An unusual sequence arrangement in the telomeres of the germ-line micronucleus in *Tetrahymena thermophila*

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The ciliated protozoan *Tetrahymena thermophila* contains two nuclei that differ dramatically in function, chromosome size and number, chromatin structure, and mode of division. It is possible that the telomeres of the two nuclei have different functions. Although macronuclear telomeric DNA has been well characterized and consists of tandem G_4T_2/C_4A_2 repeats that are synthesized by the enzyme telomerase, micronuclear telomeres have not been isolated previously. Here, we report the identification and cloning of micronuclear telomeres and demonstrate that although they contain the same terminal tandem G_4T_2 repeats as macronuclear telomeres, they are strikingly different in three respects. First, the tracts of G/C-rich telomeric repeats are approximately seven times longer in the micronucleus than in the macronucleus (~ 2.0 – 3.4 vs. ~ 0.3 – 0.5 kb, respectively) from the same cell population. Second, the immediate telomere-associated sequences (TASs) from six different micronuclear chromosome ends have an unusually high G/C content and degree of homology to one another, unlike macronuclear TASs. The TAS from at least one micronuclear chromosome is unique to micronuclear telomeres and is not present in the macronucleus. Finally, and unexpectedly, all micronuclear telomere clones contain an inner homogeneous tract of a variant G_4T_3 repeat adjacent to the distal tract of G_4T_2 repeats. The native micronuclear telomeric DNA is composed of approximately 30% G_4T_3 repeats, corresponding to 0.6–1.0 kb per average telomere, positioned centromere-proximally to most or all of the G_4T_2 repeats. Neither the G_4T_3 sequence nor any other variant repeat is found in macronuclear telomeres. Furthermore, such a homogeneous tract of a variant repeat has not been found in the telomeres of any eukaryote.

[Key Words: *Tetrahymena thermophila*; micronuclear telomere; telomeric repeats; telomere-associated sequences]

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The regions near eukaryotic chromosome ends appear to have many functions, most of which are poorly understood. It has been well established that the distinctive simple G/C-rich DNA repeats usually found at telomeres are synthesized by the enzyme telomerase (for review, see Greider 1991; Blackburn 1992, 1994), providing a means for the complete replication of the chromosome. In addition, telomeres provide a protective "cap" that prevents the natural ends from fusing, thus differentiating them from broken ends, an observation that has been long recognized (McClintock 1939, 1941; Muller and Herskowitz 1954). A broken end created by the loss of a telomere causes cell cycle arrest in yeast and, by what may be a separate mechanism, greatly increases chromosome loss (Bennett et al. 1993; Sandell and Zakian 1993). In another very different role, telomeres have the capacity to repress the expression of nearby genes (Gottschling et al. 1990). Telomeric repeats can influence silencing

when positioned chromosome internally (Stavenhagen and Zakian 1994) and, when present on a yeast circular plasmid, can greatly increase the mitotic stability of the plasmid (Longtine et al. 1992, 1993).

Telomeres have been observed cytologically to occupy defined positions within the nucleus during interphase, mitosis, and meiosis, but the particular localization can vary between organisms and cell types. The significance of these spatial orientations is not understood. In *Schizosaccharomyces pombe* (Funabiki et al. 1993), *Trypanosoma brucei* (Chung et al. 1990), and *Drosophila melanogaster* (Hochstrasser et al. 1986), telomeres localize to the periphery of interphase nuclei and frequently associate with each other. In *Saccharomyces cerevisiae*, the telomere-binding protein Rap1p and, by inference, the telomeres, is also found in clusters at the nuclear periphery (Klein et al. 1992; Palladino et al. 1993). During meiotic prophase in many organisms, the chromosome extremities have been observed to associate with one another and to localize to the nuclear envelope (Dan-

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cis and Holmquist 1979). In maize zygotene, telomeres are found at the nuclear periphery and adjacent to the nucleolus (Dawe et al. 1994; H. Bass, Z. Cande, and J. Sedat, pers. comm.). Recent observations in living *S. pombe* have suggested that telomeres may have a prominent role in a very dramatic nuclear movement during karyogamy and meiosis: The telomeres are found in a single cluster colocalized with the spindle pole body at the leading edge of the elongated moving nucleus (Chikashige et al. 1994). Thus, the list of potential telomere functions continues to expand.

Tetrahymena thermophila provides a unique system for potentially dissecting the multiple roles of telomeres. Like most ciliates, *T. thermophila* possesses two nuclei within a single cell, which differ dramatically in terms of function, chromosome content and chromatin structure, and mode of division (for review, see Blackburn and Karer 1986; Prescott 1994). Therefore, it is possible that the telomeres in the two nuclei have very different functions, possibly reflected in different structures and mode of synthesis. The macronucleus contains hundreds of different subchromosomal pieces, each having a copy number of ~50, except for the rDNA chromosome, which is amplified to ~10⁴ copies (for review, see Kapler 1993). The macronuclear chromosomes are acentric, and the macronucleus divides amitotically without chromosome condensation; the resulting daughter cells can differ significantly in their DNA content. The macronucleus is the sole source of gene expression during vegetative growth. The micronucleus, on the other hand, would appear similar to most eukaryotic nuclei, as it contains only five pairs of metacentric chromosomes, divides mitotically, and can undergo meiosis. Nevertheless, its functional repertoire is limited, as its main role is providing the germ line of the cell rather than gene expression. Upon conjugation, the diploid micronuclei in both partners undergo meiosis and cross-fertilization to form new micronuclei. In each cell, the old macronucleus is then destroyed, and a new macronucleus is differentiated from a copy of the new micronucleus. The micronucleus is transcriptionally silent throughout the life cycle except for a brief period early in meiotic prophase, and it is not known which RNAs are synthesized at this time (Sugai and Hiwatashi 1974; Martindale et al. 1985). Throughout vegetative growth, the micronuclear chromatin structure reflects the transcriptionally inert state and contains certain histone and linker proteins that are markedly different from those in the macronucleus (e.g., Allis et al. 1984; Stargell et al. 1993; Stargell and Gorovsky 1994).

The *T. thermophila* macronuclear telomeres contain ~300–500 bp of the repeated sequence G₄T₂. The synthesis of macronuclear telomeres has been well studied, and is carried out by the ribonucleoprotein telomerase, which provides the templating telomeric repeat sequence within its RNA moiety (for review, see Blackburn 1990, 1992). By introducing a telomerase RNA gene bearing an altered template sequence into the macronucleus, the repeat sequence of the distal regions of macronuclear telomeres has been changed artificially from

G₄T₂ to, for example, G₄TC (Yu et al. 1990). In these transformants, the macronuclear telomeres are lengthened and acquire the variant repeat sequence. The phenotype of these cells is drastic: The macronuclei become grossly enlarged and ultimately cease to divide (Yu et al. 1990). The presence of the specific repeat sequence G₄T₂ on the distal regions of the telomeres, therefore, is important for some poorly understood telomere function in the macronucleus, independent of telomerase recognition and elongation. It is not known what role, if any, wild-type telomeres play in chromosome segregation or in the amitotic division of the macronucleus, and the spatial organization of telomeres in the macronucleus is unknown.

