, using conditions described in reference 14. Final washes were at 65°C in 40 mM sodium-phosphate buffer (pH 7.2)–1 mM EDTA–1% SDS.

Sucrose gradient sedimentation of nucleosomes. The procedure for sedimentation of mouse J558 and HeLa-S chromatin through sucrose gradients was as previously described (48). Briefly, for each cell type, three 1-ml aliquots of nucleus suspension (2×10^8 nuclei per ml) were digested for 5 min at 30°C with 20, 200, and 2,000 U of MNase per ml, respectively. The nuclei were collected in an Eppendorf microcentrifuge at setting 4, and each was lysed in 0.5 ml of 1 mM EDTA and centrifuged at setting 14 for 5 min. The supernatant (1.5 ml) was adjusted to 5% sucrose–50 mM Tris-HCl (pH 8.0)–100 mM KCl and centrifuged through a 36-ml gradient of 5 to 30% sucrose in 50 mM Tris-HCl (pH 8.0)–100 mM KCl for 20 h in an SW28 rotor at 4°C at 27,000 rpm. Fractions of 0.8 ml were collected, precipitated with isopropanol, treated with proteinase K, extracted with phenol-chloroform, and again precipitated with isopropanol before analysis of the DNA on sequencing gels as described above.

RESULTS

Nucleosomal chromatin in mammalian telomeres. We examined telomeric chromatin in three mammalian cell lines with different telomere lengths. The BALB/c mouse plasmacytoma cell line J558 has extensive telomeric repeat regions, with most chromosome ends carrying between 20 and 150 kb of telomeric repeat DNA (Table 1) (33, 60). In addition, we used two subclones of the human HeLa cervical carcinoma cell line which differ in telomere length (17, 19, 54). One subclone (for clarity called HeLa-L here) carries fairly long telomeric repeat arrays of ~22.5 kb (Table 1). The other subclone, HeLa-S, has much shorter telomeres with only 2 to 7 kb of telomeric repeats (Table 1).

Nuclei from each cell line were digested with MNase, and

TABLE 1. Nucleosome arrays in telomeric chromatin

Cell line	Size range (kb) of telomeric repeat region[a]	Longest nucleosome oligomer[b]	
		Bulk	Telomere
J558, mouse plasmacytoma	20–150 (50)	9, 9, 9	7, 7, 9
HeLa-L, human cervical carcinoma	14–31 (22.5)	7, 8, 7	6, 6, 7
HeLa-S, human cervical carcinoma	2–7 (4)	8, 8, 9	3, 3, 4
HL-60, human promyelocytic leukemia	2.5–7 (4)	8	3
RD, human rhabdomyosarcoma	2–5 (3.5)	7	4

[a] The size range of the telomeric repeat region and the peak of the distribution (between parentheses) were determined by two methods. Telomeric *Hae*III fragments were fractionated by standard agarose gel electrophoresis and by contour-clamped homogeneous electric field electrophoresis (CHEF) and detected with a $(TTAGGG)_n$ probe. In addition, *Bgl*II fragments were fractionated by CHEF and hybridized to a subtelomeric probe (pTH2Δ) which detects chromosome ends with a *Bgl*II site ~2.5 kb proximal to the telomeric repeat region. Human telomeric repeat regions may include ~0.5 kb of TTAGGG-like sequences. The value for J558 telomeres was deduced from CHEF analysis using a $(TTAGGG)_n$ repeat probe and is in agreement with previously published values (see text).

[b] Nucleosomal arrays in MNase-digested chromatin were examined by fractionation in 1.5% agarose gels. Bulk nucleosomes were detected by ethidium bromide-UV staining and by hybridization to dispersed repetitive elements. Telomeric nucleosomes were detected by hybridization to $(TTAGGG)_{135}$ DNA. The multiple values given are derived from independent chromatin isolations. In each case, bulk and telomeric nucleosomes were examined on the same gel. For each cell line, the longest nucleosome oligomers were determined by using similar electrophoresis and hybridization conditions. In some experiments with HeLa-S cells, bands at the position of nucleosomal 5- and 6-mers were detected with the telomeric probe (see Fig. 1C). However, the restriction endonuclease sensitivity of these bands indicates that they are not derived from telomeric loci (see Fig. 2A and Results).

the isolated DNA fragments were fractionated on agarose gels. MNase fragments derived from telomeric chromatin were detected with labeled $(TTAGGG)_{135}$ DNA which predominantly anneals to telomeric loci in the cell lines used here. Telomere-related sequences also occur at several chromosome-internal sites in human and mouse DNAs (32, 33, 42, 60, 65), but the contribution of these loci to the TTAGGG repeat signal is minor. BAL 31 exonuclease experiments have shown that in BALB/c mouse and HeLa DNAs, the majority of the TTAGGG repeat signal is derived from chromosome ends (30, 33, 60).

