

Two Types of Telomeric Chromatin in *Tetrahymena thermophila*

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The telomeric d(GGGGTT)·d(AACCCC) repeat tracts (G_4T_2 repeats) in *Tetrahymena thermophila* macronuclei were shown previously to be packaged in a non-nucleosomal DNA-protein complex. Here, we demonstrate that these telomeric repeats, together with a short region of the immediately adjacent non-telomeric sequence, exist in two distinct types of chromatin. The non-nucleosomal complex (type I complex) comprises ~90 to 97% of telomeric DNA, has no apparent underlying periodic nucleosomal substructure, and includes the whole telomeric tract as well as the immediately adjacent sequence. Type II chromatin, comprising the remaining ~3 to 10% of the total telomeric DNA, consists of tightly packed nucleosomes clustered at the inner border of the telomeric tracts, with a periodicity of $154(\pm 3)$ bp. This packing is similar to that of telomeric nucleosomes in vertebrates. However, in contrast to the unstability of vertebrate telomeric mononucleosomes, the *T. thermophila* mononucleosomes were stable to micrococcal nuclease digestion. During the natural lengthening of the *T. thermophila* telomeric DNA tracts that occurs in vegetatively dividing cells, the overall ratio of type I and type II chromatin did not change. However, type I complex expanded with the length of the telomeric DNA repeat tract, and the number of telomeric nucleosomes increased from an average of one, up to three to four, per telomeric tract. This finding of telomeric nucleosomes in *T. thermophila* suggests that the difference between vertebrates and lower eukaryotes in telomeric chromatin structure is quantitative rather than qualitative. We propose that deposition of nucleosomes competes with non-nucleosomal complex formation on telomeric DNA, resulting in a sub-population of chimeric telomeres containing inner nucleosomes abutting a distal, variable length of type I complex.

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Introduction

Telomeres, the natural ends of eukaryotic chromosomes, are specialized DNA-protein structures that stabilize chromosomes and provide for their complete replication (McClintock, 1938; Muller *et al.*, 1991; reviewed by Blackburn, 1991; Sandell & Zakian, 1992; Zakian, 1995). Telomeric DNA in most eukaryotes consists of tracts of tandem repeats of a simple repeated sequence unit (reviewed by Henderson, 1995). In the ciliated protozoan *Tetrahymena thermophila*, this sequence con-

sists of d(GGGGTT)·d(AACCCC) repeats (G_4T_2 repeats; Blackburn & Gall, 1978). Sequence-specific DNA-protein interactions with these sequences are important for telomere function; for example, in *T. thermophila* and the yeast *Kluyveromyces lactis*, mutations that change telomeric DNA sequences alter telomere length regulation, nuclear and cellular morphology, and decrease cell viability (Yu *et al.*, 1990; McEachern & Blackburn, 1995; Romero & Blackburn, 1995; Gilley & Blackburn, 1996; Kirk *et al.*, 1997). Conversely, mutations in the *Saccharomyces cerevisiae* telomeric DNA-binding protein RAP1 in *S. cerevisiae* and *K. lactis* change telomeric DNA length and cause RAP1 allele-specific increases in chromosome loss, mitotic recombination and chromosome non-disjunction (Conrad

Abbreviations used: MNase, micrococcal nuclease; NTS, non-transcribed spacer; PMSF, phenylmethylsulfonyl fluoride.

et al., 1990; Lustig *et al.*, 1990; Kyrion *et al.*, 1992, 1993; Krauskopf & Blackburn, 1996).

The short (typically <1 kb) telomeres of lower eukaryotes have previously been reported to be packaged predominantly in a specialized, non-nucleosomal DNA-protein complex that covers the entire telomeric DNA repeat tract (Blackburn & Chiou, 1981; Edwards & Firtel, 1984; Gottschling & Cech, 1984; Budarf & Blackburn, 1986; Wright *et al.*, 1992). In contrast, in the telomeres of vertebrates, which are typically several kilobases in length, the bulk of the telomeric DNA is packaged as tightly spaced nucleosomes (Makarov *et al.*, 1993; Tommerup *et al.*, 1994). As there was no report of nucleosomes on the telomeres of lower eukaryotes, it was suggested that nucleosomal packaging of telomeric repeats is specific to higher eukaryotes (Makarov *et al.*, 1993; Tommerup *et al.*, 1994; Lejnine *et al.*, 1995). Subsequently, it was found that a human telomeric protein, TRF1, binds duplex human telomeric repeat DNA sequence *in vitro*, by means of a DNA-binding domain structurally related to that of Rap1p (Chong *et al.*, 1995a; Konig & Rhodes, 1997). TRF1 cytologically colocalizes with telomeres, and changes in TRF1 expression modulate telomere lengths in human cells in culture (Chong *et al.*, 1995b; van Steensel & de Lange, 1997). Human telomeric DNA remains attached to the nuclear scaffold and this nuclear association requires localization at a telomeric DNA tract to a chromosome end, providing further evidence for non-nucleosomal proteins(s) at human telomeres (de Lange, 1992).

The non-nucleosomal packaging observed in simple eukaryotes has been on telomeric repeat tracts that are much shorter than vertebrate telomeres. For example, in the polygenomic somatic macronucleus of *T. thermophila*, telomeric repeat tracts are typically a few hundred base-pairs in length (Blackburn & Gall, 1978). However, these telomeric tract lengths can vary in wild-type cells propagated under continuous vegetative growth (Larson *et al.*, 1987). When the macronucleus differentiates from a mitotic product of the germline nucleus at a given stage of development (reviewed by Bruns, 1986), its telomeres are all ~300 bp in length. Subsequent long-term, log-phase vegetative growth of *T. thermophila* results in gradual lengthening, in concert, of all the macronuclear G₄T₂ repeat tracts by 3 to 10 bp per generation until up to ~1 kb of telomeric DNA repeats are added (Larson *et al.*, 1987). In cells expressing certain mutant telomerase RNAs, and which therefore synthesize mutated telomeric DNA repeats, all macronuclear telomeres are also lengthened, or shortened, in concert (Yu *et al.*, 1990; Romero & Blackburn, 1995; E.H.B. & T. Ware, unpublished results).

Here, we report that *T. thermophila* telomeric DNA is complexed in two forms regardless of telomeric DNA length: most in the previously described non-nucleosomal DNA-protein complex (Blackburn & Chiou, 1981) and a small, hitherto

undetected, fraction in tightly packed, telomeric nucleosomes. As the mean length of the telomeric repeat tract steadily increases during cell divisions, the non-nucleosomal complex grows correspondingly, while the number of telomeric nucleosomes increases from an average of one nucleosome to an array of up to four nucleosomes. As with vertebrate telomeric chromatin, the *T. thermophila* telomeric nucleosomes are tightly spaced. We propose that the majority of *T. thermophila* telomeres contain only the non-nucleosomal complex, while a minority consist of the tightly packed telomeric nucleosomes clustered at the inner telomere-subtelomere junction plus a distal portion in the non-nucleosomal complex. These results suggest that the ratio of non-nucleosomal to nucleosomal complexes into which telomeres are packaged depends on a dynamic relationship between these two telomeric nucleoprotein forms. We propose that the telomeres of higher and lower eukaryotes do not differ in fundamental chromatin structure, but in their proportioning into different types of chromatin.

Results

Two types of telomeric chromatin package the growing *Tetrahymena* telomeres

To examine macronuclear telomeric chromatin at different stages of telomeric DNA growth, *T. thermophila* cells were mated to synchronize telomere length in the macronucleus (Larson *et al.*, 1987). The mated cells were diluted into three separate cultures and then progeny of the mating selected (see Materials and Methods). The cultures were kept continuously in early logarithmic growth phase, and every 40 generations cells were harvested and DNA prepared.

The length of telomeric DNA was determined by examining the telomeres on the high-copy number palindromic minichromosomes that carry the ribosomal RNA genes (rDNA) in the macronucleus (Figure 1A). The rDNA telomeres account for about half of the telomeres in the cell, and reflect the average mean length and length distribution of all the macronuclear telomeres (Larson *et al.*, 1987). The linear palindromic rDNA molecules also contain two arrays of mapped nucleosomes, separated by the rRNA transcription unit, as shown in Figure 1B: a subtelomeric array of three nucleosomes at each end of the rDNA minichromosome next to the telomeric G₄T₂ repeat tract (Budarf & Blackburn, 1986), and a seven nucleosome array of precisely positioned nucleosomes that span the central region of the rDNA (Palen & Cech, 1984; E.H.B. & Renata Gallagher, unpublished data). Whole-cell genomic DNA was digested with *SspI*, which cuts each rDNA subtelomeric region 105 bp from the terminal G₄T₂ (Figure 1C), Southern blotted and hybridized with 3'NTS-2, a subtelomeric probe specific for the rDNA (Figure 1C). The blots (Figure 2A) were scanned on a PhosphorImager, and the mean length of the telomeric G₄T₂

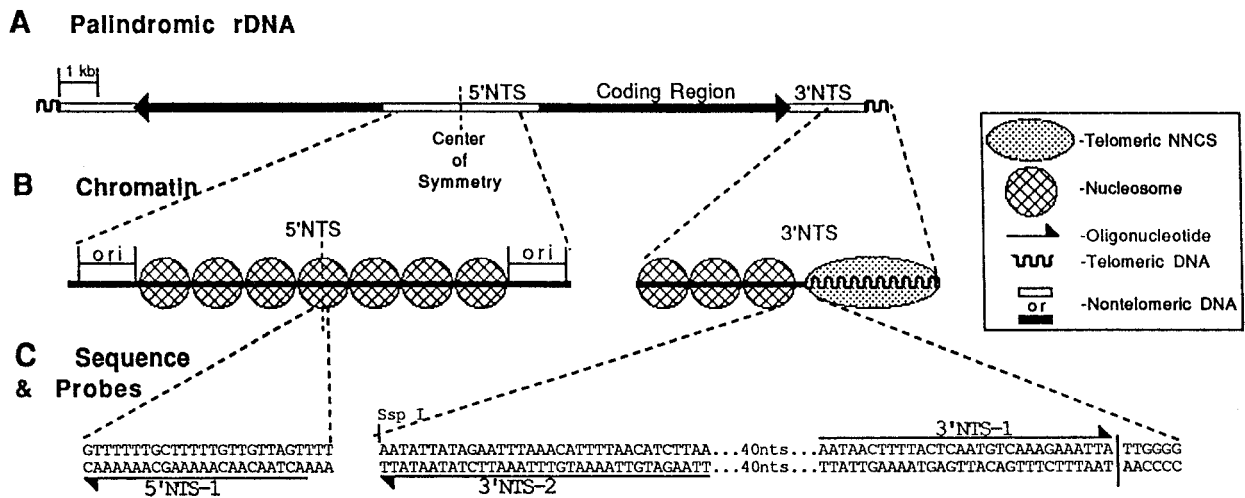


Figure 1. rDNA map, chromatin structure and rDNA-specific oligonucleotide probes. A, Diagram of rDNA structure. The rDNA 21 kb palindrome with its center of symmetry is shown. The white boxes are non-transcribed spacer regions (NTSs), the black arrows represent the transcription units, and the wavy lines are telomeres. B, Chromatin structure of the 5' and 3'NTS of the rDNA (Blackburn & Chiou, 1981; Budarf & Blackburn, 1986; Palen & Cech, 1984). Stippled oval, no-nucleosomal chromatin complex; cross-hatched circles, nucleosomes; ori, origin of rDNA replication (Kapler, 1993). C, Partial sequence of the 5' and 3' non-transcribed spacer regions (NTSs) of the rDNA and associated oligonucleotide probes. The sequence of the subtelomeric region from the recognition site of the enzyme *Ssp*I to the first telomeric repeat is shown. Arrows represent oligonucleotides that hybridize to this region, with the name referring to the oligonucleotide printed above.

repeat tracts calculated by subtraction of the 105 bp of subtelomeric DNA (Figure 2B). The three cultures showed very similar kinetics of telomeric DNA growth. Mean telomere length increased by ~3 bp/generation between 40 and 120 cell generations. After 120 generations, no further net growth of the telomeric DNA occurred (Figure 2B and data not shown), while the cells continued to divide at the same rate for several passages. These results are consistent with previous observations of telomeric DNA growth in *T. thermophila* (Larson *et al.*, 1987).