Micronuclear telomeres have not been isolated previously, primarily because of their relative scarcity in the cell, and nothing is known about their synthesis or function. Because the micronucleus is not transcribed and the macronucleus carries a single copy of the telomerase RNA gene (Greider and Blackburn 1989; Romero and Blackburn 1991), it is likely (but has not been proven) that the telomeres of both nuclei are synthesized by the same telomerase components of macronuclear origin and, therefore, that the micronuclear telomeres are also composed of G₄T₂ repeats. The micronuclear chromosomes contain numerous stretches of nontelomeric G₄T₂ tracts, a factor that has also hindered the cloning of micronuclear telomeres (Yao and Yao 1981). The chromosomal position and function of these internal tracts is not known, but frequently they are associated with a class of transposon-like elements (Cherry and Blackburn 1985). Interestingly, all internal telomere-like DNA tracts are eliminated from the chromosomes during differentiation to the transcriptionally active macronucleus.

As a first step toward understanding the functions and synthesis of micronuclear telomeres during vegetative growth and meiosis, we report here the identification and cloning of the *T. thermophila* micronuclear telomeres. We show that although the distal repeat sequence is G₄T₂ as in macronuclear telomeres, the micronuclear telomeres are unique in several respects.

Results

Identification of micronuclear telomeres

Macronuclear telomeric repeats have been well characterized in *T. thermophila* and consist virtually exclusively of G₄T₂ repeats (Budarf and Blackburn 1986; Spangler et al. 1988). Initial hybridization studies indicated that micronuclear telomeres contain the same repeat sequence (Shampay and Blackburn 1989). However, these telomeres have been very difficult to identify largely because of two reasons. First, the micronuclear genome contains ≤100 stretches of nontelomeric G₄T₂ tracts (Yao and Yao 1981). Thus, hybridization to the telomeres is obscured by the internal G₄T₂ repeats. Second, the micronucleus and macronucleus contain 20 and ~4 × 10⁴ chromosome ends, respectively. Hence, even greatly

purified micronuclei still contain high levels of contaminating macronuclear telomeres; a previous attempt to clone micronuclear telomeres by functional analysis in yeast yielded only internal micronuclear G_4T_2 tracts and macronuclear telomeres (Shampay and Blackburn 1989).

Initially, we attempted to identify micronuclear telomeres by assaying for G_4T_2 repetitive sequences that are preferentially sensitive to shortening by Bal31 exonuclease, a commonly used test for telomeric DNA (e.g., Cross et al. 1989; McEachern and Hicks 1993). Micronuclei were purified from the progeny of recently mated *T. thermophila* to minimize the loss or rearrangement of micronuclear DNA that may occur with extended passaging of vegetatively growing cells (Wyman and Blackburn 1991). DNA from the purified micronuclei was subjected to Bal31 treatment, and at various times during digestion, aliquots were removed and enzymatic activity was stopped. To monitor the rate of digestion, λ HindIII-digested DNA was included in the reaction. The samples were then digested with HindIII, and DNA fragments were separated by agarose gel electrophoresis under conditions that resolve high molecular weight products. A Southern blot of this gel was probed with a radiolabeled oligonucleotide consisting of the macronuclear telomeric sequence $(C_4A_2)_4$ (Fig. 1A). This analysis revealed two hybridizing bands, indicated by arrows in Figure 1A, whose migration changed over time relative to the stationary bands from hybridization to internal telomere-like repeats. The upper of these two candidate telomeric fragments is seen as the appearance of a band at the 4-min time point, and the lower candidate is seen as the shortening of a diffuse band from the 0- to 2-min time points. On the basis of the change in migration, ~ 1 kb was digested from each of these fragments, consistent with the rate of Bal31 digestion of ~ 250 bp/min determined from the λ HindIII-digested DNA (data not shown). These results suggested that the micronuclear telomeric G_4T_2 tracts are longer than 1 kb, which is considerably longer than macronuclear telomeres.

Previous evidence indicated that internal micronuclear G_4T_2 tracts are generally less than several hundred base pairs (Cherry and Blackburn 1985), and therefore, we reasoned that the background from internal tracts could be eliminated by digesting the micronuclear DNA with a frequently cutting restriction enzyme, and subsequently separating the smaller internal tracts from the larger telomeric fragments. The enzyme *MseI* (recognition sequence AATT) was selected because it cuts the extremely A/T-rich *T. thermophila* DNA roughly every 100 bp. After a Bal31 digestion time course, micronuclear DNA was cleaved with *MseI*. These samples were then analyzed by Southern blotting using the same $(C_4A_2)_4$ telomeric oligonucleotide as above (Fig. 1B). The results clearly revealed the true telomeres, visible as a broad smear that becomes shorter during Bal31 digestion. The bulk of the initial telomeric hybridization signal is between 2.0 and 3.4 kb. It is likely to consist almost entirely of telomeric repeats (shown below), as *MseI* cleaves ~ 100 bp from the start of the repetitive DNA. In a similar assay, purified macronuclear DNA

Figure 1. Southern blot analysis of Bal31-digested micronuclear DNA. Purified micronuclear DNA was digested with Bal31 exonuclease, and aliquots were removed and enzymatic activity stopped at the indicated times. The DNA was then digested with HindIII (A) or the frequently cutting enzyme *MseI* (B). DNA fragments were separated by agarose gel electrophoresis, and Southern blots were probed using an oligonucleotide with the telomeric repeat sequence $(C_4A_2)_4$. Two Bal31-sensitive fragments are barely detectable in A, indicated by arrows. The endonucleolytic activity of Bal31 causes a general loss of DNA with extended digestion, apparent at the last time point.

from the same cell population was run alongside micronuclear DNA, and the telomeric signal (which was nearly 100 times stronger) was centered ~ 500 bp, with no hybridization signal observed above 1.4 kb (data not shown). Hence, the smear observed in Figure 1B was not attributable to contaminating macronuclear DNA.