The results in Fig. 1 illustrate that MNase generated extensive nucleosomal ladders in mouse telomeric chromatin. Long partial products reflecting nucleosomal arrays with up to nine subunits were detected in the telomeric loci (Fig. 1A). These ladders were similar to what is seen in bulk chromatin as visualized through hybridization with the dispersed *Bam*HI repeat (Fig. 1A) and ethidium staining (data not shown). Essentially the same result was obtained with the human HeLa-L cells with long telomeres. Telomeric chromatin from HeLa-L also yielded nucleosomal ladders that were similar to the bulk chromatin products, each containing partials with up to seven subunits (Fig. 1B and Table 1). These results are consistent with a canonical nucleosomal organization in the majority of the long telomeric repeat regions in these cells.

Altered chromatin in short HeLa telomeres. A different result was obtained with HeLa-S cells, which have telomeres that range from 2 to 7 kb (Table 1). In these cells, a discrepancy was noted between the MNase patterns in bulk and telomeric chromatin (Fig. 1C). While the bulk chromatin yielded the expected MNase ladders extending to partials with seven to eight nucleosomal subunits, the telomeric pattern revealed a less extensive MNase periodicity (Fig. 1C). Al-

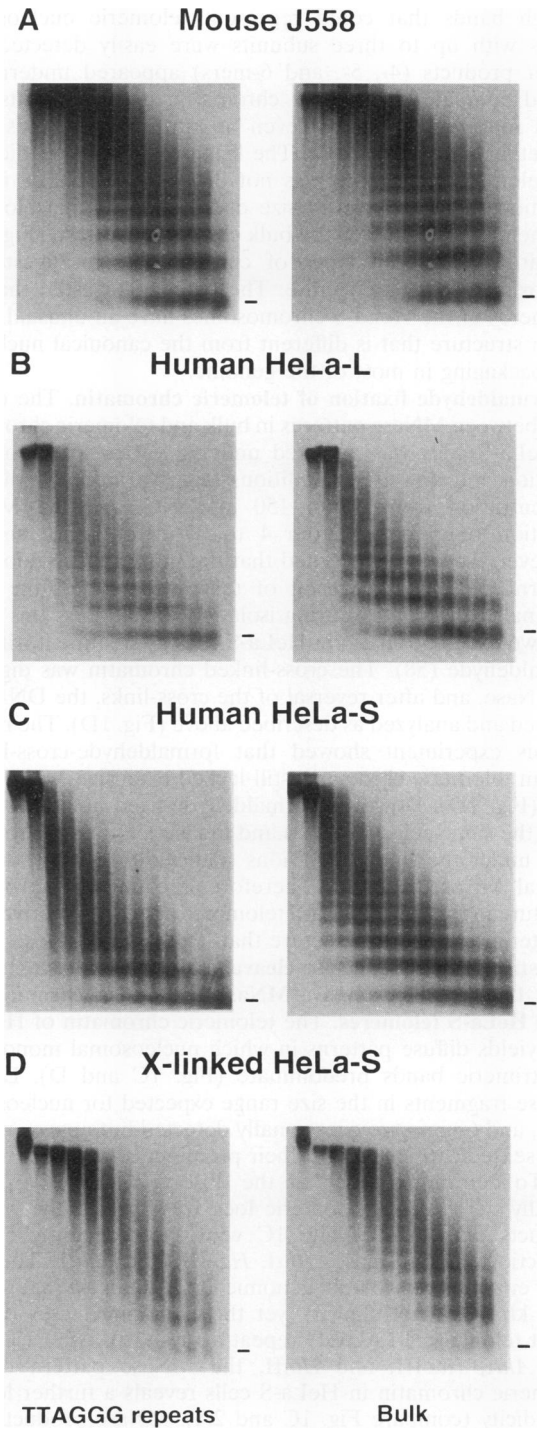

FIG. 1. Two kinds of telomeric chromatin. Nuclei from mouse J558 cells (A), human HeLa-L cells (B), human HeLa-S cells (C), and formaldehyde cross-linked HeLa-S cells (D) were treated with increasing amounts of MNase, and the resulting DNA fragments were fractionated on 1.5% agarose gels. The autoradiographs visualize telomeric DNA (left panels) as detected with an 800-bp DNA probe composed of TTAGGG repeats and bulk DNA (right panels) detected on the same membrane with probes for dispersed repeats (*Bam*HI repeats for panel A and *Alu* repeats for panels B to D). The MNase concentrations ranged from 2 to 4,000 U/ml in approximately twofold increments. The position of the mononucleosomal band is indicated to the right of each autoradiograph. Exposure times were adjusted to achieve comparable intensities.

though bands that could represent telomeric nucleosomal arrays with up to three subunits were easily detected, the longer products (4-, 5-, and 6-mers) appeared underrepresented compared with bulk chromatin, and fragments that could represent arrays of seven or more nucleosomes were altogether absent (Fig. 1C). The lack of MNase periodicity in the telomeric chromatin was not due to a difference in the digestion rate. The overall size distribution of the telomeric fragments was similar to the bulk chromatin pattern (Fig. 1C), indicating that both types of chromatin were digested at approximately the same rate. The results suggested that the telomeres of the HeLa-S chromosomes have an unusual chromatin structure that is different from the canonical nucleosomal packaging in most of the genome.