Nuclei were isolated from the same cultures and chromatin structure was probed using micrococcal nuclease (MNase) digestion. MNase is diagnostic for nucleosomes, cleaving preferentially in the DNA linkers between nucleosome cores revealing the spacing of nucleosomal arrays. In *T. thermophila*, the mean spacing of bulk macronuclear nucleosomes is 202 bp, and MNase digestion of bulk nucleosome cores protects a 145 bp limit digestion product (Gorovsky *et al.*, 1997). Similarly, in all species examined, extensive MNase digestion of bulk nucleosomes protects a well-defined limit DNA digestion product, 145 to 148 bp in size, reflecting the nucleosomal core structure (van Holde, 1989; Luger *et al.*, 1997). Previous work has shown that the entire tract of *T. thermophila* telomeric G_4T_2 repeats is protected from MNase digestion in a non-nucleosome complex (Blackburn & Chiou, 1981; Budarf & Blackburn, 1986). Nuclei were digested for 15 minutes at 30°C with 5 to 20 units/ml of MNase (see Materials and Methods). Purified DNA from these digests was electrophoresed in a 1.5% agarose gel and stained with ethi-

dium bromide. In the mock MNase treatment, DNA was not detectably digested, demonstrating that there was little or no endogenous nuclease activity under these conditions (Figure 3A, lanes 1, 8, 15 and 22). As the MNase concentration was increased, the periodic structure of bulk chromatin was first observable at five to ten units of MNase (Figure 3A, lanes 2, 10, 17 and 24) and at 200 units/ml of MNase, more than 90% of the bulk DNA was mononucleosome-sized or smaller (Figure 3A, lanes 7, 14, 21 and 28). While the ethidium bromide-staining signal approached an ~145 bp fragment, the smear of fragments below 145 bp in the 200 units/ml of MNase digestion indicated that double-stranded cuts had been introduced within the core particles at this MNase concentration (Figure 3A lanes 7, 14, 21 and 28). The ethidium bromide-staining pattern of the MNase titrations did not change significantly between generations 80 and 160 (Figure 3A; for example, compare lanes 10, 11 and 17, 18 and 24, 25).

To examine telomeric chromatin, the agarose gel was Southern blotted, immobilized with an extensive UV cross-linking procedure (see Materials and Methods), and hybridized with the ^{32}P end-labeled oligonucleotide $(C_4A_2)_4$ as a telomeric probe. The digestions with lower concentrations of MNase (5 to 50 units/ml) were characterized by a wide, heterogeneous smear of telomeric oligonucleotide hybridization signal (Figure 3B, lanes 2 to 5, 9 to 12, 16 to 19 and 23 to 26), similar to the protection from MNase described previously for *T. thermophila* telomeres (Blackburn & Chiou, 1981). We refer to this protection pattern as type I complex. Unlike bulk chromatin, the pattern of hybridization to the

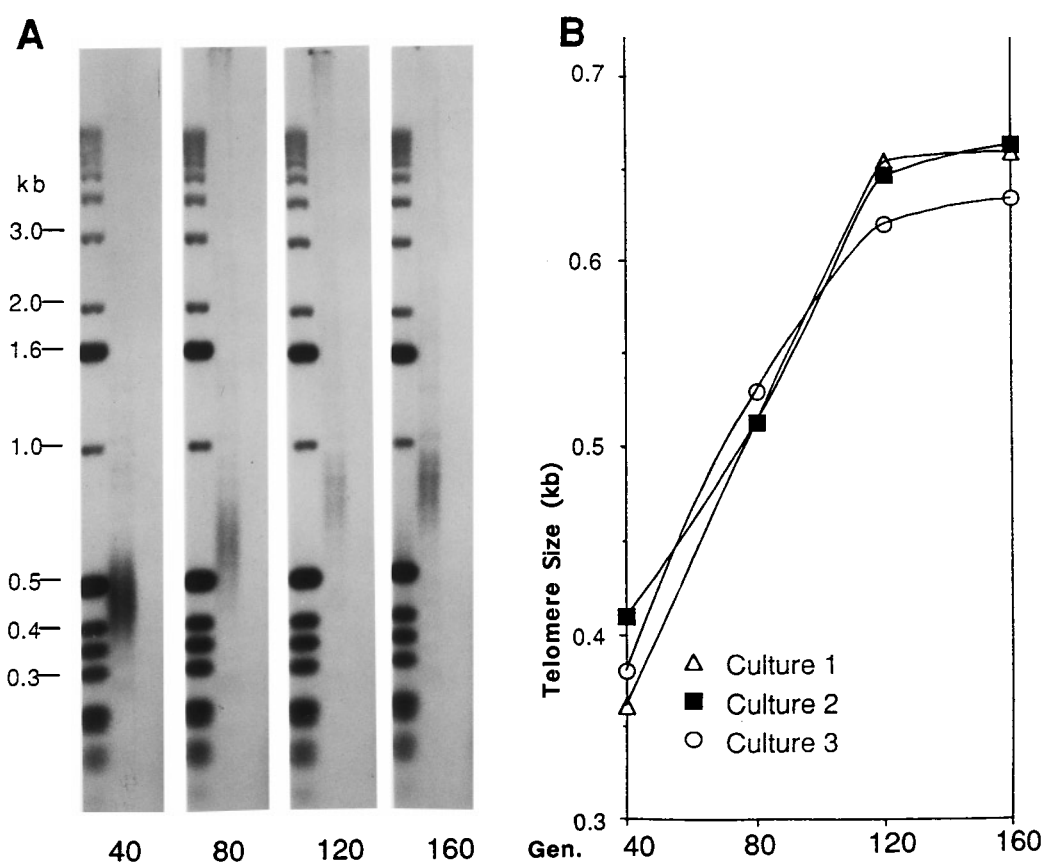


Figure 2. Increase in telomere lengths in vegetatively dividing *Tetrahymena* cells. Genomic DNAs were prepared from three separate cultures at different times during log phase growth (40, 80, 120, and 160 generations after mating), digested with *SspI*, and separated on a 1.5% agarose gel along with ^{32}P -labeled size markers (in kilobases, kb, left lane of each pair). The ^{32}P end-labeled DNA marker fragments were blotted with the genomic DNA digests. The gels were Southern blotted and hybridized using the ^{32}P -labeled 3'NTS-2 probe, specific for the rDNA telomeric restriction fragment (see Figure 1C). A, Southern blot of *SspI*-digested DNA from culture 1. B, Southern blots of DNAs from the three different cultures were analyzed using a PhosphorImager and the peak size (in kb, y-axis) of each telomere distribution plotted against the number of cell generations (x-axis).

telomeric probe changed dramatically between 40 and 120 generations (Figure 3B), with the molecular mass of the DNA in the type I complex growing as the telomeric DNA became longer. As shown in the PhosphorImager scans of the 25 units/ml of MNase digestions (Figure 4, top) the peak of the type I signal increased from ~600 bp to ~850 bp between generations 40 and 120, while the mean DNA telomeric tract length increased by 240 bp (Figure 2). As described below, when purified protein-free genomic DNA at the same concentration was digested with 25 units/ml of MNase, telomeric DNA was reduced to fragments smaller than the mean length of the telomeric tract (see below; Figure 8B, lane 6). Hence, intrinsic resistance of the telomeric DNA sequence to MNase digestion cannot account for the persistence of the telomeric type I signal during the MNase digestion of chromatin.

To eliminate the possibility that the observed growth of type I telomeric chromatin was due to subtle differences in the MNase digestions, electrophoresis, or blotting that were not detectable by

examination of bulk chromatin, we also used the same Nytran filter, after stripping the telomeric probe, to probe the chromatin structure of the 5' non-transcribed spacer region (5'NTS) of the palindromic rDNA molecule. This region contains a highly positioned seven nucleosome array, and the oligonucleotide probe 5'NTS-1 hybridizes to DNA packaged in the central nucleosome core in this array (Figure 1B and C). The 5'NTS is 8 to 10 kb away from either telomere, and separated from both telomeres by the highly transcribed rRNA gene. As expected, it remained unaffected by changes in telomere structure (Figure 3C). PhosphorImager scanning analysis demonstrated that the sizes of the 5'NTS nucleosomal DNA fragments did not vary significantly during the growth of the culture (Figure 4, bottom). The periodic structure of the 5'NTS region was easily observable at five to ten units/ml of MNase (Figure 3C, lanes 2, 10, 17 and 24). With each subsequent ~2-fold increase in enzyme concentration, the nucleosome profile was further digested, from the seven-step nucleosome ladder (Figure 3C, lanes 3, 11, 18 and 25), down to