Cloning micronuclear telomeres

To clone micronuclear telomeres, we developed a strategy to eliminate contamination from the internal G_4T_2 sequences and from macronuclear telomeres. Micronuclear DNA was digested with Bal31 such that ~ 500 bp was lost from the ends, thereby completely digesting away most contaminating macronuclear telomeres but leaving $>75\%$ of the micronuclear telomeric tracts. *NotI* linkers were then added to this DNA to place a rare

restriction enzyme site immediately adjacent to the telomeric G_4T_2 tracts, thus differentiating the telomeres from the internal G_4T_2 tracts. The DNA was digested with *NotI* and *Tsp509I*, which cuts *T. thermophila* DNA very frequently and leaves termini complementary to those produced by *EcoRI* digestion. After separation on an agarose gel, DNA in the region corresponding to 1.0–3.5 kb was purified and cloned into a *NotI*–*EcoRI*-digested λ ZapII vector, and plaques were screened by hybridization to a $(C_4A_2)_4$ telomeric oligonucleotide.

From this screen, 20 positively hybridizing plaques were identified and the inserts were sequenced. Of these, 19 clones contained *NotI* sites immediately adjacent to a tract of telomeric repeats with the linker present in the orientation indicative of ligation to a telomere. The clones varied in the number of telomeric repeats they contained (the repeats are discussed below), but all were probably the result of deletions during the cloning process in *Escherichia coli*, as the insert sizes ranged from 300 to 850 bp. All clones contained ~60–100 bp of complex telomere-associated sequence (TAS) beginning with the predicted *Tsp509I* restriction site. The clones were categorized by TAS. Six independent chromosome ends could be identified by virtue of their differing TAS, labeled TAS A through TAS F in Figure 2. Because the micronuclear genome contains 10 pairs of chromosome ends, we have identified more than half of the TASs, if each chromosome end has a unique sequence and not accounting for allelic variation in heterozygotes. For each TAS, from two to five independent clones were obtained. All of these TASs have a high degree of homology to each other, ranging from 55 to 87% identity. In addition, these sequences have an unusually high G/C content (ranging from 35 to 44% overall, and particularly high near the telomeric repeats), relative to the ~25% overall G/C content of the *T. thermophila* genome (Prescott 1994). There are no other obvious sequence features, except for an imperfect inverted repeat, indicated by open arrows in Figure 2, present in five of the six sequences.

To verify the telomeric position and micronuclear origin of the cloned inserts, we investigated whether one of the cloned sequences (TAS A) was specific to micronuclear telomeres. To obtain a hybridization probe containing no telomeric repeats, a TAS fragment was made by

PCR using the primers indicated by solid arrows in Figure 2 with a TAS A-containing clone as template. This 89-bp PCR product was then used as a probe for Southern blot analysis of DNA from separately purified micronuclei and macronuclei which had been digested with *Bal31* for various times, then cleaved by *HindIII* (Fig. 3). The fragment hybridizing to the TAS A probe was shortened and then lost during the *Bal31* time course, indicating that this sequence does reside at a telomere. Under these relatively stringent conditions, this sequence was limited to the micronuclear telomere, that is, it was not present elsewhere in the micronuclear genome as a *Bal31*-resistant band. In addition, TAS A was not found in macronuclear DNA, indicating that this sequence is eliminated during macronuclear development. As a control for the telomeric specificity of *Bal31* digestion and for the presence of macronuclear DNA on the blot, the blot was stripped and reprobed with a radiolabeled fragment from the *T. thermophila* telomerase RNA gene (Fig. 3). As anticipated, this sequence was present in the DNA from both nuclei and was resistant to *Bal31*.

The centromere-proximal telomeric repeat sequence is G_4T_3 in all clones

Upon sequencing through the TASs of the cloned micronuclear telomeres, we were surprised to find that in all 19 independent clones, the sequence immediately adjacent to the complex TAS was not G_4T_2 as expected but, rather, G_4T_3 (Fig. 4; four examples are presented). In every clone, the repetitive telomeric sequence began with at least one G_4T_3 repeat. In 17 of the 19 clones, large tracts of ≤ 45 G_4T_3 repeats were present. The number of G_4T_3 repeats within each tract varied between clones, and the average was 28 repeats. These tracts were immediately adjacent to the more distal stretches of the expected telomeric repeat sequence G_4T_2 , and the junction between the two domains (indicated by arrowheads in Fig. 4) was $G_4T_3G_4T_2$ in all 19 clones except one. In no cloned telomere did we find any interspersion of the two types of repeat; out of $>10^3$ repeats sequenced, all were confined to their distinct G_4T_3 or G_4T_2 repeat domain.

More surprising than the existence of a variant repeat in the micronuclear telomeric DNA was the homogeneity of the G_4T_3 repeats, clearly seen from the sequencing

Figure 2. Sequence of six TASs, identified from 19 different clones. Homology alignments were performed using TAS C for comparison, facilitated by the Altschul DNA sequence alignment program. Dashes represent identity, and dots represent gaps. All clones had the *Tsp509I* restriction site (AATT) at the 5' end. Solid arrows represent the position of primers used to synthesize a PCR product of TAS A. Open arrows represent the imperfect inverted repeat found in five TASs. The start of the telomeric repeats is shaded.

Figure 3. Southern blot analysis of Bal31-digested purified micronuclear (MIC) and macronuclear (MAC) DNA. DNA was digested with Bal31 exonuclease, and aliquots were removed and enzymatic activity stopped at the indicated times. The DNA was then digested with *Hind*III, and fragments were separated by agarose gel electrophoresis. A Southern blot was probed with a radiolabeled PCR product of TAS A, where indicated. This probe was then removed from the blot, and the blot was re-probed with a radiolabeled fragment of the macronuclear telomerase RNA gene, where indicated, as a control. Some overall loss of DNA occurred at the last time point, because of the endonucleolytic activity of Bal31.

data of the four clones in Figure 4. Accurate sequencing data were obtained from ≥ 200 repeats in the cloned G_4T_3 domains, and none contained any sequence other than G_4T_3 . In addition, we searched for pattern changes among the longer sequencing products from copying the C-rich strand and found no indication of a single base change. Thus, it is highly likely that none of the total of 476 repeats found in the cloned G_4T_3 domains contained any variation in sequence. Similarly, all information that we obtained from reading either telomeric DNA strand indicated that there were no sequence variations among the 529 repeats of the G_4T_2 domains.

G₄T₃ repeat content and distribution in native micronuclear telomeres

As mentioned above, it was evident from the insert sizes that all the telomere clones obtained had lost telomeric repeats by unknown processes during cloning. Therefore, to analyze the overall G_4T_3 repeat content and distribution in native micronuclear telomeres, we used differential hybridization to G_4T_2 and G_4T_3 oligonucleotides to

distinguish between the two different repeat sequences. Two plasmids were used as controls: one containing a 21-repeat G_4T_2 insert (p G_4T_2 -21), and the other containing an 18-repeat G_4T_3 insert (p G_4T_3 -18). Thus, the total length of the telomeric DNA tract was 126 bp in each plasmid. Optimal specificity was obtained with two oligonucleotides having the sequences $GT_2(G_4T_2)_3G$ and $T_3(G_4T_3)_3$, designed to maximize mismatches to the nonspecific telomeric repeat, washed at a temperature of 55°C (see Materials and methods).