Formaldehyde fixation of telomeric chromatin. The difference between MNase patterns in bulk and telomeric chromatin of HeLa-S cells was detected under a variety of chromatin isolation and digestion conditions (e.g., variation of the salt concentration from 50 to 150 mM KCl and the MNase digestion temperature from 4 to 37°C; data not shown). However, we were concerned that the more diffuse telomeric pattern could be the result of rearrangement of the most terminal nucleosomes during isolation. To address this question, we fixed chromatin in HeLa-S cells by treatment with 1% formaldehyde (58). The cross-linked chromatin was digested by MNase, and after reversal of the cross-links, the DNA was isolated and analyzed as described above (Fig. 1D). The results of this experiment showed that formaldehyde-cross-linked human telomeric chromatin still lacked extensive MNase ladders (Fig. 1D). However, formaldehyde-fixed bulk chromatin from the same cells (Fig. 1D) and mouse telomeric chromatin fixed under the same conditions (data not shown) produced normal MNase patterns. Therefore, the unusual chromatin structure in the short HeLa-S telomeres appears to derive from an altered chromatin structure that is present in vivo.

Restriction endonuclease cleavage of MNase products confirms the lack of extensive MNase ladders in chromatin of short HeLa-S telomeres. The telomeric chromatin of HeLa-S cells yields diffuse patterns in which nucleosomal mono-, di-, and trimeric bands predominate (Fig. 1C and D). Longer MNase fragments in the size range expected for nucleosomal 4-, 5-, and 6-mers are occasionally detected but appear underrepresented compared with their prominence in bulk chromatin. To determine which of the observed MNase products actually derive from telomeric loci, we digested the MNase products displayed in Fig. 1C with the frequently cutting restriction endonucleases *Alu*I, *Hae*III, and *Hin*fI. Together these enzymes cleave bulk genomic DNA to an average size of <0.5 kb (data not shown), yet these endonucleases do not digest telomeric TTAGGG repeat arrays (19). After cleavage with *Alu*I, *Hae*III, and *Hin*fI, the MNase pattern of the telomeric chromatin in HeLa-S cells reveals a further loss of periodicity (compare Fig. 1C and 2A). MNase products that may represent mono-, di-, and trinucleosomal chromatin stretches are still detected in Fig. 2A, indicating the presence of some nucleosomes in the short telomeres of HeLa-S. However, the faint 4-, 5-, and 6-mer bands that were detected in Fig. 1C are removed by digestion of the MNase products with *Alu*I, *Hae*III, and *Hin*fI. (Note that several discrete higher-molecular-weight fragments can be observed in the first eight lanes of Fig. 2A. The presence of these fragments in DNA that has not been cleaved with MNase [first lane] indicates that they do not represent nucleosomal bands.) These results suggest that the faint 4-, 5-, and 6-mer products observed in Fig. 1C are not derived from telomeric loci and confirms the unusual organization of the chromatin in HeLa-S telomeres.

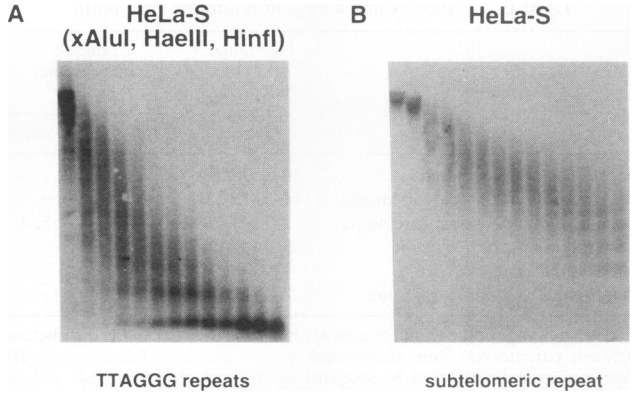

FIG. 2. Subtelomeric chromatin in HeLa-S. (A) Telomeric pattern in MNase products from HeLa-S chromatin that were subsequently digested with *Alu*I, *Hae*III, and *Hin*fI to remove subtelomeric sequences. The MNase samples and their analysis by blotting were identical to those in Fig. 1C. (B) Subtelomeric nucleosomal organization detected by the subtelomeric repeat probe pTH2Δ in MNase-digested HeLa-S chromatin (same samples as in Fig. 1C).

Nucleosomal organization of subtelomeric regions. Although the altered telomeric chromatin structure was detected with telomere-specific probes, we considered that subtelomeric chromatin might contribute to the diffuse MNase patterns. Results of two sets of experiments argue against this possibility. First, the digestion of MNase products with restriction endonucleases should dissociate much of the subtelomeric sequences from telomeric fragments. However, as shown in Fig. 2A, treatment with *Alu*I, *Hae*III, and *Hin*fI does not improve the periodicity of the telomeric MNase pattern. Since digestion with these enzymes removes most, if not all, subtelomeric sequences from the telomeric tracts (1, 9, 13, 16, 19), it is unlikely that subtelomeric chromatin contributes much to the pattern seen in Fig. 2A.