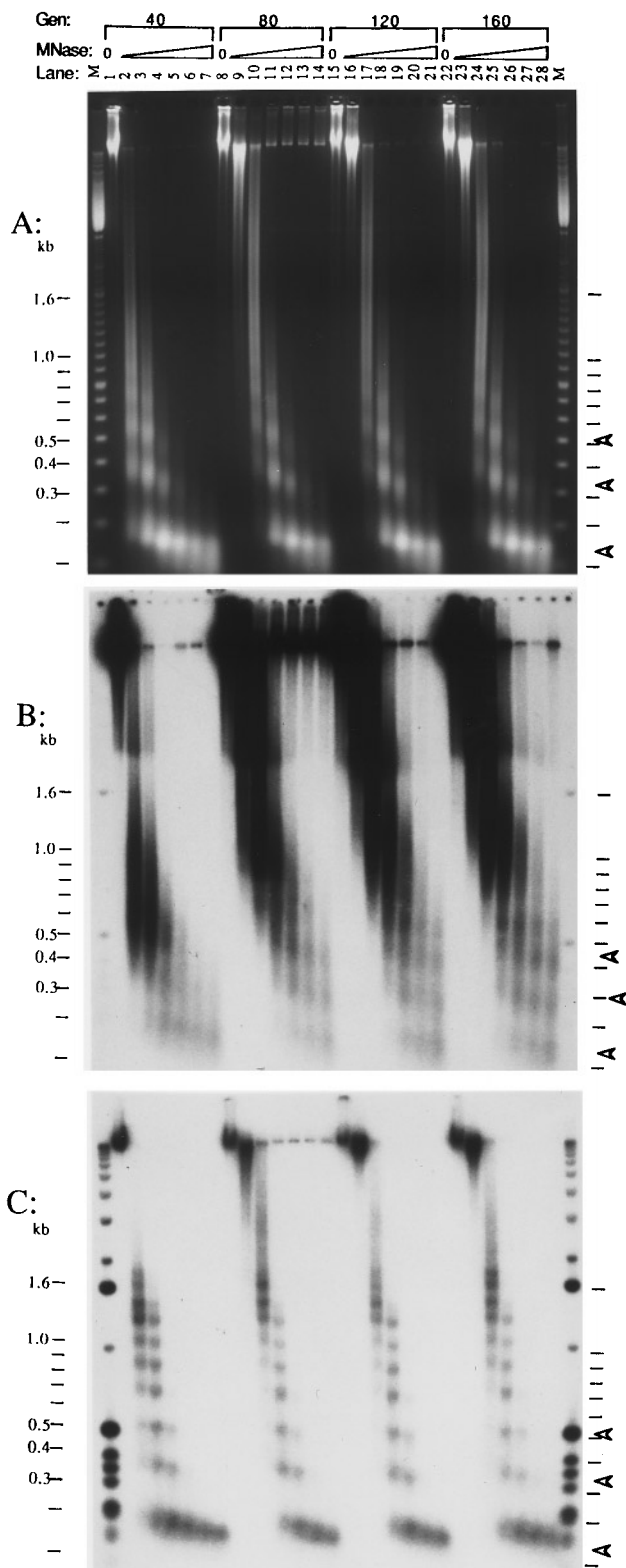


Figure 3. Micrococcal nuclease (MNase) titrations of nuclei isolated at different times after mating. Freshly isolated nuclei from log phase *Tetrahymena* cells were prepared after 40, 80, 120 and 160 generations of log phase growth after mating. Nuclei were treated with varied concentrations of MNase for 15 minutes (see Materials and Methods). DNA purified from these MNase-treated nuclei were separated on a 1.5 % agarose gel. The loadings (units/ml of MNase) were as follows:

over 90% mononucleosome core particles (Figure 3C, lanes 5, 13, 20 and 27). In summary, with long-term passaging, of *T. thermophila*, the type I-protected telomeric region grew as the telomeres lengthened, with no detectable effect on either bulk chromatin structure or the chromatin structure of the central region of the rDNA molecule.

Unexpectedly, after higher concentrations of MNase (50 to 200 units/ml) had digested away the great majority of telomeric signal, the dominant remaining signal that hybridized to the telomeric probe was a short ladder of bands (Figure 3B, lanes 5 to 7, 12 to 14, 19 to 21 and 26 to 28). This ladder (referred to as type II telomeric chromatin) suggested a subunit structure of this MNase-resistant chromatin associated with the telomeric sequence. Evidence that the type II chromatin consists of telomeric nucleosomal arrays is described below. At 40 generations, when the mean telomeric DNA length was 380 bp (Figure 2B), the ladder barely had two steps (Figure 3B, lane 7). As the telomeric repeat tract grew to 520 bp and 620 bp (Figure 2B), the ladder grew to three and then four strong steps (Figure 3B, lanes 14 and 15). Hence, the mean telomeric DNA tract length increased upon prolonged culturing out of 120 generations, and the type II signal responded by growth of the ladder. In contrast, there was no comparable change in the bulk or 5'NTS nucleosomes Figure 3A and C).

T. thermophila germline nuclei contain cross-hybridizing, non-terminal telomere-homologous sequences, including G₄T₂ repeat tracts found in non-telomeric locations (Cherry & Blackburn, 1985; Kirk & Blackburn, 1995). However, the total amount of hybridization signal observed in type II chromatin was too high to be accounted for by these low-abundance, internal sequences in the cell. More importantly, the increase in the number of steps in the type II ladder as the telomeric DNA grew, and the lack of any comparable effect on bulk or 5'NTS nucleosomes (Figure 3A and C), strongly argued that type II chromatin originates

0, lanes 1, 8, 15 and 22; 5, lanes 2, 9, 16 and 23; 10, lanes 3, 10, 17 and 24; 25, lanes 4, 11, 18 and 25; 50, lanes 5, 12, 19 and 26; 100, lanes 6, 13, 20 and 27; and 200, lanes 7, 14, 21 and 28. The MNase digestions of the 40 generation timepoint nuclei, which contained half the number of nuclei as the others, required half the concentration of MNase to digest chromatin to a similar extent (compare lanes 1 to 7, 8 to 14). M, 100 bp DNA ladder size markers, with sizes shown in kb. Open arrowheads, positions of telomeric mono-, di- and trinucleosomes (bulk, type II and 5'NTS chromatin in A, B and C, respectively). A, Agarose gel (1.5%) stained with ethidium bromide. B, Southern blot of the gel shown in Figure 3A probed with ³²P-labeled (C₄A₂)₄ oligonucleotide, the telomeric probe (see Figure 1C). C, Southern blot of the gel shown in Figure 3A probed with ³²P-labeled 5'NTS-1, which hybridizes to the 5'NTS of the rDNA (see Figure 1C).

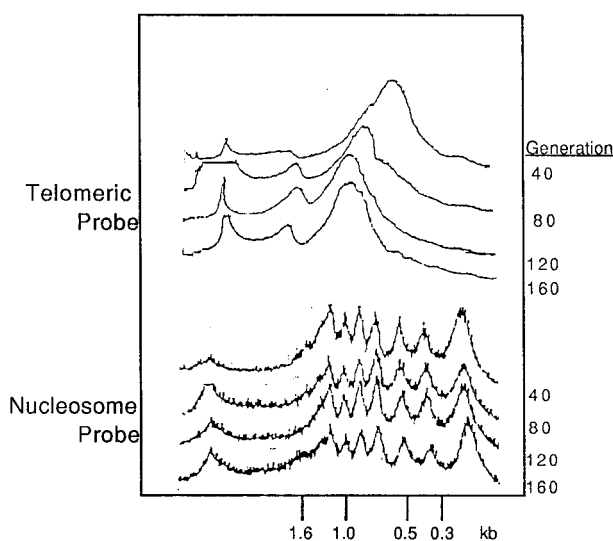


Figure 4. PhosphorImager scanning analysis of telomeric chromatin growth. The Southern blots in Figure 3B and C were scanned and the profiles of the 25 units/ml of MNase digestion (80, 120 and 160 generation time-points), and 10 units/ml of MNase digestion (40 generation time point) compared for both the nucleosomal and telomeric probes. The 10 units/ml of MNase digestion was used for the 40 generation sample to compensate for the twofold lower concentration of nuclei in this sample. Flat-topped peaks (undigested nuclear material) were cut off for ease of comparison of the profiles.

from macronuclear telomeric repeats rather than from these low-abundance, non-terminal sequences.

Spacing of the type II chromatin ladder

The fastest-migrating band of the type II ladder hybridizing with the telomeric probe comigrated at ~145 bp with the limit MNase product of bulk DNA. However, the spacing of the type II ladder (arrowheads, Figure 3) was tighter than that of the corresponding bulk nucleosome ladder. In the type II ladder, three subunits protected an ~460 bp fragment (Figure 3B, lanes 14, 21 and 28). We confirmed previous findings that duplex G_4T_2 repeat DNA shows normal mobility relative to duplex DNA size standards in agarose gel electrophoresis (Budarf & Blackburn, 1987; data not shown). Thus the close spacing of the type II ladder was not an artifact caused by aberrant migration of the telomeric DNA sequence.

The positions of the hybridization peaks corresponding to the steps of the type II ladders were used to determine that the periodicity of the type II ladder is $154(\pm 3)$ bp (where 3 bp is the standard deviation of the regression of the slope; see Materials and Methods). As internal controls, the spacing of the bulk and the 5'NTS nucleosomes were determined in the same way, and were $197(\pm 3.5)$ bp and $179(\pm 7.6)$, respectively, consistent

with published values (Gorovsky *et al.*, 1978; Palen & Cech, 1984). Thus the spacing of the telomeric type II subunits is significantly tighter than that of bulk nucleosomes in the macronucleus and germline nucleus of *T. thermophila*, or of nucleosomes in the 5' and 3'NTS or the rDNA (Palen & Cech, 1984; Budarf & Blackburn, 1986; Gorovsky, 1986).

Co-sedimentation of the limit digest product of type II chromatin with mononucleosomes

To test whether the hydrodynamic properties of type II telomeric chromatin were consistent with those of nucleosomes, nuclei were prepared from a fresh log phase culture of *T. thermophila* cells. After digestion of nuclei with MNase, soluble chromatin, released by hypotonic disruption of the nuclei, was separated by centrifugation through a 5% to 27% (w/w) linear sucrose gradient. DNA was purified from the fractions and electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide to determine the sedimentation of bulk nucleosomes (Figure 5A). The distribution of telomeric chromatin was analyzed by Southern blotting and hybridization with the telomeric $(C_4A_2)_4$ probe (Figure 5B). Consistent with previous findings (Blackburn & Chiou, 1981), a heterogeneous population of type I chromatin fragments sedimented ahead of trinucleosomes on the gradient (Figure 5B, lanes 2 to 6). In addition, hybridization of the telomeric probe to a ladder of three type II telomeric chromatin fragments was detected (Figure 5B, fractions 7 to 10), the smallest of which coincided with the position of the ~145 bp limit digestion product of bulk mononucleosomes (Figure 5A and B, lanes 9 and 10). Telomeric hybridization to this mononucleosome-sized band, relative to the second and third bands in the type II ladder, was weak because of under-representation of the mononucleosomes in this experiment. This was determined to result from the poor relative solubility of smaller telomeric chromatin fragments that was often observed in nuclear preparations (data not shown). We concluded that in sucrose gradient sedimentation this type II particle behaved as expected for a telomeric mononucleosome.