To quantitate the level of hybridization obtained with either probe and to determine the levels of cross-hybridization to the nonspecific sequences, two identical dot blots were prepared having duplicate spots of fivefold serial dilutions of p G_4T_2 -21, p G_4T_3 -18, and control Bluescript plasmid alone. First, as the control for the molar quantity of plasmid on the dot blot, hybridization to a radiolabeled oligonucleotide corresponding to the T7 promoter in the Bluescript plasmid was used (Fig. 5; only the 20-ng spot is shown in the bottom row). The T7 promoter probe was then removed, and the same blots were re-probed with either the G_4T_2 - or G_4T_3 -specific oligonucleotide (Fig. 5, top three rows). For both oligo-

Figure 4. Sequence analysis of telomeric repeats from four different micronuclear telomere clones. The T7 promoter sequence was used as the sequencing primer to synthesize through the TAS of the G-rich strand. The transition from G_4T_3 to G_4T_2 repeats is indicated by arrowheads for clones 11, 3, and 10. In clone 6, the transition was located more distally, and the results from the longer products were more difficult to read. In this case, the transition was determined from sequencing reactions of the opposite strand (not shown).

Figure 5. Dot blot hybridization analysis to determine hybridization signal intensity and cross-reactivity of G_4T_2 and G_4T_3 repeat-specific oligonucleotides. Duplicate dot blots were prepared carrying serial dilutions in the amounts indicated of a Bluescript plasmid containing 21 G_4T_2 repeats (p G_4T_2 -21), a Bluescript plasmid containing 18 G_4T_3 repeats (p G_4T_3 -18), and the Bluescript plasmid alone. The blots were first probed with a radiolabeled oligonucleotide of the T7 promoter (T7pr) sequence as a control for the molar quantity of plasmid on the blots. The spots containing the 20-ng dilutions were quantitated by PhosphorImager in Table 1, and an autoradiograph is shown (T7pr). The probe was then washed off the blots, and the same blots were re-probed with radiolabeled oligonucleotides having the sequence 5'GT₂(G₄T₂)₃G as the G_4T_2 -specific probe and 5'T₃(G₄T₃)₃ as the G_4T_3 -specific probe. The spots containing the 20-ng dilutions were quantitated by PhosphorImager in Table 1, and autoradiographs of the entire blots are shown.

nucleotides, the levels of cross-hybridization to the Bluescript vector and to the nonspecific repeat sequence were negligible under these conditions ($\leq 1\%$). The relative hybridization signal for each telomeric probe was normalized to the molar amount of plasmid DNA on the blot, based on the T7 promoter control. The radioactivity from the 20-ng spots of this (Fig. 5) and a duplicate set of blots (autoradiograph not shown) was quantitated using a PhosphorImager (Table 1). Table 1 shows that the relative G_4T_2 /T7 oligonucleotide hybridization was 1.63 for p G_4T_2 -21, and the relative G_4T_3 /T7 oligonucleotide hybridization was 0.63 for p G_4T_3 -18. Thus, hybridization of the G_4T_2 probe to its specific telomeric repeat sequence resulted in a signal intensity 2.6 times greater than that obtained from hybridization of the G_4T_3 probe to its corresponding repeat sequence. This value was used in subsequent quantitations to determine the G_4T_3 repeat content and distribution of native micronuclear telomeres.

Purified micronuclear DNA was subjected to a gradual time course digestion with Bal31 (using duplicate zero time points), and samples were cleaved with *MseI*, and divided in half for duplicate Southern blots. The blots were probed with either the G_4T_2 or G_4T_3 oligonucleotide and washed under conditions to maximize specificity (Fig. 6). The test plasmid dot blots quantitated in Table 1 were included in the same bag with the corre-

Figure 6. Southern blot analysis of Bal31-digested micronuclear DNA to determine the G_4T_3 repeat content and distribution in native micronuclear telomeres. Purified micronuclear DNA was digested with Bal31 exonuclease, and aliquots were removed and enzymatic activity stopped at the indicated times. For reproducibility, two aliquots were removed before the addition of enzyme (zero time point). DNA samples were then digested with the restriction enzyme *MseI*, and each sample was divided in half and separated by electrophoresis in the same agarose gel so that duplicate blots could be prepared. Southern blots were probed with radiolabeled oligonucleotides having the sequence 5'GT₂(G₄T₂)₃G as the G_4T_2 -specific probe and 5'T₃(G₄T₃)₃ as the G_4T_3 -specific probe. The broken line to the left of the G_4T_2 autoradiograph indicates the 2.0- to 3.4-kb region of the zero time point samples (lanes 1,2) quantitated by a PhosphorImager in Table 2. The solid bars (left) indicate the position of the Bal31-digested samples quantitated by a PhosphorImager in Table 2, and the numbers indicate which lane was quantitated in this region. The signals from identical positions of both blots were quantitated.

Table 1. Quantitation of hybridization to plasmid dot blot

p G_4T_2 -21			p G_4T_3 -18		
probe			probe		
G_4T_2 ^a	T7pr ^a	G_4T_2 /T7pr	G_4T_3 ^a	T7pr ^a	G_4T_3 /T7pr
7.84	4.51	1.74	3.99	6.69	0.60
7.51	4.84	1.55	4.22	7.33	0.58
8.17	5.00	1.63	5.01	7.17	0.70
8.12	5.04	1.61	4.60	7.00	0.66
Mean		1.63			0.63
S.D. ^b		0.08			0.06

^aPhosphorImager (Molecular Dynamics, CA) units, $\times 10^5$

^bStandard deviation.

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sponding micronuclear DNA blot during hybridization and washes. Thus, there could be no potential variation in signal intensity because of differences in ionic strength or temperature. The specificity of the probes is further seen on the blots in Figure 6 by the lack of hybridization of the G_4T_3 oligonucleotide to most chromosome-internal G_4T_2 repeat tracts. Only two Bal31-resistant fragments were strongly detected by the G_4T_3 probe. The origin of these chromosome-internal G_4T_3 tracts is unknown, and like their G_4T_2 counterparts, they were not detectable in macronuclear DNA (data not shown).