A second argument against a subtelomeric origin of the diffuse patterns comes from direct examination of subtelomeric chromatin. Human chromosomes contain several classes of subtelomeric repetitive elements (9, 13, 16, 19). One of these repeats (detected by clone pTH2Δ) is at least 4 kb in length and occurs directly adjacent to the telomeric repeat region at several chromosome ends in HeLa cells (19). We used the pTH2Δ probe to determine whether the unusual chromatin structure of HeLa-S telomeres extends into this subtelomeric domain. In Fig. 2B, the nucleosome structure of subtelomeric DNA was revealed by hybridization of pTH2Δ to MNase-digested HeLa-S chromatin. Long ladders of partial MNase products extending to the position of 7-mers were detected (Fig. 2B), indicating that the subtelomeric chromatin contained extensive nucleosomal arrays.

Remarkably, the overall digestion rate of the pTH2Δ subtelomeric chromatin was very slow compared with bulk and telomeric chromatin (compare Fig. 1C and 2B; data not shown). The exceptional MNase resistance was also observed with digestion of protein-free genomic DNA (Fig. 3), indicating that some feature of the subtelomeric DNA sequence (most likely its G+C content of ~80% [19]) inhibits MNase digestion. Because of the MNase resistance of the subtelomeric DNA sequence, it is likely that the nuclease had already removed most of the telomeric sequences from the subtelomeric nucleosomal products detected in Fig. 2B. Thus, the

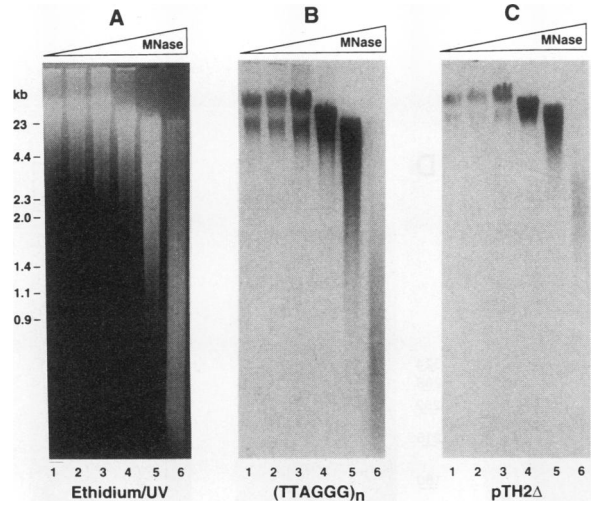

FIG. 3. MNase digestion of naked telomeric and subtelomeric DNA. Purified, protein-free DNA from HeLa-S cells was digested with MNase, analyzed by electrophoresis in a 1.0% agarose gel (A), and then hybridized with double-stranded TTAGGG repeat DNA (B) and subsequently with probe pTH2Δ for subtelomeric DNA (C). Lanes 1 to 6 contain DNA digested with 0, 0.2, 0.5, 1.5, 5, and 15 U of MNase per ml, respectively.

detected nucleosomal arrays detected in Fig. 2B probably extend proximally into the subtelomeric chromatin. These data demonstrate the presence of extensive nucleosomal arrays in subtelomeric DNA. Therefore, the unusual chromatin at the ends of HeLa-S chromosomes is derived from and restricted to the telomeric repeat domain.

Detection of unusual telomeric chromatin correlates with short telomere length. Comparison of HeLa-S and HeLa-L telomeric chromatin (Fig. 1B and C) suggested that the unusual chromatin structure is specifically detected in short telomeres. The correlation with telomere length was corroborated by the results with two additional human cell lines (RD and HL-60) which, like HeLa-S, carry telomeres that are in the range of 2 to 7 kb (data summarized in Table 1). While the MNase pattern for bulk chromatin in these cells showed the expected extensive periodicity, RD and HL-60 telomeres displayed the same unusual chromatin structure as HeLa-S telomeres. Thus, the data in Table 1 indicate that the unusual telomeric chromatin structure is specifically detected in cell lines with telomeres in the size range of 2 to 7 kb.

Sequence specificity of MNase and a predominance of homogeneous TTAGGG repeats in mammalian telomeres. Telomeric nucleosomes were subjected to high-resolution analysis on denaturing (7 M urea) polyacrylamide gels, and the size-fractionated DNA was detected by blotting and hybridization with a radiolabeled RNA complementary to TTAGGG repeats. As shown in Fig. 4, MNase-digested telomeric DNA from mouse cells produced strikingly repetitious ladders of doublet bands with a 6-nucleotide (nt) periodicity. Homogeneous 6-nt telomeric DNA ladders were also detected in naked mouse DNA (Fig. 4A, lane 3), in DNA from human chromatin (Fig. 4D), and with a $(TTAGGG)_4$ probe for the C-rich telomeric strand (data not shown). Horz and Alternburger (31) have shown that the sequence 5′TAG3′ is preferentially attacked by MNase (at the TpA phosphodiester bond), especially when surrounded by G/C-rich flanking sequences as in the $(TTAGGG)_n$ arrays. Furthermore, their data suggest that the nuclease removes the 5′-terminal A residue after the primary cleavage event. These activities are expected to generate a ladder of doublets with 6-nt periodicity composed of $(AGG\ GTT)_n$ and $5'GGGTT(AGGGTT)_n$, in full agreement with the doublet 6-mer ladder observed in the G-rich strand of MNase-digested telomeric repeats (Fig. 4 and 5).