Nucleosome-like protection on one face of the telomeric DNA revealed by DNase I digestion of type II chromatin

To further characterize the structure of telomeric chromatin, nuclei were digested with DNase I, and the spacing of DNase I cleavages in telomeric chromatin was determined. In digestions of nuclei with high concentrations of DNase I, the majority of the telomeric hybridization signal was digested away, and only sub-nucleosomal core fragments (<145 bp) were substantially represented, as shown by Southern blotting analysis of the native DNA fractionated by agarose gel electrophoresis (data not shown; see Materials and Methods). Hence at

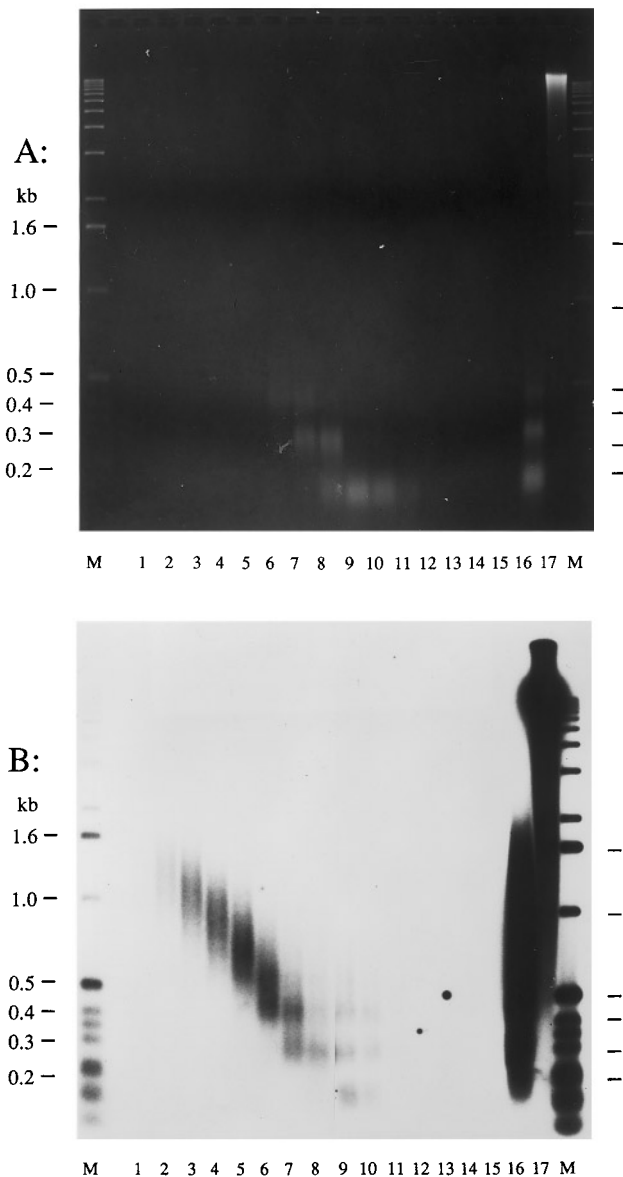


Figure 5. Sucrose gradient analysis of chromatin. MNase-digested nuclei were lysed and the soluble chromatin loaded onto a 5% to 27% (w/w) sucrose gradient. Fractions were collected from the bottom, the DNA purified from them, and separated on a 1.5% agarose gel. A, Ethidium-stained agarose gel. Fraction 1, bottom of gradient; fraction 15, top of gradient; lane 16, aliquot of the MNase-digested sample that was loaded onto the sucrose gradient; lane 17, DNA from nuclei incubated with buffer only (undigested sample). B, The gel was blotted and probed with ^{32}P -labeled $(\text{C}_4\text{A}_2)_4$. The marker lanes (M) show the 1 kb ladder of DNA fragments ^{32}P -labeled by polynucleotide kinase. Lanes 9 and 10 are an ~ 5 -fold lighter exposure of the gel than the rest of the lanes, to allow comparison of the position of the under-represented telomeric mononucleosome with the higher-order species.

these higher concentrations of DNase I, the digestion patterns were likely to reflect type II chromatin rather than the type I complex, which would

have been digested away. The purified DNA samples from DNase I digestions of either control protein-free DNA (Figure 6A) or nuclei (Figure 6B) were also fractionated on denaturing polyacrylamide Lutter gels (Materials and Methods). These gels suppress gel electrophoretic migration differences due to differences in base composition, allowing resolution of mixed sequence DNA according to fragment length (Lutter, 1979). The DNA was electroblotted to Nytran, immobilized and probed with the telomeric $(\text{C}_4\text{A}_2)_4$ probe (Figure 6A and B). At low DNase I concentrations, the pattern in cleavage in denaturing gel electrophoresis showed maxima spaced 6 bp apart (Figure 6B, lanes 2, 3), similar to the pattern in naked DNA (Figure 6A). However, the most extensive DNase I digestions revealed clusters of cleavage maxima spaced ~ 10 to 11 bp apart (Figure 6B, lanes 4 to 7). Such a periodicity of DNase I cleavages has been reported for many instances of DNA bound to a nucleosomal, other protein or inorganic surface (Noll, 1974; Finch *et al.*, 1975; Rhodes & Klug, 1980; Serrano *et al.*, 1993). In the area of the gel with highest resolution, it was possible to map the cleavage maxima to within one or two nucleotides, by digital scanning of the autoradiogram of the 25 units/ml of DNase I lane (Figure 6B, lane 4) and counting bands relative to an established marker ladder (Figure 6B and data not shown). This analysis revealed major cleavages separated by 52 or 53, 62 or 63 and 72 or 74 nucleotides, indicative of a 10.5 base cleavage periodicity. These results are consistent with those reported for *Tetrahymena* nucleosomes (Mathis & Gorovsky, 1978; van Holde, 1989). The DNase I cleavage pattern also contained a weak cleavage at ~ 60 bp and stronger cleavages at ~ 70 and ~ 80 bp (Figure 6B). This pattern in particular is characteristic of bulk nucleosome cores in *Tetrahymena* (Gorovsky *et al.*, 1977; van Holde, 1989), and provides further evidence consistent with nucleosomal packaging of a fraction of the telomeric DNA.

In summary, the hydrodynamic properties in sucrose gradients, the protection of a core-sized particle of ~ 145 bp, the multiple-step ladder of subunits, and the 10.5 base cleavage periodicity of DNase I cleavage were all consistent with packaging of a fraction of *T. thermophila* telomeric DNA in nucleosomes.

Unchanged relative abundance of types I and II telomeric chromatin during growth of telomeric DNA

Telomeric nucleosomes in mammalian systems have been reported to be more susceptible to MNase digestion than bulk nucleosomes (Makarov *et al.*, 1993; Lejnine *et al.*, 1995). In contrast, in the MNase digestion titrations of *T. thermophila* nuclei, the type II signal was highly resistant to further MNase digestion, although the type I signal disappeared quickly. PhosphorImager scanning of the titrations in Figure 3 showed that over 90% of the

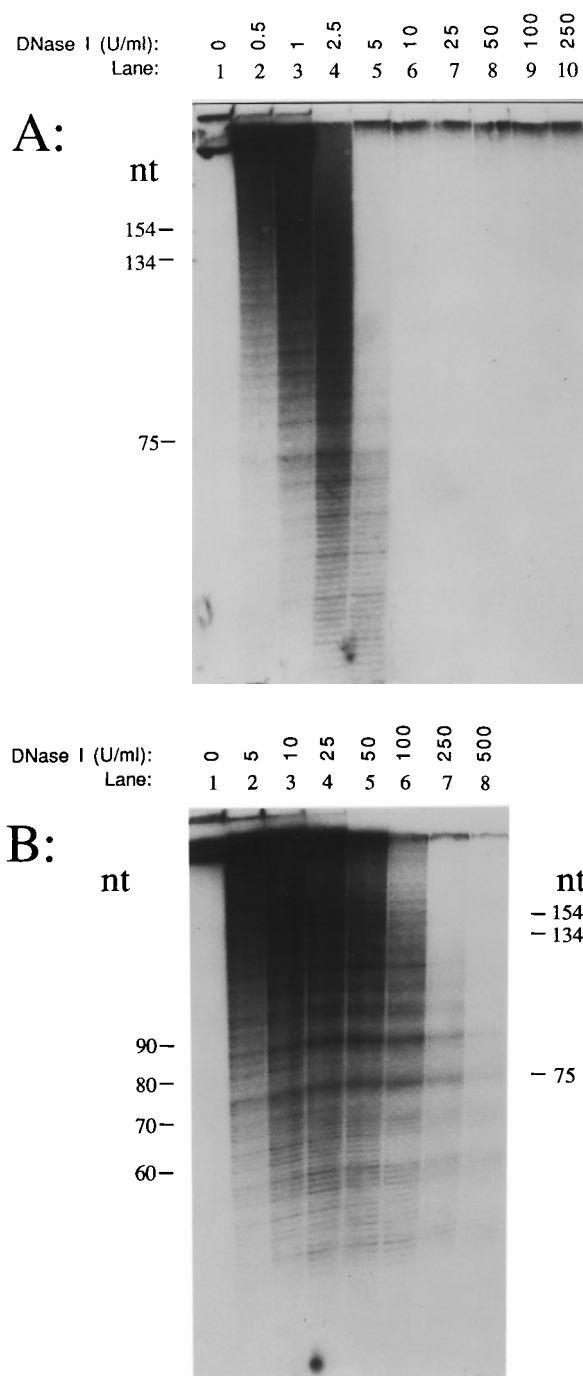


Figure 6. DNase I cleavage of telomeric chromatin. Genomic *Tetrahymena* DNA or nuclei were digested with various concentrations of DNase I (shown in units/ml above each lane) and loaded onto denaturing 8.25% polyacrylamide gels with a 6:1 (w/w) acrylamide to bis ratio (Lutter gel, see Materials and Methods). The gels were electroblotted and filters probed with the ^{32}P -labeled telomeric oligonucleotide $(\text{C}_4\text{A}_2)_4$. Positions of single-stranded DNA size marker fragments (in nt) are shown on the left of A and B. A, Lutter gel showing DNase I digests of purified *Tetrahymena* DNA with DNase I concentrations up to 250 units/ml. B, Lutter gel showing DNase I digests of nuclei with concentrations up to 500 units/ml.

signal from type I telomeric chromatin was lost between 10 and 50 units/ml of MNase. In contrast, between 50 and 200 units/ml of MNase, when essentially only type II chromatin remained, the type II chromatin signal decreased less than two-fold (Figure 3B and data not shown). This result suggested that the type II signal is relatively resistant to MNase digestion compared with both bulk nucleosomes and type I chromatin.

The rate and extent of MNase digestion of telomeric DNA in type II chromatin were directly compared with a control DNA sequence in a canonical nucleosome, the 5'NTS-1 sequence (see Figure 1C). Chromatin analysis has shown that this 5'NTS-1 DNA sequence is packaged as nucleosomes on 100% of the rDNA molecules (E.H.B. & Renata Gallagher, unpublished results). The total hybridization signals in each lane of the blots probed with the telomeric probe or the 5'NTS probe (Figure 3B and C) were determined by PhosphorImager scanning. The 5'NTS-1 nucleosomal signal dropped to 30% of maximum over the course of the titration, while the signal from telomeric probe hybridization dropped to about 3% (Figure 7). Since the type I signal was lost early in the titration, this residual signal reflected the amount of type II chromatin. If the type I complex were no more stable than the canonical 5'NTS nucleosome, then the 3% signal of telomeric DNA in type II chromatin could reflect an actual abundance of ~10% of the telomeric DNA in type II chromatin. However, as described below, the type II telomeric nucleosomes are in fact more resistant to MNase digestion than canonical nucleosomes. Therefore, we conclude that between 3 and 10% of telomeric DNA is in type II chromatin. The results for the 40, 80, 120 and 160 generation points varied only slightly from generation to generation, showing no correlation with telomere length (Figure 7). Hence, the fraction of telomeric DNA in type II chromatin was not detectably changed as the telomeric DNA grew.