The results from this experiment were used first to determine the overall G_4T_3 repeat composition in full-length native micronuclear telomeres. Before Bal31 digestion, the bulk of the telomeric DNA fragments was 2.0–3.4 kb in length (Fig. 6, lanes 1,2; region marked by broken line). This region of both blots in the two zero time point lanes was quantitated by PhosphorImager analysis (Table 2). After correcting for the 2.6-fold difference in relative signal between the two oligonucleotides (from Table 1), it was determined that 28% of the total telomeric DNA consists of G_4T_3 repeats, corresponding to 0.6–1.0 kb per telomere. Equivalent values (ranging from 24 to 36%) were consistently obtained from different experiments using the same DNA and also a different micronuclear DNA preparation from another stock of the same strain. Similar results were also obtained from five other *T. thermophila* laboratory strains, including the most divergent C3 strain (data not shown).

As described above, all 19 telomere clones contained one or more centromere-proximal G_4T_3 repeats adjacent to the distal G_4T_2 tracts. Because the largest cloned G_4T_3 tract (~0.3 kb) was less than half the size determined to be present in the average native telomere, we addressed the possibility that the remaining 0.3- to 0.7-kb G_4T_3 tracts present in the native micronuclear DNA are lo-

Figure 7. Schematic diagram illustrating two possible configurations of native micronuclear telomeres having ~30% G_4T_3 repeat composition. Large solid bars indicate G_4T_3 tracts, large shaded bars indicate G_4T_2 tracts, and small open bars indicate complex TASSs. (A) A single G_4T_3 tract confined to the centromere-proximal region is depicted. (B) A G_4T_3 tract corresponding to the largest cloned G_4T_3 tract (~45 repeats) is depicted centromere proximally, and the remaining G_4T_3 repeats are arbitrarily spaced more distally. Below each configuration is shown the resulting telomeric fragment after digestion of 1.35 kb by Bal31, indicated by a vertical broken line, and the predicted percent G_4T_3 composition. The 1.15 kb is the last position that was analyzed by Southern blot PhosphorImager quantitation, because of the strong hybridization signal from the internal telomere-like repeats masking the signal of the smaller telomeric fragments. A possible derivation of telomere clone from each configuration of native telomere is also depicted; the lines represent regions that would have been deleted during the cloning process.

Table 2. Quantitation of G_4T_2 and G_4T_3 hybridization to Bal31-digested micronuclear DNA

Gel lane ^a	Bal31 digestion time (sec)	Quantitated sample (kb)	G_4T_3 ^b	G_4T_3 , corrected ^c	G_4T_2 ^b
1	0	2.0–3.4	74.2	193	489
2	0	2.0–3.4	69.3	180	450
1	0	2.50	6.7	17.5	33.4
2	0	2.50	5.6	14.6	36.3
3	50	2.20	4.8	12.5	40.2
4	100	2.05	3.8	9.9	31.5
5	150	1.90	2.1	5.5	25.6
6	200	1.75	1.5	3.9	13.2
7	250	1.55	1.9	4.9	10.2
8	300	1.35	2.4	6.2	8.8
9	350	1.15	3.4	8.8	4.9

^aFig. 6, lane from which sample was quantitated.

^bPhosphorImager (Molecular Dynamics, CA) units, $\times 10^3$.

^cPhosphorImager units $\times 2.6$ correction factor from Table 1, $\times 10^3$.

cated more distally. In addition, as the most distal 500 bp of telomeric DNA had been digested before cloning, it was possible that large G_4T_3 tracts could have been present near the chromosome termini. Schematic diagrams of two possible native telomere configurations are presented in Figure 7, which depicts an average 2.5-kb telomeric fragment that contains 100-bp complex TAS and ~700-bp G_4T_3 repeats. In figure 7A, all G_4T_3 repeats are positioned centromere proximally. In this case, digestion of 1.35 kb by Bal31 (as in Fig. 6, lane 9) would result in a final fragment having ~70% G_4T_3 composition. Alternatively, if the native telomeres contain large tracts of G_4T_3 repeats more distally, shown arbitrarily positioned in Figure 7B, the same extent of Bal31 digestion would result in a fragment having considerably less G_4T_3 repeat composition, in this example, <30%.

By quantitating the relative repeat signal during the course of Bal31 digestion in Figure 6, we investigated the position of the G_4T_3 tracts in native telomeres. Samples

from each lane of the two blots in Figure 6 were quantitated by PhosphorImager analysis. To avoid artifactual signals and those from internal telomere-like repeats, samples of a narrow size range were chosen, which corresponded approximately to the average telomeric fragment, decreasing in size roughly linearly during Bal31 digestion (indicated by bars to the left of the G_4T_2 blot in Figure 6). The data presented in Table 2 indicate that although the hybridization signal obtained with each probe decreased during digestion (as expected because of the increased separation of the smaller sized fragments in the gel), a much greater proportion of the G_4T_3 signal remained at the later time points; 55% of the original G_4T_3 signal was still present at the last time point, whereas only 14% of the G_4T_2 signal remained. In addition, the G_4T_3 repeat composition at the last Bal31 time point (Fig. 6, lane 9) was calculated as a percentage of the combined $G_4T_2 + G_4T_3$ signals. At the final time point, when 1.35 kb has been lost from the telomeres, the remaining 1.15-kb fragments have a G_4T_3 composition of 64%. These results are consistent with an internal localization of the G_4T_3 repeats, as depicted in Figure 7A. This determination has limitations, however, because of inherent telomere length variability, possible nonuniform rates of Bal31 activity, and due to the inability to analyze telomeric fragments shorter than ~ 1.0 kb in Figure 6, because of masking by the internal telomere-like repeats. Therefore, we cannot completely rule out the existence of some distally located G_4T_3 repeats. Within these limitations, we conclude that the G_4T_3 tracts are positioned centromere proximally to most or all of the G_4T_2 repeats in native micronuclear telomeres.

Discussion

Here, we report the identification and cloning of micronuclear telomeres from *T. thermophila*, the first ciliate micronuclear telomeres to be isolated. Previous results from *T. thermophila* (Shampay and Blackburn 1989) and the hypotrichous ciliate *Oxytricha fallax* (Dawson and Herrick 1984) have indicated the probability that ciliate micronuclear telomeres contain the same repeat sequence as their macronuclear counterparts. We report that the distal telomeric repeat sequence in the *T. thermophila* micronucleus is the same as that found in the macronucleus, but the micronuclear telomere sequence is nonetheless unique in several respects. Thus, the three *T. thermophila* organelle genomes have telomeres that are distinct in sequence and in sequence arrangement: the long telomeres containing inner G_4T_3 repeat tracts of the micronucleus; the short G_4T_2 telomeres of the macronucleus; and the mitochondrial telomeres having unusually complex repeat sequences (Morin and Cech 1988).