The detection of a homogeneous 6-nt MNase telomeric ladder in human cells (Fig. 4D and 5D) is in agreement with previous indications that human telomeres are predominantly composed of long arrays of precise TTAGGG repeats and underscores the fidelity of human telomerase (1, 9, 19, 44, 45, 52). Since the same homogeneous 6-nt ladder is detected in MNase-cleaved J558 DNA, the remarkably long telomeres of these mouse cells are apparently also composed mostly of tandemly repeated precise TTAGGG repeats.

Short repeat size and MNase hypersensitivity of telomeric nucleosomes. Superimposed on the 6-nt doublet ladder, MNase digestion of telomeric chromatin yielded classes of preferential partial digestion products. Makarov et al. (39) determined that telomeric nucleosomes in rat liver contain ~157 bp DNA, which is much less than the DNA of bulk nucleosomes in the same tissue. Using in vitro-generated telomeric TTAGGG repeat ladders as size standards, we estimated that human and mouse telomeric nucleosomes have a similarly short repeat size of 150 to 165 bp (Fig. 4 and 5 and data not shown). However, the size heterogeneity of the telomeric MNase products precluded a more precise evaluation of their DNA content.

Telomeric mononucleosomes were found to be highly sensitive to overdigestion with MNase. The effect is illustrated in Fig. 4B and C, which show that mouse telomeric mononucleosomes were progressively degraded by MNase concentrations that hardly affected the composition of bulk core particles. While the bulk core particles were fairly resistant to a range of MNase concentration, no metastable telomeric core particle was detected in the 146-nt range or at another molecular size. The same hypersensitivity was observed with human telomeric nucleosomes derived from HeLa-S (Fig. 4D and E) and HeLa-L (data not shown) cells. The increased rate at which MNase digested the telomeric mononucleosomes was not due only to the sequence of telomeric DNA because the overall digestion rate of naked telomeric DNA was approximately the same as for bulk genomic sequences (Fig. 3). Therefore, some interaction between telomeric DNA and the histone octamer, or the presence of a specific (histone) protein or protein modification, must contribute to the increased MNase sensitivity of the telomeric mononucleosomes.

Cosedimentation of telomeric and bulk nucleosomes. Since the mammalian telomeric nucleosomes described above were unusual with respect to their short repeat size and MNase hypersensitivity, we needed to verify that the particles observed in telomeric chromatin were indeed nucleosomal. To compare the physical characteristics of telomeric and bulk nucleosomes, we fractionated MNase-digested J558 and HeLa-S chromatin fragments through sucrose gradients. In Fig. 5, fractions of two 5 to 30% sucrose gradients were inspected for the presence of telomeric and bulk nucleosome oligomers. The patterns obtained after annealing of the dispersed *Bam*HI repeat and a TTAGGG repeat probe to fractionated mouse chromatin were strikingly similar if not identical (Fig. 5A and B). The two probes revealed the same differential sedimentation rate of various nucleosome oligomers and mononucleosomal particles. Similarly, in the experiment with the HeLa-S chromatin, mononucleosomes and nucleosomal dimers from telomeric and bulk chromatin cosedimented (Fig. 5C and D). Note the typical 6-nt ladder pattern in Fig. 5D, indicating that these nucleosomal fragments were in fact derived from homoge-

