Developmentally regulated telomere addition in *Tetrahymena thermophila*

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ABSTRACT

To investigate the developmentally programmed telomere addition that accompanies chromosome fragmentation during macronuclear differentiation in *Tetrahymena thermophila*, five representative telomeric regions from the macronucleus were cloned and characterized in detail. The sequences adjacent to the telomeric (C₄A₂:T₂G₄) repeats on these five macronuclear ends had no significant sequence homology or shared secondary structure. Two developmentally independent examples of one macronuclear telomere had a 5 base pair difference in the position of the junction between the telomeric repeats and the adjacent sequences. A telomere-adjacent sequence, in the form of a synthetic oligonucleotide, was unable to prime the addition of telomeric repeats *in vitro*. The implications of these results for the mechanisms underlying developmentally programmed chromosome fragmentation and telomere addition in *Tetrahymena* are discussed.

INTRODUCTION

Developmentally programmed formation of new telomeres in somatic nuclei is a striking aspect of cellular differentiation in ciliated protozoa and some nematodes (1-4). Like other ciliated protozoa, *Tetrahymena thermophila* is unicellular but contains two distinct types of nuclei: a germline micronucleus and a somatic macronucleus. The micronucleus is diploid, undergoes normal mitosis and meiosis, and is generally transcriptionally silent during vegetative growth. The macronucleus is polyploid, divides amitotically, and is transcriptionally active (5). The macronucleus is derived from the micronucleus during the sexual phase of the life cycle (reviewed in 6).

Studies of DNA renaturation kinetics indicate that 10-20% of the sequence complexity of the micronuclear genome is

absent from the macronucleus of T. thermophila (7). This conclusion is supported by comparisons of randomly chosen regions of the micronuclear genome with their macronuclear counterparts (8,9,10,11,12). Most of the eliminated sequences are moderately repetitive (8,9,10,12), and are dispersed on all five micronuclear chromosomes (8,10,12). In the majority of the DNA elimination events occurring during macronuclear development, the sequences flanking the eliminated region become joined together (11,12). However at a few hundred sites the sequences adjacent to the eliminated regions are not joined together. Instead they become stable, free ends in the macronucleus (13,14,15). Thus, macronuclear development involves specific chromosome breakage as well as DNA sequence elimination. An activity that adds telomeric repeats to telomeric sequence primers in a non-templated fashion has been identified in Tetrahymena cell extracts (16,17). Non-telomeric G+C-rich sequences were tested and did not prime telomere addition by this activity in vitro. However, there is substantial evidence that the telomeric repeated sequence $(C_4A_2:T_2G_4)_n$, present at the ends of all macronuclear DNA molecules in Tetrahymena (18), is added de novo to the newly created DNA ends following chromosome breakage (15,19,20).

Yokoyama and Yao (21) found the 5-nucleotide sequence 5'-TTATT-3' present within a few base pairs of the telomeric repeats in each of three telomeric regions they cloned from Tetrahymena macronuclei. We report here the characterization of five additional cloned telomeric restriction fragments from the Tetrahymena macronucleus. The sequence 5'-TTATT-3' was present in only three of these additional macronuclear ends. In all macronuclear telomeres that have been characterized to date, we can identify no other conserved feature of the DNA sequence or secondary structure. In addition, for one particular macronuclear telomere, we present evidence that the site of telomere addition is not uniquely determined at the nucleotide level. These results are in agreement with a model for chromosome breakage in which the sequences that identify a breakage site are located entirely in micronuclear chromosomal regions that are eliminated during macronuclear development.

Such a sequence has recently been identified by Yao et al. (15), who characterized several micronuclear chromosome breakage sites and identified a conserved 15-nucleotide sequence (Cbs sequence) located within regions destined to be eliminated during macronuclear development.

The potential for variability at the sites of chromosome breakage and the lack of DNA sequence conservation between different macronuclear ends raise the question of what is recognized by the enzyme(s) that add telomeric repeats to newly created DNA ends. As an initial step in identifying the requirements for addition of telomeric repeats to a non-telomeric sequence, we tested the sequence adjacent to the telomeric repeats in one of our clones for the ability to function as a substrate for the addition of telomeric repeats in vitro.

MATERIALS AND METHODS

Strains and culture conditions

Tetrahymena thermophila strain B (lines B1868 and B1979) were provided by D. Nanney, University of Illinois. Stocks were maintained in liquid culture at room temperature in 1% PPYS (1% Proteose Peptone (Difco), 0.1% Yeast Extract (Difco), 0.001% Sequesterine (Ciba-Geigy)) and were diluted into fresh media every three weeks. To obtain large cultures of Tetrahymena, cells from a stock culture were inoculated into flasks containing 2% PPYS (same as 1% PPYS, all concentrations doubled) and were incubated at 30°C with gentle aeration.