One fundamental difference between the telomeric DNA from the two *T. thermophila* nuclei is length. The bulk of micronuclear telomeric DNA (including both types of repeats) ranges in size from 2.0 to 3.4 kb, whereas macronuclear telomeres are only 0.3–0.5 kb. It is worth noting that the macronuclear telomere length is

quite typical for a unicellular organism [e.g., yeasts (Shampay et al. 1984; McEachern and Blackburn 1994) or *Chlamydomonas* (Petracek et al. 1990)], whereas the micronuclear telomere length more closely reflects that of typical higher eukaryotes [e.g., *Arabidopsis* (Richards et al. 1992) or *Ascaris* (Muller et al. 1991)]. It is possible that the length difference in the two *T. thermophila* nuclei is the result of different selective pressures. In the macronucleus, shorter telomeres may be advantageous, supported by the observation that cells with shorter macronuclear telomeres have a growth advantage over cells with longer telomeres (Larson et al. 1987). In the micronucleus, perhaps the larger stretches of telomeric DNA are required for some unknown specific mitotic or meiotic telomere function. Interestingly, in humans, telomere length has also been shown to be significantly greater in the germ line than in somatic cells (for review, see de Lange 1994), and it has been proposed that this length difference is the result of a telomerase activity, which is greater in germ cells. The telomere length disparity seen in the two *T. thermophila* nuclei may reflect differences in the regulation of telomerase activity or other telomeric proteins. It is known that macronuclear telomeres are synthesized by telomerase during vegetative growth and de novo during development in mating cells (Yu et al. 1990; Yu and Blackburn 1991), and some evidence indicates that telomerase activity is increased during mating (Greider and Blackburn 1985; Avilion et al. 1992). Whether micronuclear telomere synthesis follows the temporal pattern of macronuclear telomere synthesis or whether the micronucleus has its own timing for telomere synthesis, independent of macronuclear events, is unknown. The latter possibility is suggested by the fact that DNA replication in the two nuclei occurs at different times during the cell cycle, as does nuclear division (McDonald 1973). We cannot exclude the possibility that telomerase acts on the micronuclear telomeres only during mating, and that the longer telomeres in this nucleus are necessary to compensate for inactive telomerase during vegetative growth. Studies of the mechanism and timing of telomere synthesis in the micronucleus will now be facilitated by the identification of the micronuclear telomere sequence.

Another difference between the telomeres of the two *T. thermophila* nuclei concerns TASs. All macronuclear TASs identified have an extremely low G/C content, $\sim 5\%$, and appear to have no sequence relationship to one another (Yokoyama and Yao 1986; Spangler et al. 1988; Yu and Blackburn 1991). In contrast, the 50 bp immediately adjacent to the telomeric repeats in all six cloned micronuclear TASs has a high G/C content of $\sim 50\%$, high relative even to the $\sim 25\%$ overall G/C content of the micronuclear genome (Prescott 1994). In addition, all micronuclear TASs identified have a high degree of homology to each other, ranging from 55 to 87% identity.

The one TAS that was tested, TAS A, hybridized only to micronuclear telomeres and was not present in the macronucleus. These results indicate that TAS A is among those sequences eliminated during macronuclear

differentiation. Approximately 10–20% of the micronuclear genome is eliminated during macronuclear development in conjugating *T. thermophila* (for review, see Karrer 1986). This process involves the temporally regulated, precise excision of specific sequences and families of sequences, followed by their active degradation. Because TAS A is not found in macronuclear DNA, and the adjacent G_4T_3 repeats also have not been found in any macronuclear telomere clone or by hybridization to macronuclear DNA, it is plausible that all micronuclear telomeric DNA is lost during differentiation. It is possible that some aspect of the micronuclear telomere structure is detrimental to the macronucleus and, therefore, is specifically eliminated. Alternatively, perhaps there is a mechanistic link between the elimination of the micronuclear telomeres and the chromosome-internal G_4T_2 tracts, although the reason for the loss of these or any other sequences is not known.

The most novel feature of the *T. thermophila* micronuclear telomeres is the presence of long homogeneous tracts of a variant repeat. Analysis of the G_4T_3 content and distribution in native micronuclear telomeres indicates the presence of 0.6- to 1.0-kb G_4T_3 sequence per telomere, most likely localized centromere proximally. We cannot exclude the possibility that the native micronuclear telomeres have some interspersions of the two types of repeat or contain yet another variant repeat sequence, which is undetectable in our hybridization assay designed specifically for G_4T_3 and G_4T_2 repeats. However, the complete lack of any configuration other than homogeneous G_4T_3 tracts followed by homogeneous G_4T_2 tracts in every cloned micronuclear telomere argues against significant interspersed regions or additional variant repeats.

Analysis of micronuclear DNA from five other *T. thermophila* strains indicated a similar G_4T_3 repeat composition (24–36%), although the variant G_4T_3 repeat was not detected in micronuclear telomeres from a closely related species, *Tetrahymena malaccensis* (K.E. Kirk and E.H. Blackburn, unpubl.). It should be noted that *T. thermophila* macronuclear telomeres are extremely homogeneous, consisting virtually exclusively of G_4T_2 repeats. Of >400 macronuclear telomeric repeats sequenced, only one G_5T_2 variant was found (Budarf and Blackburn 1986; Spangler et al. 1988). Given the homogeneity of the micronuclear G_4T_3 tracts, their conservation in all *T. thermophila* strains examined, and their absence in macronuclear telomeres, it is tempting to speculate that the G_4T_3 tracts might play a role in some specialized telomere function of the micronucleus, such as mitotic chromosome segregation or meiosis, or in telomere length regulation. Perhaps there are specific G_4T_3 -binding proteins in the micronucleus. It is interesting to note that although G_4T_3 repeats are not normally found in wild-type macronuclear telomeres, they can be tolerated in vivo when synthesized onto the ends of macronuclear chromosomes by a template mutation of the telomerase RNA gene. Of several variant telomeric repeat sequences that have been synthesized in this manner in vivo, the G_4T_3 variant is the only se-

quence that is not lethal (Yu et al. 1990; D. Gilley and E.H. Blackburn, pers. comm.).