Susceptibility of telomeric chromatin to micrococcal nuclease

To measure the relative susceptibility of the type I complex and type II nucleosomes compared with the bulk nucleosomes, we used more extensive MNase digestion. A fresh mating was conducted and progeny cells were passaged in log phase until the telomeric repeat tracts were ~500 bp long (see Materials and Methods). Nuclei were extensively digested with MNase as described in Materials and Methods (Figure 8). Type I complex was continuously digested throughout the titration and the mean size of telomeric DNA protected in type I complex approached the mean length of telomeric DNA in the culture (~500 bp; asterisk in Figure 8B). Naked telomeric DNA was slightly less susceptible to MNase digestion than naked bulk DNA throughout the digestion series (Figure 8A and B, lanes 1 to 8; for example, compare lanes 4

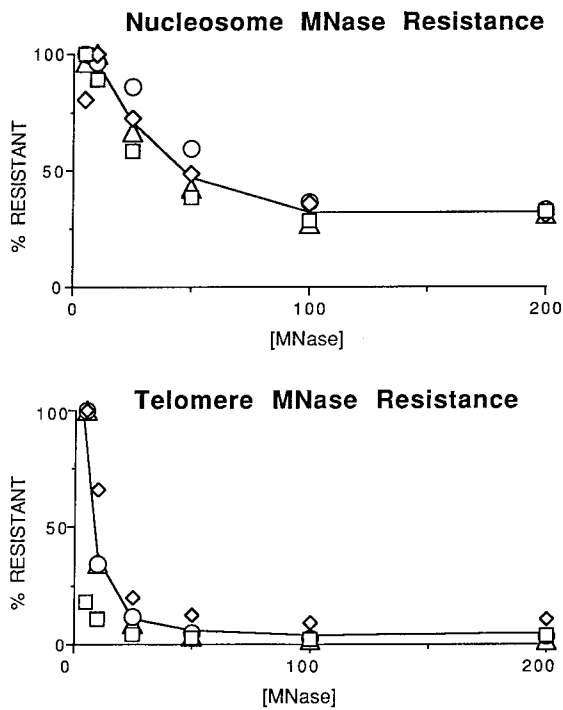


Figure 7. Resistance to micrococcal nuclease digestion of nucleosomes and telomeric chromatin. Freshly isolated nuclei from log phase *Tetrahymena* cells were prepared at 40, 80, 120 and 160 generations after mating and treated with various concentrations of MNase for 15 minutes (see Materials and Methods). DNA purified from these MNase-treated nuclei were separated on a 1.5% agarose gel, Southern blotted and hybridized using the ³²P-labeled 5'NTS-1 rDNA probe (top panel; see Figures 1C, 3C), or the ³²P-labeled telomeric oligo (C₄A₂)₄ (bottom panel; see Figures 1C, 3B). The blots were analyzed using a PhosphorImager and the fraction of hybridization signal remaining after digestion with MNase plotted as the percentage of total signal resistant to MNase digestion (% Resistant, y-axis), against the concentration of MNase used (units/ml, x-axis). The data plotted are for samples shown in Figure 3 at 40 generations (squares), 80 generations (diamonds), 120 generations (circles) and 160 generations (triangles). The five units/ml of MNase samples were set at 100%, since recovery of DNA from undigested control samples was sometimes incomplete because of the high molecular mass of the DNA, but reproducible for the five units/ml of MNase samples.

and 5 between Figure 8A and B). Hence, the higher overall susceptibility of telomeric type I chromatin compared with the 5'NTS chromatin (see Figure 7) was not due to an intrinsic high susceptibility of the duplex G₄T₂ repeat DNA sequence. The ratio of type I to type II signal was unaffected by raising the hybridization wash temperatures to 50°C, which removed over 50% of the original signal (data not shown). In additional experiments, dissociation of proteins was reduced by digestion at lowered temperatures or by formaldehyde cross-linking. Even after extensive MNase digestion, some telomeric DNA remained as a wide, hetero-

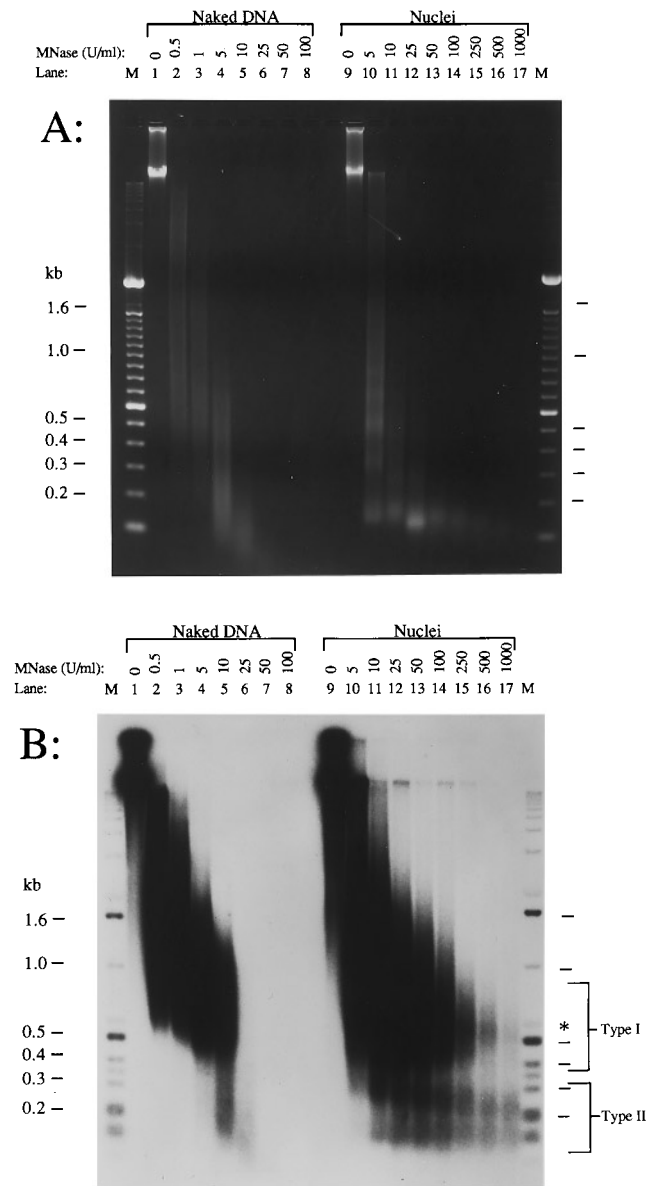


Figure 8. Resistance of type II telomeric nucleosomes is not attributable to inherent resistance of the telomeric DNA sequence to MNase. Purified whole-cell *Tetrahymena* DNA (Naked DNA, lanes 1 to 8) or isolated nuclei (Nuclei, lanes 9 to 17) were digested with the MNase concentrations indicated above each lane and the purified DNA from the digests fractionated in a 1.5% agarose gel, stained with ethidium bromide (A), Southern blotted and hybridized with the telomeric probe (C₄A₂)₄ (autoradiogram in B). Marker DNA lanes (M) show a mixture of 10 bp and 1 kb ladder DNA marker fragments (A), with the 1 kb marker fragments ³²P-labeled by polynucleotide kinase and blotted (B). Asterisk (*), position of the type I chromatin peak; square brackets, size ranges of types I and II telomeric chromatin. In this experiment, the type I signal persisted longer than in the experiment shown in Figure 3.

geneous size distribution in these experiments (data not shown), providing further evidence that most of the telomeric DNA is in type I chromatin.

The type II chromatin ladder in Figure 8B contained two clear steps and was visually well separated from type I chromatin (Figure 8B, square brackets). Type II chromatin showed very little further digestion even in the last three increases in MNase concentration (from 250 to 100 units/ml; Figure 8B, bracket, lanes 15 to 17). In contrast, by 50 units/ml of MNase, over 90% of bulk nuclear DNA was reduced to mononucleosome-sized fragments (Figure 8A, lane 13), and naked DNA at twice the concentration digested with 20-fold less MNase had no detectable telomeric signal remaining (Figure 8A, lane 7). Hence, unlike telomeric nucleosomes of higher eukaryotes, type II telomeric nucleosomes are at least as stable as bulk nucleosomes to MNase. Furthermore, the type II dinucleosome to mononucleosome ratio changed only slightly even with very high concentrations of MNase. This provided further evidence that the type II ladder is not the result of breakdown of higher-order species. Instead, as discussed below, we propose that the type II ladder results from the digestion of type II chromatin away from flanking chromatin that is more sensitive to MNase: specifically, type I telomeric chromatin on its outer border and the neighboring, more widely spaced, subtelomeric nucleosomes on its inner border (see Discussion).

Type II telomeric chromatin does not consist of subtelomeric nucleosomes

Subtelomeric nucleosomes are located adjacent to the telomeric repeats in the rDNA (Budarf & Blackburn, 1986). If some telomeric repeats were included in the subtelomeric nucleosome closest to the telomeric repeat array, the telomeric probe would act as an indirect end-label for these subtelomeric nucleosomes, as described previously in other systems (Gottschling & Cech, 1984; Budarf & Blackburn, 1986; Price, 1990). However, several lines of evidence established that the type II chromatin signal reflects nucleosomes on telomeric DNA, rather than end-labeled neighboring subtelomeric nucleosomes.

First, the spacing of the nucleosome steps in the telomere blot was inconsistent with the known spacing of the three 5'NTS subtelomeric nucleosomes of the rDNA, which account for about half of all the subtelomeres in the cell. These subtelomeric nucleosomes, cover 530 bp (Budarf & Blackburn, 1986; data not shown), rather than the ~460 bp covered by three of the more tightly packed type II nucleosomes (Figure 3; three open arrowheads in B).

Second, MNase digests of nuclei were Southern blotted and probed with two subtelomeric rDNA oligonucleotides. As shown in Figure 9, the probe 3'NTS-1 which hybridizes to the immediate subtelomeric nucleotides of the rDNA (see Figure 1C), detected both the type I chromatin pattern and up to four steps of the type II ladder (Figure 9, open arrowheads). The lanes marked with asterisks in

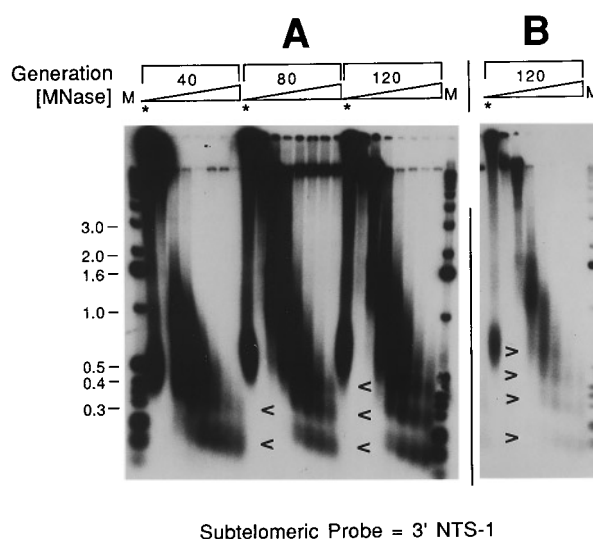


Figure 9. Strong hybridization of the subtelomeric probe to type II chromatin relative to type I chromatin at all telomeric DNA lengths. MNase digests of nuclei, and *SspI* digests of purified DNA (lanes marked with an asterisk), all from the 40, 80 and 120 generation time-points, were run on a 1.5% agarose gel, Southern blotted and probed with the 3'NTS-1 subtelomeric probe (see Figure 1C). A, Sets of lanes from left to right contain samples from the 40, 80 and 120 generation timepoints. The MNase concentrations used in each set of lanes were (left to right): 0, 5, 10, 25, 50, 100 and 200 units/ml. B, Shorter exposure of the 120 generation lanes of the same gel. Open arrowheads, positions of type II telomeric nucleosomes. *SspI* digests (lanes with asterisks) are included to show the telomeric DNA lengths (see Figure 1).