DNA Isolation

Tetrahymena micronuclei and macronuclei were prepared as described by Howard and Blackburn (12), except that the filtration of the micronuclear preparation was omitted, and nuclear DNAs were further purified according to Cherry and Blackburn (22). Macronuclear DNA was subsequently fractionated by Hoechst 33258-CsCl density-gradient centrifugation to remove rDNA (23).

Cloning

Macronuclear end clones pTtMe1, pTtMe2, pTtMe3 and pTre3 were isolated as follows: macronuclear DNA was digested briefly

with either Bal-31 (New England Biolabs, Inc.) or S1 nuclease (Bethesda Research Laboratories) to create blunt ends at the termini, using conditions suggested by the manufacturer. Nuclease digestion was stopped by the addition of EDTA, followed by extraction with phenol-chloroform and precipitation of the DNA with ethanol. The nuclease-treated macronuclear DNA was subsequently digested to completion with BamHI, BglII or HindIII. The plasmid vector pBR322 was digested with PvuII to produce a blunt end, and either BamHI or HindIII to produce an appropriate sticky end, and the fragment to be used in cloning was purified by elution from an agarose gel using the method of Vogelstein and Gillespie (24). Macronuclear DNA was ligated to the pBR322 fragment and the ligation products were transformed into E. coli strain DH-1. Recombinant plasmids containing telomeric sequences were identified by colony hybridization (25), using a hybridization probe consisting entirely of the telomeric repeated sequence $(C_4A_2:T_2G_4)_n$.

Macronuclear end clone pTtMe4 was originally isolated by Janis Shampay (pers. comm.), using a yeast linear plasmid vector (26). The recombinant macronuclear end fragment was subcloned into pBR322 using essentially the method described above.

Blots and Hybridizations

Agarose gel electrophoresis and transfer of DNA to nitrocellulose or nylon (Nytran; Schleicher and Schuell) filters was carried out using standard procedures (27). DNA probes were labelled with $\alpha^{32}P$ by either nick translation (28) or random hexamer-primed labelling (29). Hybridizations generally were carried out in 5 X SSPE (1X SSPE= 0.15 M NaCl, 15 mM NaPO₄, pH 7.7, 1 mM EDTA) and 1 X Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin) at 65°C. Filters were washed twice for 15 minutes in 1 X SSC (1X SSC= 0.15 M NaCl, 15 mM NaCitrate, pH. 7.0), 1% SDS at 65°C, and twice for 15 minutes in 0.1 X SSC, 0.1% SDS at room temperature.

To test for homology between the cloned macronuclear end sequences, each plasmid was digested with a restriction enzyme (AhaIII) that cuts frequently within the cloned region. The digests were electrophoretically separated on gels and blotted, and the blots were probed with the following: pBR322 (to

identify vector fragments), a fragment consisting entirely of $C_4A_2:T_2G_4$ repeats, or each entire plasmid clone. The blots were washed under moderately stringent conditions (~12 degrees below the T_m).

Bal-31 digestions for blots

Bal-31 digestions were carried out using buffer conditions suggested by the manufacturer (New England Biolabs, Inc.). In one experiment (pTre3 probe) macronuclear DNA was digested with Bal-31 (0.65 u/ μ g DNA) at 30°C, and aliquots from the reaction were stopped at times ranging from 0-20 minutes. In a second experiment (all other probes) macronuclear DNA was digested with BAL-31 (0.44 u/ μ g DNA) at 24°C, and aliquots from the reaction were stopped at times ranging from 0-10 minutes. In both experiments, Bal-31 digestion was stopped by the addition of EDTA, followed by extraction of the DNA with phenol-chloroform and precipitation with ethanol.

Figure 1. Summary of restriction mapping data for five macronuclear end clones. For restriction enzymes that cut frequently within the cloned macronuclear sequences (AhaIII, SspI), only some sites are shown. Telomeric repeats, . Macronuclear DNA, . pBR322 DNA, . Fragments used as hybridization probes, . Restriction sites: AhaIII=V. BamHI=||. BglII=↓. EcoRI=∇. HindIII=V. HinfI=↑↓. SspI=↓.

DNA sequence analysis

DNA sequences were determined by the method of Maxam and Gilbert (30). In preparation for sequencing, DNA fragments were either 5'-end-labelled using [$\gamma^{32}\text{P}$]-ATP and polynucleotide kinase (30), or 3'-end-labelled with terminal transferase (31), except that [$\alpha^{32}\text{P}$]dideoxy ATP was used according to the manufacturer (Amersham) instead of cordycepin triphosphate.