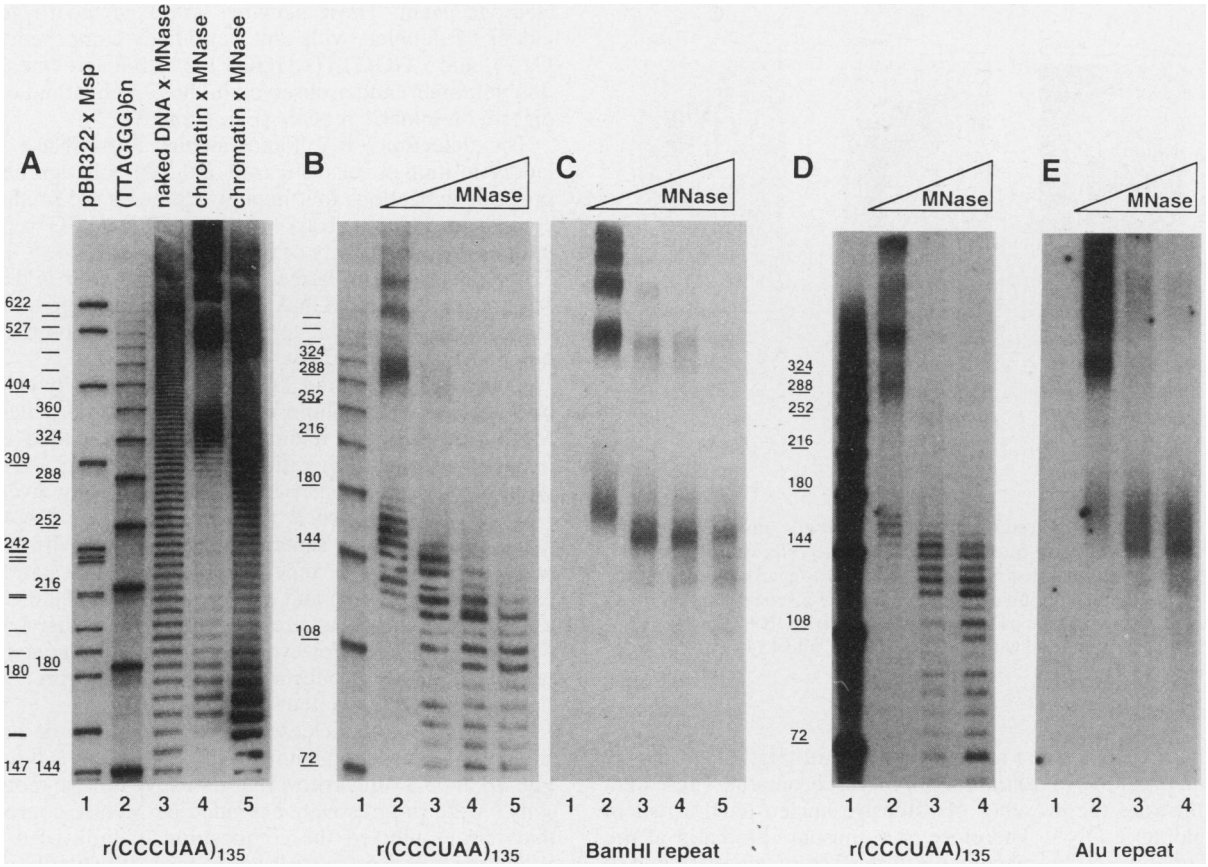

FIG. 4. Characterization of telomeric nucleosomes on denaturing polyacrylamide gels. MNase digestion products of naked mouse (J558) DNA, chromatin from J558 cells, and HeLa-S chromatin were analyzed on 7 M urea–5% polyacrylamide gels, electroblotted, and hybridized with a telomeric $(CCCUAA)_{135}$ riboprobe or probes for dispersed repetitive elements (*Bam*HI for mouse DNA in panel C and *Alu* for human DNA in panel E). (A) MNase digestion products from mouse J558 naked telomeric DNA (lane 3) and J558 telomeric chromatin (lanes 4 and 5) digested with MNase at 15 U/ml (lane 3), 10 U/ml (lane 4), and 160 U/ml (lane 5). (B) MNase hypersensitivity of mouse telomeric mononucleosomes. Mouse (J558) telomeric chromatin digested with 200, 800, 2,000, and 4,000 U of MNase per ml in lanes 2 to 5, respectively. (C) Detection of bulk nucleosomes in the same samples. (D) MNase hypersensitivity of HeLa-S telomeric mononucleosomes. HeLa-S telomeric chromatin was digested with 240, 1,900, and 3,750 U of MNase per ml in lanes 2 to 4, respectively. (E) Detection of bulk nucleosomes in the same samples. Exposure times were adjusted to achieve approximately equal intensities. The marker DNA in lane 1 of panel A is end-labeled *Msp*I-digested pBR322 DNA. Lane 2 of panel A and lanes 1 in panels B to E contain a telomeric $(TTAGGG)_{6n}$ marker ladder (see Materials and Methods) that is detected by hybridization with telomeric probes. Molecular sizes (in nucleotides) are indicated to the left of the autoradiographs.

neous TTAGGG repeat arrays. In this and other experiments, we have been unable to detect a difference in the sedimentation properties of telomeric and bulk nucleosomes, suggesting that the MNase digestion pattern observed in telomeric chromatin was indeed due to nucleosomes.

DISCUSSION

Telomeres are generally thought to function as specialized nucleoprotein complexes containing telomere-specific proteins. The DNA in this complex, identified in many eukaryotes, often displays conserved features such as its repetitive nature, a strand bias toward G residues, and a 3′ overhang (7, 67; for different telomeric DNAs, see references 4, 37, and 41). From the similarities of their target DNAs, it is anticipated that telomeric proteins are also evolutionarily conserved in many eukaryotes. In support of this idea, widely divergent unicellular organisms have telomeres with an unusual chromatin structure without canonical nucleosome arrays (8, 11, 26, 66). It was recently proposed that mammalian chromosomes depart from this rule and carry telomeres with canonical chromatin (39). However, we find that similar to the situation in ciliates and yeasts, human telomeres also have an unusual chromatin structure without extensive nucleosomal arrays. Therefore, we suggest that an altered chromatin structure is a common theme at eukaryotic chromosomal telomeres.