A telomere configuration in which there are distinct, homogeneous arrays of two different telomeric repeats, such as that found in the *T. thermophila* micronucleus, has not been identified previously. Most eukaryotic telomeres contain uniform arrays of a single telomeric repeat. Exceptions include *S. cerevisiae* telomeres that are entirely made up of irregular repeats, and the telomeres of *Paramecium tetraurelia* (Forney and Blackburn 1988) and certain *Candida tropicalis* strains (McEachern and Blackburn 1994), which are composed of two distinct variants seemingly randomly dispersed. In some eukaryotes, the distal telomeric DNA is uniformly composed of a single telomeric repeat, but the proximal regions contain dispersed variants. For example, in *Arabidopsis thaliana*, the outermost repeats are present in a homogeneous tract and, moving inward, a stretch of heterogeneous variant repeats is found, followed by a stretch of highly degenerate repeats, until the complex TAS is reached (Richards et al. 1992). In humans, the distal telomeric repeats are homogeneous, but several variant repeats are scattered among the more inner repeats (Brown et al. 1990). It is not known whether the variant or degenerate telomeric repeats found in the inner domains of some organisms have a function or whether they are merely tolerated mutations.

Telomeric DNA can be envisioned as having two domains with a somewhat indeterminate border: one outer domain in which the “faithful” telomeric repeats are the result of relatively recent telomerase-mediated synthesis, and an inner, possibly degenerate, region that is replicated solely by conventional DNA polymerases. It seems probable that the G_4T_3 tracts in the *T. thermophila* micronuclear telomeres are part of an inner domain that is not a product of telomerase activity during vegetative growth. What is the origin of the G_4T_3 variant repeats in the micronuclear telomeres? One possibility is that they are the evolutionary remnant of an ancient telomerase enzyme that synthesized G_4T_3 repeats. In this model, however, it is surprising that no mutations have appeared in the G_4T_3 repeat tracts, unless there is a very strong selection against any such mutations. Another possibility is that they arose directly from the amplification and homogenization of a telomeric repeat mutation that occurred originally in the inner domain of a micronuclear telomere. Several mechanisms have been invoked for the creation and maintenance of homogeneous repetitive DNA sequences (Smith 1976; Dover 1982; Lohe and Brutlag 1987), and one or more of these may have been used throughout the evolution of the micronuclear telomeres. An alternative hypothesis is that the micronucleus has two different, sequential telomerase activities that function to synthesize its telomeres: one bearing a G_4T_3 -templating RNA expressed uniquely in the micronucleus, and the second carrying the macronuclear-expressed G_4T_2 version. Therefore, the micronucleus would necessarily possess two telomerase RNA genes. We have searched for a second gene by Southern blot hybridization to the macronucleus-expressed ver-

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sion and have not found any indication of a second copy in the micronucleus (K.E. Kirk and E.H. Blackburn, unpubl.).

The results presented here provide the foundation for future investigations into the regulation of germ-line micronuclear telomere length and synthesis. In addition, it should be possible to alter the outermost telomeric repeat sequences of micronuclear chromosomes and study the effect of such mutations on micronuclear mitotic divisions and on meiosis.

Materials and methods

Strains and growth conditions

Routine growth conditions for *T. thermophila* have been described previously (Orias and Bruns 1975). Strains PB-9R [ChxA2/ChxA2 (cy-s)] mating type II and VII were cultured in 2% PPYS, cells were starved and mated as described previously (Wyman and Blackburn 1991), and progeny were selected by growth in 15 µg/ml of cycloheximide. For large-scale growth, cells were inoculated into 40 liters of prewarmed 2% PPYS containing 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin B, and antifoam in a 60-liter fermentor to an initial concentration of 10^3 /ml. Cells were grown at 30°C with 25 lb air pressure and 125 rpm stirring to a final cell density of 1.4×10^5 /ml. *E. coli* strain XLI-blue MRF' (Stratagene) was used for propagation of a phage library, *E. coli* strain SOLR (Stratagene) was used for the excision of Bluescript plasmids from the phage vector, strain DH5α was used for amplification of pG₄T₂-21, and strain XLI-blue (Stratagene) was used for amplification of pG₄T₃-18.

Purification of nuclei

Micronuclei were purified by a modification of procedures described previously (Gorovsky et al. 1975; Allen et al. 1983). A 40-liter culture of 1.4×10^5 cells/ml was prepared ~75 generations postmating. Cells were concentrated across a Millipore membrane, centrifuged at 3000 rpm for 5 min using a Sorvall GS-3 rotor, and washed in 0.2 M sucrose, 10 mM Tris-HCl (pH 7.5), and 2 mM MgCl₂. Remaining procedures were carried out at 4°C. Cells were resuspended in 1.8 liters of medium A (Allen et al. 1983), and 18 ml of *n*-octanol was added slowly with stirring. Cells were lysed in a 1-liter Waring Blendor in two batches, and nuclei were released from the cell debris by five cycles of blending for 30 sec at 5-min intervals. The lysate was then centrifuged in a Sorvall GSA rotor at 3000 rpm for 7 min, and the supernatant (enriched for micronuclei) was blended as before. This material was centrifuged at 6000 rpm for 15 min, and the pellets containing micronuclei and contaminating macronuclei were resuspended in 48 ml of medium A. Separation of micronuclei and macronuclei was achieved on Percoll (Sigma) gradients, essentially as described previously (Allen et al. 1983). The nuclei were layered onto 8×24 ml of preformed 25% Percoll gradients and centrifuged at 3000 rpm for 18 min in an HB-4 rotor. All except the bottom 5 ml of the gradient was discarded, and the nuclei were washed once in medium A containing 0.2% NP-40 and centrifuged as above. The pellet was resuspended in 20 ml of medium A, layered onto 4×24 ml of preformed 50% Percoll gradients, and centrifuged at 3000 rpm for 8.5 min. The micronuclear fractions were drawn from the top one-third of the tubes, and the macronuclei were collected from the bottom of the tubes. The micronuclei were washed and separated again on a second 50% Percoll gradient. The final fractions were washed twice in medium A and resuspended in 10 ml of 60 mM KCl, 15

mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, and 15 mM Tris-HCl (pH 7.4). A 10% yield of micronuclei was obtained (based on 1 micronucleus/cell-starting material), generating a 300- to 400-fold purification from macronuclei.

Isolation of DNA

Nuclei were lysed by adding 18 ml of 1.5% sarcosyl, 0.5 M EDTA, and 10 mM Tris-HCl (pH 7.4) and incubating for 30 min at 50°C. Ten milliliters of 0.5 M EDTA, 10 mM Tris-HCl (pH 7.4), containing 0.2 mg/ml of pronase, was then added and incubated overnight. The macronuclear DNA sample was extracted with phenol-chloroform and ethanol precipitated. The micronuclear DNA sample was separated from contaminating macronuclear rDNA by Hoechst-CsCl gradient. For every 24 ml of DNA solution, 0.5 mg of Hoechst 33342 and 48 ml of saturated CsCl were added and centrifuged on a VTi 50 rotor at 42,000 rpm for 24 hr at 18°C. The micronuclear DNA band was collected, and the Hoechst dye was removed by extraction with isopropanol; DNA was ethanol precipitated. Yield was ~1 pg of DNA/micronucleus.