In vitro telomere addition assay

Tetrahymena cell extracts were prepared as described by Greider and Blackburn (16) and were generously provided by Carol Greider. The assay was carried out using a range of concentrations of substrate oligomer in the reaction. The lowest corresponded to the concentration of $(\text{T}_2\text{G}_4)_4$ typically used by Greider and Blackburn ($0.25\ \mu\text{M}$; ref.16), and the highest was 40-fold greater. For each assay, the oligomer was combined with Tetrahymena nuclear extract, [$\alpha^{32}\text{P}$]-dGTP, unlabelled dTTP and reaction buffer as described (16). After incubation at 30°C for 90 minutes the reactions were stopped with EDTA, extracted with phenol-chloroform and precipitated with ethanol. DNA pellets were resuspended in gel loading buffer and analyzed by electrophoresis on a 6% polyacrylamide denaturing gel.

RESULTS

Cloning macronuclear DNA ends

Five telomeric restriction fragments from Tetrahymena macronuclear DNA were cloned into pBR322 as described in the Materials and Methods. The restriction map deduced for each (Fig.1) showed that the sequences adjacent to the telomeric repeats in these five clones differ from one another. No common pattern of restriction sites was found, such as that found in yeast (32), indicating that there is no large conserved sequence located internally to the telomeric repeats. In addition, no homology was detectable by hybridization between the cloned sequences, with the exception of the telomeric $\text{C}_4\text{A}_2:\text{T}_2\text{G}_4$ repeats.

To determine whether the cloned sequences identified by $\text{C}_4\text{A}_2:\text{T}_2\text{G}_4$ homology represented macronuclear telomeres, macronuclear DNA was treated with Bal-31 nuclease and hybridized

with the cloned telomere region probes shown in Figure 1. Telomeric sequences should be progressively shortened by Bal-31 digestion of genomic macronuclear DNA. Macronuclear DNA was digested with Bal-31 for times ranging from 0-10 minutes, then digested with a restriction endonuclease. The resultant fragments were separated by agarose gel electrophoresis, blotted, and the blots were probed with fragments isolated from the non-C₄A₂:T₂G₄ portion of each of the five clones. Where possible, the restriction endonuclease used to digest the Bal-31-treated macronuclear DNA was chosen so that the probe would hybridize to two DNA fragments: the putative telomeric fragment containing the C₄A₂:T₂G₄ repeats, and an internal fragment to provide a control for endonucleolytic degradation of the DNA. The results of this analysis are presented in Figure 2.

At the zero time point, each probe hybridized to restriction fragments of the size predicted if the clone represented a macronuclear end. In every case, the fragment which (by comparison to the plasmids) was expected to contain the telomeric repeats was heterogeneous in size, as is characteristic of macronuclear telomeres in *Tetrahymena* (33,34). Most significantly, with increasing time of Bal-31 digestion the heterogeneous bands were progressively shortened, while the internal bands remained unchanged. This result demonstrates that the cloned sequences represent true macronuclear ends.

Genomic Arrangements of Macronuclear End Sequences

On the basis of the restriction mapping data, we identified one of the clones (pTre3) as the terminal BglIII fragment of the rDNA. This was subsequently verified by hybridization to an rDNA probe (data not shown) and by DNA sequence analysis (see below). To examine the genomic distribution of the four non-rDNA clones, we used restriction fragments from those clones to probe micronuclear and macronuclear genomic blots. The genomic restriction maps deduced for each clone are presented in Figure 3. In each case, the macronuclear restriction map was precisely colinear with the map of the cloned sequence. In addition, in all macronuclear restriction digests using enzymes that cut at sites further from the telomere than the positions of the hybridization probes, only a single band was detected with each

Figure 2. Results of tests for Bal-31 sensitivity. Bal-31 digestions were performed as described in the text. The numbers above the lanes of the autoradiograms indicate the number of minutes of Bal-31 digestion for each DNA sample. The hybridization probes used in this analysis are indicated in Figure 1.

probe. This is consistent with the conclusion that each of the cloned telomeres is found on only a single species of subchromosomal macronuclear DNA molecule.

Probes from pTtMe1, pTtMe2 and pTtMe4 hybridized to a simple pattern of restriction fragments in micronuclear genomic digests (data not shown), suggesting that these three macronuclear end sequences are each present as a unique sequence in the micronucleus. In each case the micronuclear map is

Figure 3. Micronuclear and macronuclear genomic restriction maps for the non-rDNA macronuclear end clones. In the restriction maps presented here, only restriction sites whose positions could be determined unambiguously are indicated. The arrangement of sequences homologous to the pTtMe3 probe in the micronucleus is complex (see text).

colinear with the macronuclear map at all positions which can be unambiguously determined, and in each case there are micronuclear restriction sites that map to positions several kb beyond the site that would correspond to the macronuclear DNA end (Figure 3). Thus these macronuclear ends are non-telomeric in the micronucleus, and are not associated with any chromosomal rearrangements except fragmentation during macronuclear development.