Mammalian telomeres contain MNase-hypersensitive nucleosomes with a short repeat size. Perhaps not surprisingly, we found that the exceedingly long telomeric repeat region (~20 to 150 kb) at the ends of chromosomes in *Mus musculus* is packaged in nucleosomes. Our data show that this repetitive DNA is largely composed of tandem TTAGGG repeats, resulting in a predominant TTAGGG specific pattern after digestion with MNase. The nucleosomal packaging of the TTAGGG repeats is inferred from several biochemical and physical properties of the chromatin. The size of the MNase products is in the range expected for nucleosomes, and the MNase digestion rate of telomeric chromatin is similar to that of bulk chromatin. Furthermore, telomeric chromatin fragments cosediment with bulk nucleosome oligomers, indicating similar physical properties. Makarov et al. provided extensive evidence for nucleosomes in rat telomeres (39), and our data

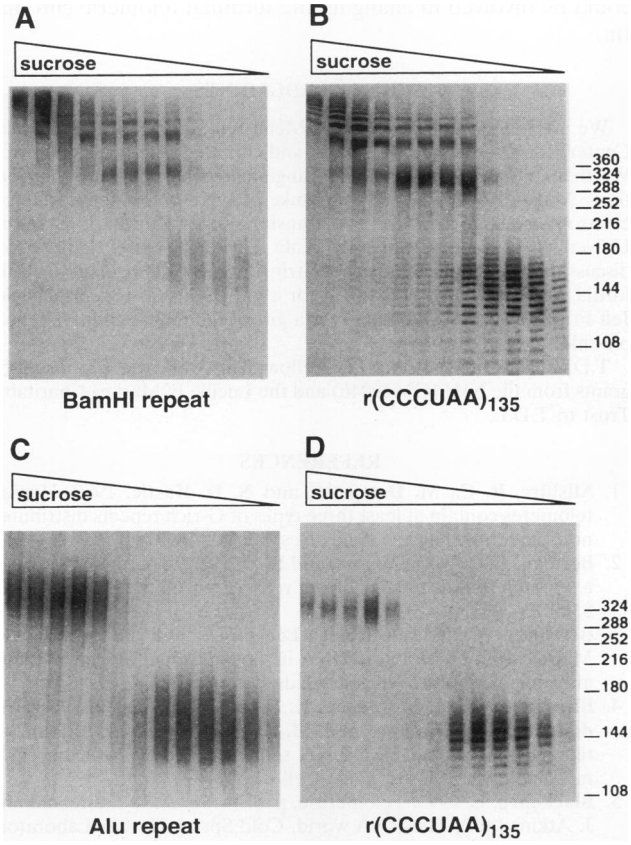

FIG. 5. Cosedimentation of telomeric and bulk nucleosomes on sucrose gradients. MNase-digested chromatin samples from mouse J558 (A and B) and human HeLa-S (C and D) cells fractionated by differential sedimentation through 5 to 30% sucrose gradients. DNA from pertinent fractions was electrophoresed on 7 M urea–5% polyacrylamide gels and then hybridized with probes for dispersed repeats (*Bam*HI in panel A and *Alu* in panel C) and a telomeric $(CCCUAA)_{135}$ riboprobe (B and D). To the right of the autoradiographs in panels B and D, the migration positions and fragment sizes (in nucleotides) are indicated for $(TTAGGG)_{6n}$ telomeric marker ladders migrating in parallel.

demonstrate nucleosomes on human and mouse telomeres. Thus, it seems that all mammalian telomeres contain at least some nucleosomal chromatin. Further analysis is required to establish whether these nucleosomes indeed contain standard histone octamers and whether telomeric histones carry specific modifications.

Telomeric nucleosomes in mammalian cells have two distinguishing features. First, these nucleosomes are among the smallest subunits reported so far (references 39 and 63 and this report). Their second distinctive characteristic is the greater sensitivity of telomeric mononucleosomes to degradation by MNase. MNase digestion of bulk nucleosomes yields core particles composed of the histone octamer and approximately 146 bp of DNA which persist as metastable units before further degradation (63). By contrast, the mononucleosomes from mouse and human telomeres are readily and continuously trimmed to yield subnucleosomal DNA fragments smaller than 146 bp. This hypersensitivity was not an intrinsic feature of the TTAGGG sequence, since MNase cleaved naked TTAGGG repeats at about the same rate as bulk DNA. Furthermore, it appears that the hypersensitivity is restricted to mononucleosomal particles, because the overall MNase digestion patterns of telomeric chromatin were similar to those of bulk chromatin. One possibility is that the DNA termini in telomeric core particles are more accessible to MNase, leading to their excessive degradation once the mononucleosome is released. Whether the effect is due to histone interactions with the unusual sequence of the telomeric repeats or dependent on a specific protein (for instance, a modified histone) remains to be determined. In this regard, it should be noted that the formation of stable nucleosomes in vitro is sequence dependent and can be enhanced by certain reiterated motifs (e.g., $[A/T]_3NN[G/C]_3NN$ [57]; for a review, see reference 62). Thus, it would be of interest to study the histone binding of vertebrate and other telomeric DNAs.

Unusual chromatin in short human telomeres. Inspection of the relatively short telomeres typical of most human tumor cell lines revealed an unusual chromatin structure in the TTAGGG repeat region. The altered chromatin was conspicuous on agarose gels, where MNase products display an abrogated ladder with reduced periodicity beyond the nucleosome trimer, merging into a diffuse pattern of heterogeneous fragments. The diffuse pattern of MNase products was detected with telomere-specific probes and persisted after degradation of bulk DNA with frequently cutting restriction endonucleases, indicating that the unusual structure originated from the telomeric repeat regions. Neighboring subtelomeric chromatin appeared to have a normal nucleosomal organization. The anomalous telomeric MNase products were found in three cell lines with telomeres in the range of 2 to 7 kb and in formaldehyde-fixed chromatin, arguing that the telomeric chromatin is in an altered state in living cells. Furthermore, the nucleosomal structure of short human telomeres is not a peculiarity of the TTAGGG repeats themselves, since the same sequence is packaged in more canonical chromatin in other cells.