Figure 9 show the sizes of the telomeric *SspI* restriction fragments of the rDNA in this culture at 40, 80 and 120 generations. At lower MNase concentrations, the mean size of the type I chromatin digestion products was slightly longer than the corresponding telomeric G_4T_2 repeat tract (compare also Figures 2 and 4), consistent with the protection of a short region of subtelomeric sequence together with the telomeric DNA tract. In contrast, the probe 3'NTS-2, only 40 bp further into the subtelomere (see Figure 1C), detected only the three previously described subtelomeric nucleosomes with the expected spacing, i.e. longer than that of type II telomeric nucleosomes (Budarf & Blackburn, 1986; data not shown). The inclusion of the 3'NTS-1, but not the 3'NTS-2, sequence in type I and type II chromatin showed that both the telomeric type I complex and the innermost type II nucleosome extend a short distance (<50 bp) inward from the telomeric repeats themselves.

Third, the MNase products shown in Figure 9 were secondarily digested with *AluI*, which cuts in the subtelomeric rDNA sequence 163 bp away from the telomeric G_4T_2 repeats and would therefore cut a subtelomeric nucleosome array (Budarf & Blackburn, 1986). Such secondary *AluI* digestion did not disrupt the type II ladder seen with

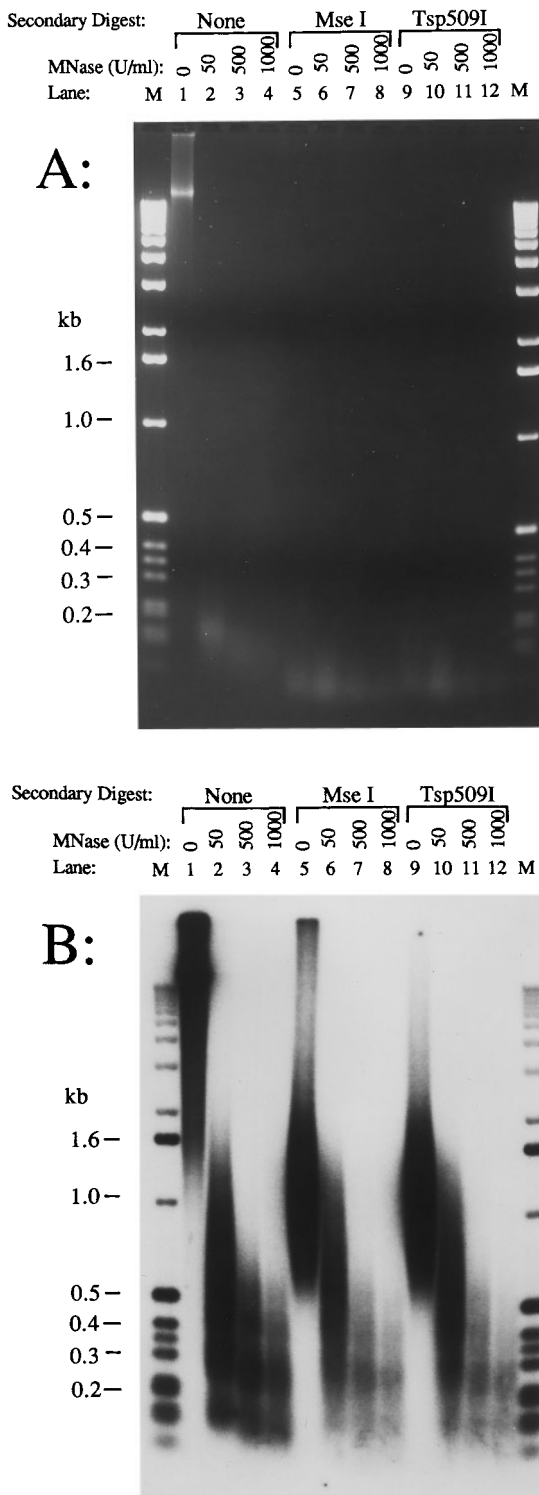


Figure 10. Type II chromatin signals are not lost upon digestion with restriction enzymes that cut very frequently in subtelomeric regions. Isolated nuclei from an 80 generation sample were digested with MNase at the concentrations indicated above each lane, the DNA purified and subsequently digested with the secondary restriction enzymes (*MseI* or *Tsp509I* lanes), or incubated in control buffer (lanes marked None) as indicated. The digested DNAs were fractionated on a 1.5% agarose gel, stained with ethidium bromide (A) and hybridized with the telomeric sequence probe (B). The marker lanes (M) show the 1 kb ladder DNA fragments

the 3'NTS-1 probe (data not shown). We also tested whether the type II signal represented subtelomeric nucleosomes in non-rDNA as well as rDNA macronuclear telomeres. DNA purified from MNase-digested nuclei was secondarily digested with the frequently cutting restriction enzymes *MseI* and *Tsp509I*. These enzymes each cut bulk genomic macronuclear DNA at sites averaging <75 bp apart (Figure 10A, lanes 5 and 9). In the rDNA, *MseI* and *Tsp509I* cut six and five times, respectively, within the first 200 bp next to the telomeric G₄T₂ repeat tract, and in several other sequenced subtelomeric regions one or the other enzyme cuts within 50 bp of the telomeric repeat tract (Yokoyama & Yao, 1986; Yao *et al.*, 1987; Spangler *et al.*, 1988). Therefore, any MNase digestion products protected by subtelomeric nucleosomes are expected to be fragmented after secondary cutting with these enzymes. However, with a telomeric repeat probe, the type II chromatin ladder was largely intact (Figure 10B, lanes 6 to 8 and 10 to 12; note that lanes 1 to 4 were loaded with twice the amount of DNA as that in lanes 5 to 12).

Long-distance sliding of nucleosomes has been described for nucleosomes depleted for histone H1 at high temperatures or high salt concentrations (Spandafora *et al.*, 1979; van Holde, 1989; Watkins & Smerdon, 1985), and short-range sliding has been shown for nucleosomes at very low ionic strength (Dong *et al.*, 1990; Meersseman *et al.*, 1991, 1992; Pennings *et al.*, 1991). However, both types of movement are highly constrained at low temperature (Meersseman *et al.*, 1992). In additional MNase digestion of nuclei performed at 0°C, telomeric type II nucleosomes were still clearly evident (data not shown). To bias against nucleosome movement, standard conditions for all the digestions performed in this work included moderate ionic strengths (60 mM KCl, 15 mM NaCl), moderate temperature (all preparative manipulations of nuclei at 0°C or 4°C and digestion at 30°C), and pH at or slightly above neutrality to avoid depletion of H1. Under these conditions, nucleosomes in the 5'NTS and 3'NTS of the rDNA remained in their characteristic positions on more than 95% of molecules (Pan *et al.*, 1995; P.C., unpublished results; E.H.B. & Renata Gallagher, unpublished results). Therefore it is unlikely that the arrays of up to four type II nucleosomes observed on the telomeric DNA were created by artifactual mobilization of subtelomeric nucleosomes occurring *in vitro*. Taken together, the results established that the type II chromatin signal results from nucleosomes on telomeric DNA, and not from end-labeled subtelomeric nucleosomes.

³²P-labeled by polynucleotide kinase and blotted along with the *Tetrahymena* DNA samples. The lanes containing the secondarily digested *MseI* or *Tsp509I* digested samples contained half the DNA of the lanes that were not secondarily digested.

Type II nucleosomes may frequently cluster at the inner region of the telomeric repeat tract

We analyzed the Southern blotting results obtained with the immediately subtelomeric 3'NTS-1 probe (Figure 9) using calculations similar to those performed for the telomeric probe (see Figure 7). The fraction of total hybridization signal in type II chromatin detected with the 3'NTS-1 probe was similar to that obtained with the telomeric (C₄A₂)₄ repeat probe (about 3%). However, the lengths of DNA protected by the vast majority of the type II nucleosomes (monomers, dimers and trimers; see Figures 3 and 9) were shorter than most of the telomeric repeat tracts. Therefore, if telomeric nucleosomes were randomly distributed across the entire rDNA telomeric tract, most would not have been detected by the 3'NTS-1 probe, which is located next to the telomeric repeats. This high level of efficiency of detection of the type II ladder with the 3'NTS-1 probe strongly suggests that at least a significant proportion of the telomeric nucleosomes cluster at the subtelomere-telomere border. It has been similarly suggested that in humans the TRF1-telomeric DNA complex occupies the distal ends of telomeres and nucleosomes occupy the remaining, inner stretch of the telomeric repeat tracts (Chong *et al.*, 1995b). These results on *T. thermophila* telomeres provide direct evidence consistent with such a model.

Discussion

Here we have demonstrated that *T. thermophila* macronuclear telomeres contain two distinct types of chromatin: the type I telomeric complex protecting the entire G₄T₂ repeat tract and the immediately adjacent subtelomeric region, and 3 to 10% of the total telomeric DNA in tightly spaced nucleosomes (type II telomeric chromatin). These results have implications for the interactions between telomeric proteins and nucleosomes, and for telomeric chromatin in other systems, and provide the first information on the dynamics of telomeric chromatin structures as the telomeric length changes.

Telomeric DNA in the type I complex was at least ten times more susceptible to MNase digestion than DNA in a canonical nucleosomal core particle. Therefore, it is highly unlikely that it contains underlying nucleosomes. In the yeasts *S. cerevisiae* and *K. lactis*, the RAP1 protein is the major DNA-binding component of the type I complex associated with the duplex telomeric DNA (reviewed by Fang & Cech, 1995; Krauskopf & Blackburn, 1996). In human telomeres, a duplex DNA sequence-specific binding protein, TRF1, with *myb* homology in its DNA-binding domain, binds two tandem human TTAGGG repeats (Chong *et al.*, 1995b). In the fission yeast *Schizosaccharomyces pombe*, another *myb*-related protein, TAZ1, also packages the duplex telomeric repeats (Cooper *et al.*, 1997). It is likely that similarly, tandem arrays of a *T. thermophila* RAP1/TRF1/TAZ1

functional homolog are involved in forming the type I complex. An *in vitro* binding activity with the expected duplex DNA sequence specificity has been found in *T. thermophila* nuclear extracts (P.C. and E.H.B., unpublished data).

The spacing (154(±3) bp) of the type II telomeric nucleosomes of *T. thermophila* resembles the unusually tight (150 to 160 bp) spacing of vertebrate telomeric nucleosomes (Gorovsky, 1986; van Holde, 1989; Makarov *et al.*, 1993; Tommerup *et al.*, 1994; Lejnine *et al.*, 1995). However, in contrast to vertebrate telomeric DNA, the majority of which is packaged in such nucleosomes, type II telomeric nucleosomes of *T. thermophila* comprise only a minority of the total telomeric complexes, and are found even on short telomeres. The *T. thermophila* nucleosomal dimers and trimers were highly resistant to MNase digestion. The stability of these short telomeric nucleosome arrays to MNase may result from a combination of the stability of the mononucleosomes and their tight packing, with the shortness of the linker DNA limiting access to MNase between nucleosome cores. In addition, *T. thermophila* telomeric nucleosome cores were no more susceptible to MNase than a nucleosome core particle in the 5'NTS. In contrast, the telomeric nucleosomal monomer of rat is unstable to MNase digestion compared with longer arrays of these nucleosomes (Makarov *et al.*, 1993). This difference in telomeric mononucleosome stability may be due to differences between *T. thermophila* and vertebrate histones (Gorovsky, 1986) and/or their telomeric DNA repeat sequences (G₄T₂ in *T. thermophila* and AG₃T₂ in vertebrates).