Bal31 nuclease digestion

Typical Bal31 nuclease digestions contained 7 µg of DNA placed into 35 µl of buffer containing 12 mM CaCl₂, 12 mM MgCl₂, 0.2 M NaCl, 20 mM Tris-HCl (pH 8.0), and 1 mM EDTA, pre-equilibrated at 30°C. Five microliters was removed and added to 0.1 volume of 0.2 M EGTA (zero time point). To the remaining DNA, 0.4 units of Bal31 nuclease (New England Biolabs) was added and vortexed, and the mixture was incubated at 30°C. Aliquots (5 µl) were removed at various times and added to EGTA to stop the reaction. The samples were then diluted up to 40 µl in the appropriate buffer and restriction enzyme.

Cloning micronuclear telomeric DNA

Standard techniques (Sambrook et al. 1989) and manufacturers' suggestions were used. Forty micrograms of micronuclear DNA was digested with 2 units of Bal31 for 1 min at 30°C, and the extent of telomere shortening was verified to be ~500 bp by Southern blot analysis. The DNA ends were then made flush by T4 DNA polymerase and ligated to 8 µg of *Not*I linkers. DNA was digested with *Not*I and *Tsp*509I (New England Biolabs). The fragments were separated by 0.8% agarose electrophoresis and the region of the gel corresponding to 1.0–3.5 kb was cut out, 2.5 µg of *E. coli* DNA was added as carrier, and the DNA was purified using GeneClean (Bio 101, Inc.). The λ Zap II vector (Stratagene) was prepared as follows: 1 µg of undigested λ DNA was digested with *Not*I, treated with calf intestinal phosphatase, and digested with *Eco*RI. The purified micronuclear telomeric DNA was ligated to the λ Zap II vector for 2 days at 14°C, and the DNA was packaged according to manufacturer's recommendations. The entire unamplified library (2000 plaques) was transferred to Hybond-N+ (Amersham) membrane and screened with a radiolabeled (C₄A₂)₄ oligonucleotide according to manufacturer's suggestions. Twenty positive plaques were identified (one was later found to be nontelomeric). The Bluescript plasmids containing inserts were excised from the phage DNA following Stratagene's protocol.

Sequencing telomeric clones

Standard procedures were used to prepare plasmid DNA (Sambrook et al. 1989). Attempts to sequence through telomeric re-

peaks on both strands, using the M13 reverse sequencing primer (–24) and the M13 sequencing primer (–20) were only partially successful using a standard sequencing kit (U.S. Biochemical). Unambiguous results were obtained using a 7-deaza dGTP kit (U.S. Biochemical) to read the C-rich strand, primed by the T7 promoter primer; therefore, most sequencing data were derived from copying the C-rich strand.

Plasmid construction

pG₄T₂-21 was constructed by ligating a *Bam*HI fragment containing 21 G₄T₂ repeats [originally cloned from a macronuclear telomere (Challoner and Blackburn 1986)] into the *Bam*HI site of Bluescript II SK(–) (Stratagene). pG₄T₃-18 was constructed as follows. A micronuclear telomere clone [TAS C; (G₄T₃)_{–37}, (G₄T₂)_{–12}] was digested with *Not*I. The linearized plasmid was then subjected to a *Bal*31 digestion time course to progressively digest away the G₄T₂ repeats and some of the G₄T₃ repeats. DNA samples from four time points were treated with T4 DNA polymerase and T4 DNA ligase, and used to transform *E. coli*. Twelve transformant plasmids were sequenced, and one containing a tract of only 18 G₄T₃ repeats was chosen for further use.

Dot blot assay

pG₄T₂-21 and pG₄T₃-18 DNAs were prepared using a plasmid kit (Qiagen) following the manufacturer's protocol. Solutions containing 2.0, 0.40, and 0.08 µg/ml of each plasmid in H₂O were boiled and placed on ice, and an equal volume of ice-cold 10× SSC (Sambrook et al. 1989) was added. One hundred microliters of each dilution was loaded onto the dot blot apparatus containing Hybond-N+ membrane (Amersham), and the DNA was bound to the membrane using a UV Stratalinker (Stratagene). The denatured plasmids were hybridized to a series of G₄T₂- or G₄T₃-specific radiolabeled oligonucleotide probes having different lengths and repeat permutations (not shown), and the maximum specificity was obtained with oligonucleotides 5'-GTT(GGGGTT)₃G and 5'-TTT(GGGGTTT)₃. Hybridization of these two oligonucleotides took place at 30°C, and washes were done at increasing temperatures in increments of 5°C. Greater than 10-fold specificity did not occur until above 45°C, and nearly all hybridization signal was lost at 60°C for both oligonucleotides (not shown).

Hybridization probes

Oligonucleotide probes were 5' end-radiolabeled using T4 polynucleotide kinase following standard protocol (Sambrook et al. 1989). 5'(CCCCAA)₄ was used as a probe for the Southern blot in Figure 1 and as a probe to screen the micronuclear telomere library. 5'-GTT(GGGGTT)₃G was used as the G₄T₂-specific probe, and 5'-TTT(GGGGTTT)₃ was used as the G₄T₃-specific probe in Figures 5 and 6, and the T7 promoter primer 5'-TAATACGACTCACTATAGGGAGA was used in Figure 5. The TAS A probe used in Figure 3 was prepared as follows. Oligonucleotides 5'-AATTATAAATAAAATAGGTATAG and 5'-ACAACCTTTGAGGTTGTTTCGG were used as primers for the micronuclear TAS A clone in a PCR using reagents from the GeneAmp kit (Perkin Elmer Cetus). The amplified product was gel purified using GeneClean (Bio 101, Inc.) and was radiolabeled using reagents from the Multiprime DNA Labeling System (Amersham). The telomerase RNA probe used in Figure 3 was a gel-purified 570-bp *Xho*I fragment from JB101 (Yu et al. 1990), which was multiprime labeled.

Hybridization conditions

All hybridizations were carried out in 0.5 M Na₂HPO₄, 7% SDS, and 1% BSA (Church and Gilbert 1984) overnight. The blots in Figure 1 were hybridized at 48°C and washed in 0.2 M Na₂HPO₄, 2% SDS, at 48°C. The blots in Figure 3 were hybridized at 65°C and washed as above at 60°C. The G₄T₂ and G₄T₃ blots in Figures 5 and 6 were hybridized at 30°C and washed in 0.05 M Na₂HPO₄, 2% SDS, at 55°C. The blot using the T7 promoter probe in Figure 5 was hybridized at 25°C and washed in 0.2 M Na₂HPO₄, 2% SDS, at 25°C.

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