Although the short human telomeres yielded a strikingly diffuse MNase pattern, they also produced fairly abundant nucleosomal monomers and dimers that cosedimented with bulk nucleosomes on sucrose gradients. These nucleosomes originated from the part of the telomere that is composed of precise TTAGGG repeats because their MNase digestion pattern showed the 6-mer doublet ladder typical of this DNA sequence. Other parts of the genome that anneal to telomeric probes, such as some chromosome-internal loci and the most proximal part of the telomeric region, contain sequence deviations that are incompatible with the observed MNase ladder. In addition to an abundance of TTGGGG and TGAGGG repeats in these loci, greater than 20% of their sequence represents repeats with altered length (e.g., TAGGG, TTAGGGG, and TTAAGGG) which should completely disrupt the homogeneous phasing of the ladder (9, 16, 32, 65).

Since these data indicate that short human telomeres contain nucleosomes, what then is the origin of the diffuse MNase pattern of this chromatin? We considered it unlikely that the aberrant MNase products are caused by the hypersensitivity to MNase or by the small and heterogeneous size of the DNA in telomeric nucleosomes. These explanations would not be consistent with the fact that the long telomeres in mouse and HeLa-L cells, which also have small, heterogeneous, MNase-hypersensitive nucleosomes, showed a canonical nucleosomal organization.

Instead, our data are more consistent with the presence of nucleosome-free regions in the unusual telomeric chromatin. We cannot predict the number and size of the nucleosome-free regions or their location with respect to the telomere terminus. A number of different configurations can be imagined, ranging

from organization with a largely nonnucleosomal structure perhaps at the telomere terminus to an arrangement in which the entire telomere is packaged in stretches of nucleosomes interspersed with nucleosome-free regions. We note that even short and infrequent disruptions in nucleosomal arrays may result in a considerable loss of MNase periodicity similar to what we observed.

Two kinds of telomeric chromatin. Long (20- to 150-kb) telomeres in mouse and rat cells appeared to be packaged in closely spaced long nucleosomal arrays and do not show the diffuse chromatin detected in short human telomeres. This difference is not due to species-specific variations, since human HeLa cells with long (~22-kb) telomeres also display a more canonical chromatin structure. Thus, while all short human telomeres showed an altered chromatin state, normal chromatin predominates in telomeres that are 20 to 150 kb in length.

Why was the unusual chromatin not detected in long mammalian telomeres? We favor a model in which all mammalian telomeres contain a short (few kilobases) domain with altered telomeric chromatin. In telomeres that are relatively short (e.g., 2 to 7 kb) such as the telomeres of HeLa-S cells, the unusual structure of this domain would dominate the overall chromatin patterns. We propose that the much longer telomeric repeat regions, such as the telomeres of mouse and rat cells, also contain a few kilobases of altered chromatin. In these cells, the unusual chromatin may have escaped detection (reference 39 and this report) because the majority of the telomere is packaged in canonical nucleosomal arrays. According to this proposal, two distinct chromatin structures can coexist in mammalian telomeres. The altered chromatin would be confined to a relatively small and constant domain of a few kilobases, while the remainder of the telomere would constitute a domain with more canonical chromatin that varies in size with telomere length. We envisage that the unusual chromatin pattern originates from a terminal telomeric domain, extending for several kilobases from the telomere terminus. Although other locations are not excluded, a terminal specialized domain would be in keeping with the normal nucleosomal organization of subtelomeric DNA, with the structure of telomeric chromatin in other eukaryotes, and with the expected activities of telomeres. It is of interest to note that the unusual chromatin proposed to delineate a terminal domain of mammalian telomeres is rather similar in appearance to the structure of centromeric chromatin in *Schizosaccharomyces pombe* (49).

What induces the altered chromatin? One possibility is that DNA near the telomere terminus is structurally distinct from the more proximal TTAGGG repeats. Examples of such alterations are the gaps found at the ends of *Tetrahymena* telomeres (6) and the novel base (β-D-glucosyl-hydroxymethyl-dU) specific to telomere termini in *Trypanosoma brucei* (3, 23). We note that the mere proximity of a DNA end also could change the terminal chromatin. For instance, nucleosomes at a chromosome end might be inherently more mobile and slide to form the altered telomeric chromatin. In this regard, the telomeric chromatin is somewhat reminiscent of mechanical or salt-induced alterations in chromatin (59, 61, 64). Under these conditions, nucleosomes are thought to slide together into closely spaced groups, leaving the rest of the DNA nucleosome free. While our cross-linking experiments argue against nucleosome movements during the isolation and processing of the chromatin, we cannot exclude the possibility that telomeric nucleosomes are mobile in vivo. Finally, the binding of a telomere terminus protein, for instance a protein similar to the telomeric proteins in hypotrichous ciliates (12, 27, 50, 51), could be involved in changing the terminal telomeric chromatin.

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