Type II nucleosomes appear to be clustered at the telomere-subtelomere border, regardless of telomere size. As the telomeric G₄T₂ repeat tracts gradually lengthened during cell divisions, the average number of type II nucleosomes in the telomeric array increased. Since the telomeric DNA tract length increases gradually, rather than by multiples of 154 bp, we propose that type II chromatin occurs on chimeric molecules, each containing an inner nucleosomal portion and the remaining outer region in a type I complex (Figure 11). Whether the relative lengths of these regions on the chimeric telomere change as telomeres lengthen is unknown; the low fraction of chimeric telomeres precluded an accurate assessment of this.

We propose that the telomeric type I complex preferentially occupies the outer end of the telomeric DNA tract, and thus confines telomeric nucleosomes to the innermost part of the telomeric repeats. The telomeric DNA-protein complex regulates access of the telomeric terminus to telomerase (Yu *et al.*, 1990; Kyrion *et al.*, 1993; McEachern & Blackburn, 1995; Krauskopf & Blackburn, 1996), and is thought to be important for other telomeric functions (reviewed by Blackburn, 1994; Kirk *et al.*, 1997). Nucleation of binding of the type I complex proteins could involve a protein like the terminus-binding proteins that specifically bind the single-

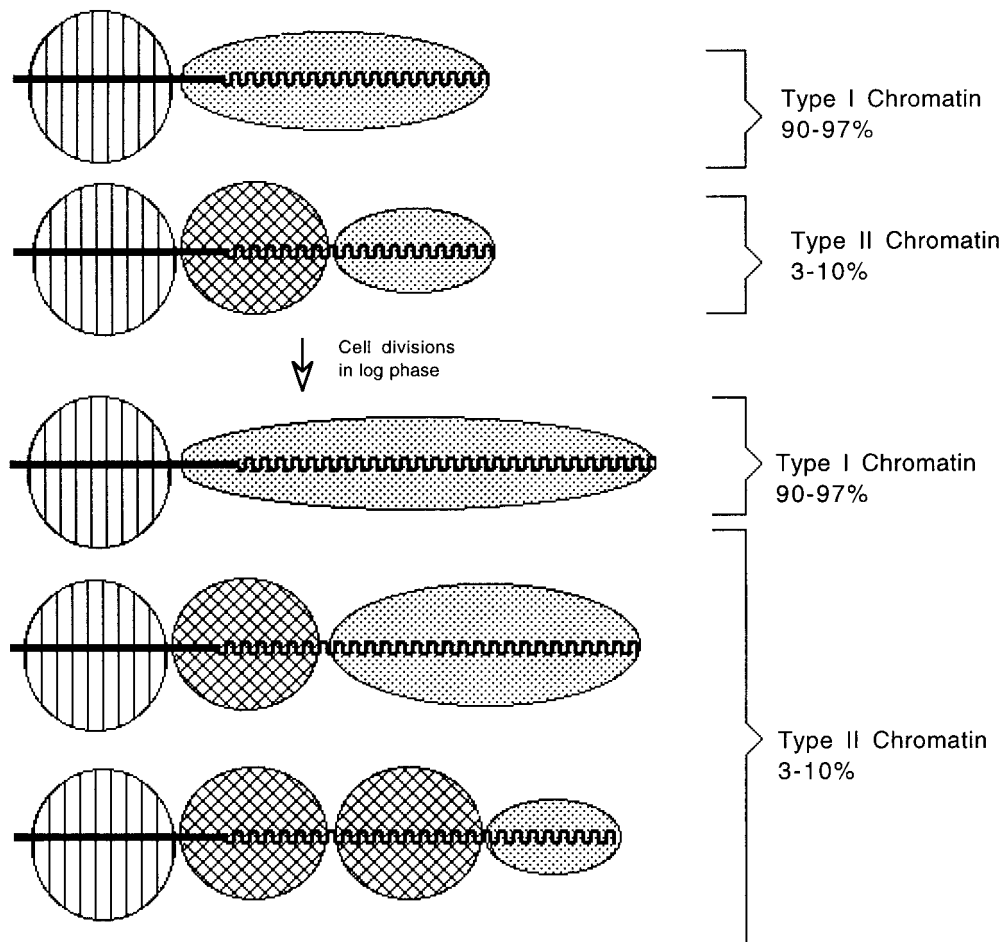


Figure 11. Model for telomeric chromatin structure of growing telomeres. Telomeres in *Tetrahymena* consist of two populations at all telomere lengths. Type I chromatin consists of telomeres in which the entire T_2G_4 repeat tract (wavy line) together with the immediately adjacent subtelomeric region (straight line) are protected in a non-nucleosomal protein complex (stippled oval). We propose that positioned subtelomeric nucleosomes (vertically hatched circles) may limit the extent of expansion of the complex into the subtelomeric region. Type II chromatin consists of telomeres that are chimeric in structure. A type II telomere contains nucleosomes (cross-hatched circles) clustered tightly at the inner region of the telomeric DNA repeat tract and onto the immediate subtelomeric sequence, and non-nucleosomal proteins (type I chromatin) on the remaining distal portion of the telomeric DNA repeat tract.

stranded overhang at the ends of telomeres in hypotrichous ciliates (reviewed by Fang & Cech, 1995). The positioned nucleosomes in the *T. thermophila* rDNA subtelomere (Budarf & Blackburn, 1986) may stop telomeric type II nucleosomes from sliding back into the subtelomeric region. Hence we envision that the type II telomeric nucleosomes are trapped and "squeezed" between these positioned subtelomeric nucleosomes and the expanding non-nucleosomal telomeric complex at the extreme terminus. We propose a similar combination of effects causes the tight packing of telomeric nucleosomes in vertebrates.

We suggest a new model for telomeric chromatin in which proteins of the type I complex must compete with nucleosomes along the whole length of telomeric DNA. In this model, the differences between the telomeres of lower and higher eukaryotes are quantitative, rather than qualitative. We propose that in *T. thermophila* this competition largely favors type I complex formation; in contrast,

in vertebrates the balance is in favor of nucleosome deposition. The fraction of *T. thermophila* telomeres in type II chromatin remained the same over all telomere lengths examined, suggesting that in this system the balance between type I and type II chromatin is not driven by telomeric DNA length, at least within the length range analyzed.

The type I complex does not protect a fixed DNA size at the resolution of these studies; with telomere lengthening during vegetative cell divisions, the size of the type I protected complex grew concomitantly to encompass the new stretch of telomeric repeats. A short region (<50 bp) of subtelomeric DNA sequence also remains protected: in this respect the telomeric type I complex of *T. thermophila* is similar to the non-nucleosomal telomeric complexes in *Oxytricha nova*, *Euplotes crassus* and *S. cerevisiae*, which protect ~100 bp, <80 bp and ~50 bp of adjacent non-telomeric sequence respectively from MNase digestion (Gottschling & Cech, 1984; Price, 1990; Wright *et al.*,

1992). We propose that on telomeric repeat tracts the interaction between the DNA and type I complex protein(s) is driven by both sequence-specific protein-DNA recognition and protein-protein interactions. The combination of these two binding energies must therefore be sufficient to displace nucleosomes on the majority of telomeres, and also to drive binding so that the type I complex extends over a short portion of the adjacent subtelomeric DNA. However, these binding interactions are insufficient to displace the neighboring positioned nucleosomes on the subtelomere. In *T. thermophila*, because 3 to 10% of telomeric DNA is packaged as nucleosomes, this competition (or destabilization of nucleosomes) must be incomplete. In addition, we consistently noted that even when a significant fraction of the population of telomeric DNA repeat tracts became long enough (~500 bp) to accommodate three closely spaced type II nucleosomes, which occupy only ~460 bp, only two were visible (e.g. see Figure 3, lanes 5 to 7 and Figure 10, 40 generation samples). This lag between acquisition of sufficient telomeric DNA length and adding new steps to the type II ladder also suggests the type II chromatin has to compete with type I complex proteins for binding to telomeric DNA.

Because the type II nucleosomes package only 3 to 10% of the total telomeric DNA, it can be further predicted if each nucleosome deposition event on telomeres were independent, ~96% of the telomeres that have nucleosomes would carry only one nucleosome (see Materials and Methods). Specifically, random nucleosome deposition predicts that telomeres with one nucleosome should be 28 times more abundant than telomeres with two nucleosomes, and telomeres bearing three or more nucleosomes would be undetectable. This contrasts with the high frequencies of tri- and dinucleosomes evident even after MNase digestions (Figure 3B, lanes 21 and 28, Figure 8B, lanes 15 to 17, and Figure 9, 80 and 120 generations titrations). Hence we infer that the assembly or establishment of the type II nucleosome arrays involves cooperative deposition of nucleosomes into the short arrays we observed.

Our data do not distinguish whether the distribution of telomeres between type I and type II chromatin reflects a stable population, with nucleosomes present on a small proportion of the telomeres at all times, or a dynamic situation with nucleosomes present on all or most telomeres only during a short period of the cell-cycle. Since chromatin undergoes disruption and maturation after every replication fork moves through (van Holde, 1993) a dynamic model, in which the type I and type II chromatin states may readily interconvert, is attractive. Recent results in *S. cerevisiae* in which mutating a chromatin assembly factor affects telomere functions thought to be mediated by the telomeric RAP1 complex may be a manifestation of such an interplay between the different chromatin states of the telomere (Enomoto *et al.*, 1997).

Materials and Methods

Tetrahymena thermophila strains and cell culture

Strains SB1915 and SB1565 were used to generate progeny for the log phase growth experiments (Larson *et al.*, 1986). *T. thermophila* strains were kept in loosely capped tubes at room temperature in 1% PPYS medium (1% (w/v) protease peptone, 0.2% (w/v) yeast extract, 0.003% (w/v) sequestrine; Ciba Geigy) and were transferred to fresh medium every four weeks.

The two *T. thermophila* strains to be mated, each of different mating type, were first grown separately in 50 ml cultures in 2% PPYS at 30°C with gentle swirling to a density of $\sim 2.5 \times 10^5$ cells/ml. The cells were harvested by centrifugation, washed and resuspended in less than 50 ml of Dryl's solution (1.7 mM sodium citrate, 1.2 mM NaH₂PO₄, 1 mM Na₂HPO₄, 2 μM CaCl₂), and starved for 24 hours at 30°C in the Dryl's solution. For mating, equal numbers of cells of each mating type were mixed in a volume of less than 200 ml and incubated in a 600 ml T-flask laid on its side without agitation for ~24 hours to allow mating to occur. The cells were then re-fed by addition of 4% PPYS stock to a final concentration of 1% PPYS. After 24 hours the cultures were diluted ~1:1000 into 2% PPYS containing 15 μg/ml cycloheximide to select for progeny cells. They were passaged for one more day in the cycloheximide-containing medium before being propagated in 2% PPYS.

To propagate cells under logarithmic growth conditions, cultures were diluted 1:1000 daily to ensure that their concentration never exceeded 3.0×10^5 cells/ml. Growth rate was determined weekly by counting cells in a hemocytometer. Growth-rate remained high during the course of the experiments reported here (doubling time ~2.4 hours). Cultures 1, 2 and 3 were triplicate cultures maintained in parallel under identical conditions. For each experiment, unless otherwise indicated results are shown for only one culture, but at least one other culture was analyzed in parallel and gave the same results.

Preparation of *T. thermophila* nuclei

For analysis of chromatin, nuclei were prepared essentially as described (Zaug & Cech, 1980; Blackburn & Chiou, 1981) with minor variations as noted. All steps were carried out on ice or at 4°C. Cells taken from the main log phase culture were grown to a density of 1×10^5 to 4×10^5 cells/ml, harvested by a ten minute spin in a GSA rotor at 2500 rpm and resuspended at approximately ten times their original density in TMS buffer (10 mM Tris (pH 7.5), 10 mM MgCl₂, 3 mM CaCl₂, 250 mM sucrose), with 0.1 mM PMSF, 1 mM DTT. 1% (v/v) NP40 was added to a final concentration of 0.16% or 0.19% (v/v) and the cells lysed for 20 minutes. Sucrose was added to 0.815 g/ml of resuspended, lysed cells and stirred until completely dissolved. Nuclei were pelleted by a 30 minute spin at 9000 g in a swinging bucket rotor (HB-4, 7500 rpm). Nuclei were washed once with buffer A (60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM β-mercaptoethanol, 15 mM Tris-HCl (pH 7.4), 2 mM CaCl₂, 0.1 mM PMSF) and resuspended again in ~10 volumes of buffer A (Blackburn & Chiou, 1981).

MNase digestions of DNA and nuclei

Freshly isolated nuclei were resuspended (or the DNA dissolved) in buffer A and incubated at 0°C. A 2× con-

centration of each MNase concentration was made in buffer A from a concentrated stock. MNase (Pharmacia) was kept as a concentrated stock (15 units/ μ l) at -20°C in 1 mM CaCl_2 , 20% (w/v) glycerol, 50 mM Tris (pH 8.0). To start the reaction, an equal volume of nuclei (or DNA) and the appropriate $2\times$ MNase concentration, or control buffer, were mixed gently for ten seconds and then incubated for 15 minutes at 30°C . For the MNase digestions shown in Figure 8, nuclei were digested very extensively by raising the concentration of the enzyme to up to five times the highest concentration used for Figure 3, and halving the concentration of nuclei in the digestions. Thus the digestion conditions used for Figure 8 represented tenfold more extensive digestion conditions than for the highest MNase concentration shown in Figure 3. Reactions were stopped by adding EGTA to a final concentration of 17 mM, mixing and returning to ice. An equal volume of DNA Prep Solution (20 mM Tris (pH 8.0), 0.5 mg/ml Proteinase K, 1% (w/v) SDS, 5 mM EDTA) was added immediately to the nuclei (or DNA) and the samples incubated at 37°C for at least four hours. The samples were phenol/chloroform-extracted once, precipitated with ammonium acetate and ethanol, lyophilized and resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA).

DNase I digestions

The DNase I digestions were performed in a similar fashion to the MNase digestions, with the following modifications. The nuclei were resuspended in buffer A. Dilutions of DNase I enzyme (RNase-free, DNase I; Boehringer Mannheim) were made in buffer A containing 20 mM MgCl_2 . The nuclei and appropriate $2\times$ DNase I dilution were pre-equilibrated separately at room temperature for two minutes before the reaction was begun by mixing for ten seconds by gentle pipetting (no bubbles). After incubation for one minute at room temperature, reactions were stopped by adding EDTA to 26 mM. The subsequent preparation of DNA was the same as for the MNase digestions. To monitor the overall extent of digestion, the purified, undenatured DNA from this digestion was electrophoresed on a 1.5% agarose gel. The gel was stained with ethidium bromide, Southern blotted, crosslinked extensively and hybridized with the telomeric probe. At 100 units/ml of DNase I, most of the bulk DNA was digested to nucleosome core particle-sized fragments within the one minute digestion, and the vast majority of the telomeric signal was digested to a heterogeneous distribution of fragments with no pause at the telomeric DNA peak length of ~ 600 bp, suggesting that there was little barrier to DNase I access to most of the telomeric DNA; at 200 units/ml of DNase I, a small fraction of the total telomeric signal remained centered at ~ 145 bp (data not shown).

Lysis of nuclei and solubilization of chromatin for sucrose density-gradient analysis

After stopping the MNase digestion, the nuclei were pelleted at $9000g$ at 4°C , resuspended in $0.1\times$ TE buffer (pH 7.4) containing 1 mM PMSF, allowed to swell for 30 minutes and vigorously mixed. The insoluble fraction (lysis pellet) was removed by brief centrifugation at $>10,000g$ in a microcentrifuge at 4°C , releasing the soluble chromatin into the supernatant (lysis supernatant). Soluble chromatin in the clear supernatant was loaded onto a 5% to 27% (w/w) linear sucrose gradient and cen-

trifuged. The gradients were spun for 18 hours at $28,000\text{ rpm}$ in an SW41 swinging bucket rotor, fractions collected from the bottom of the tube and the absorbance of odd-numbered fractions measured at 260 and 280 nm in a Shimadzu UV160U spectrophotometer.

Preparation of whole-cell DNA

Cells were grown in 2% PPYS at 30°C to a density of 3.0×10^5 cells/ml, with gentle swirling on a gyratory shaker. The cells were pelleted at full speed in an IEC HN-SII bench-top centrifuge for six minutes or by a short spin in a tabletop microcentrifuge, depending on the volume of culture. Approximately 90% of the medium was removed and one volume of NDS (0.5 M EDTA, 10 mM Tris-HCl (pH 9.5), 1% SDS) was added to the pelleted cells. This mixture was incubated at 55°C for at least 20 minutes, another volume of 2 mg/ml self-digested Pronase was added, and the proteinase-DNA mixture returned to 55°C for at least four hours. An equal volume of water was added, and at this point the sample was sometimes frozen at -20°C for several weeks with no detectable effect on the quality of the DNA. DNA was extracted twice with phenol/chloroform and then extracted twice with chloroform, precipitated with two volumes of ethanol, then reprecipitated with ammonium acetate and ethanol. The pellet was washed with 70% ethanol and resuspended in TE buffer.

Agarose gel electrophoresis and blotting

DNA was electrophoresed in 1.5 to 2.0% agarose gels containing 0.5 $\mu\text{g/ml}$ ethidium bromide, at a constant voltage of 50 V, usually until the bromophenol blue dye had migrated at least 10 cm. Immediately before loading for electrophoresis, samples were treated with 100 ng of RNase A in gel loading buffer for five minutes or less at room temperature. Southern blotting procedures were as described (Sambrook *et al.*, 1989) with a few alterations. For efficient retention of fragments smaller than 2 kb on the membranes, the gels were not depurinated. The ethidium bromide-stained gels were denatured for one hour in 0.5 M NaOH, 1.5 M NaCl. The DNA was transferred to Nytran (Schleicher & Schuell) by the standard wick method in $10\times$ SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) for 12 to 18 hours. The gels were checked for complete transfer under UV light. Normally no detectable signal remained in the gel, but rarely some staining at limit mobility was observed. The filter was then crosslinked in a Statalinker 1800 (Stratagene) using the auto-crosslink setting, dried at 60°C for one hour and recrosslinked at room temperature by repeating the auto-crosslink cycle. Telomeric DNA below 300 bp was preferentially lost from the filter if only a single UV crosslinking step was performed (data not shown). DNA marker ladders were purchased from Pharmacia (1 kb ladder), BRL (100 bp ladder) and New England Biolabs (Lambda *HindIII* ladder). Labeled markers were ^{32}P 5'-end labeled using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (ICN; $>7000\text{ Ci/mmol}$) and T4 Polynucleotide Kinase (U.S. Biochemicals or New England Biolabs).

Lutter gels

These gels were prepared using a crosslinking ratio of acrylamide to bis of 6:1 (8% (w/v) acrylamide, 1.25% (w/v) bisacrylamide) and contained TBE buffer (Lutter, 1979). For various double-stranded markers this resulted

in strands running within one nucleotide of each other (data not shown). Following electrophoresis, the gel was electroblotted at 4°C at 90 to 100 V (3 to 4 A), the blot crosslinked and probed with ³²P end-labeled (C₄A₂)₄ oligonucleotide as for the Southern blotting experiments.

Hybridization conditions

Hybridization conditions were used as described previously (Church & Gilbert, 1984) with the following buffers: hybridization buffer was 1% (w/v) bovine serum albumin, 1 mM EDTA, 0.5 M NaHPO₄ (pH 7.2), 7% SDS. Wash buffer was 1 mM EDTA, 40 mM NaHPO₄ (pH 7.2), 1% SDS. Prehybridizations were done for approximately one hour at 30°C. The appropriate probe was then added at room temperature and allowed to hybridize overnight (18 to 24 hours). The blot was washed twice at room temperature for 15 to 20 minutes each. The blot was then washed in a much larger volume (~500 ml) for an additional 15 to 20 minutes at 30°C. Oligonucleotide probes were stripped by incubation in wash buffer at ~50°C. For removal of the telomeric probe, the membrane was soaked in 0.4 M NaOH for more than one hour at 40°C.

PhosphorImager scanning and laser densitometry

Quantification of lanes on blots and scans of the signal distribution within lanes were performed on a Molecular Dynamics PhosphorImager using ImageQuant 3.0 software. In some cases, for further analysis the raw data were transferred to Cricket Graph 1.3, Aldus Superpaint 3.0 or Microsoft Excel 4.0. Plotting the positions of the type II hybridization signal peaks for the steps of the type II ladders against the logarithm of the molecular mass of the marker fragments (taken from Figure 3A and data not shown), and by using a linear regression function, yielded a spacing of 154(±3) bp (where 3 bp is the standard deviation of the regression of the slope). The positions of peaks in the type II chromatin signal taken from Figure 3B were also determined by using a normalization function to eliminate the contribution of the fragment length to the intensity of hybridization of each peak to the telomeric probe, allowing the intensity of each peak to be gauged solely as a function of its molar abundance. To generate the data shown in Figure 7, the total counts for each lane were normalized to the maximal signal in the titration, which in all cases was at five to ten units/ml of MNase, rather than no MNase, because some presumably undigested DNA was trapped in the well.

Ethidium bromide-stained agarose gels were photographed using Polaroid 55 Negative/Positive Film. Analysis of signal distributions from photographic negatives was performed on a LKB 2202 Ultrascan laser densitometer.

Calculation of expected frequencies of nucleosome monomers, dimers, trimers, etc for cooperative versus non-cooperative deposition

We assumed that if each nucleosome deposition into telomeric sequence is independent, the probability that a telomere has *j* nucleosomes is:

$$\left\{ \frac{k!}{(k-j)!j!} \right\} p^j (1-p)^{k-j}$$

where *p* is the probability for one deposition event and *k*

is the maximum number of nucleosomes. Therefore the probability that a telomere has at least one nucleosome, $p(j \geq 1)$, is equal to:

$$1 - (1-p)^k$$

Assuming that telomeric nucleosomes are as stable as the 5'NTS nucleosomes, it was found experimentally that $p(j \geq 1) = 0.10$ for type II chromatin. (If telomeric nucleosomes are less stable, $p(j \geq 1)$ will be lower.) For example, for a three nucleosome array:

$$1 - (1-p)^3 = 0.1$$

Therefore, $p \leq 0.04$; i.e. ~96% of telomeres would be expected to have only one nucleosome per telomere if deposition were non-cooperative.

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