The arrangement of the clone pTtMe3 in the micronucleus is more complex. Hybridization to different nullisomic strains of *Tetrahymena*, which lack both copies of various combinations of micronuclear chromosomes but have independently derived normal macronuclei (35), showed that sequences homologous to the pTtMe3

probe are present on at least two different micronuclear chromosomes (data not shown). We have not determined which one of those loci is processed to generate a macronuclear end.

The bulk of the macronuclear genome is replicated to a final ploidy level of ~45C during macronuclear differentiation, except for the rDNA, which is amplified from a single micronuclear copy to $\sim 10^4$ copies per macronucleus (36,37). For each cloned non-rDNA macronuclear telomere, the intensity of hybridization of the cloned probe to comparable amounts of micronuclear and macronuclear DNA on dot blots and Southern blots were similar. From this we would argue that none of these sequences is significantly amplified above ~45C in the macronucleus.

Telomere-Associated DNA Sequences

To compare the cloned macronuclear telomeres further, and to identify any regions of limited homology not detectable on blots, we determined their nucleotide sequences for a distance of approximately 100 nucleotides adjacent to the telomeric repeats (Fig. 4). Computer and manual searches identified no regions of significant homology. Although there is the potential for extensive secondary structure within these sequences, we can identify no particular structure which is conserved among all five. With the exception of the telomeric repeats, the only obvious characteristic shared by these different macronuclear ends is that the sequences adjacent to the repeats are extremely A+T rich. The A+T content of the sequence for approximately 100 bp immediately adjacent to the telomeric repeats for all five clones varies from 83% to 95.5%, and is significantly above the average for the genome (77%) (7).

The pattern of hybridization of a $C_4A_2:T_2G_4$ probe to genomic blots of DNA from macronuclei independently developed from genetically identical micronuclei appears to be invariant (34). Probes specific to pTtMel_{2,3} or 4 were hybridized with macronuclear DNA from both genetically identical and different laboratory strains of *T. thermophila*. These sequences were also in similar sized restriction fragments in the different macronuclei, apart from the small variations in size attributable to different numbers of telomeric $C_4A_2:T_2G_4$ repeats

Figure 4. Nucleotide sequences of five macronuclear end clones. For each macronuclear end clone, the DNA sequence for approximately 100 nucleotides adjacent to the telomeric repeats is shown. The sequences are aligned so that nucleotide number one in each case is the first nucleotide that is clearly not a part of the telomeric repeated sequence (C₄A₂:T₂G₄)_n. The sequences read from 5' to 3', with the telomeric repeats at the 5' end.

described elsewhere (38) (data not shown). At the level of sensitivity of these experiments, these results indicate that telomere addition occurs reproducibly at the same sites each time a new macronucleus develops.

To determine whether or not telomere addition sites are uniquely defined at the nucleotide level, we compared the DNA sequence of the junction between the telomeric repeats and the adjacent sequences in two examples of the rDNA telomere that were formed in two independent macronuclear development events. pTre3 was compared with the previously cloned rDNA telomere pTrel (19). These two rDNA telomeres are from the same highly inbred B strain of *T. thermophila*. Stocks B1868 and B1979 are the products of 18 and 19 generations of inbreeding respectively of a single original B strain isolate. Hence, the micronuclear genomes in this homozygous strain should be identical, so that any differences detected between them in the macronucleus almost certainly result from variability in genome rearrangement during macronuclear development. Although >100 adjacent base pairs of rDNA sequences were identical, a 5 base pair difference was found in the position at which the telomeric repeats begin in the two cloned rDNA telomeres (Fig. 5). This difference in the position of the junction site in developmentally independent examples of the same macronuclear end shows that developmentally programmed chromosome breakage and/or telomere addition in

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B1868 mic TTTTAAAAATAAGTTAAACCCCAATTTAATAATTTCTTTGACAT
B1868 mac (pTrel)      ....CCCCAACCCCAA --- --TAATTTCTTTGACAT
B1979 mac (pTre3)      ....CCCCAACCCCAATTTAATAATTTCTTTGACAT
                                telomeric
                                C4A2
                                repeats
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Figure 5. Sites of addition of telomeric repeats in two independently developed rDNA ends. The sequence adjacent to the telomeric repeats is shown for each macronuclear rDNA end clone, and the corresponding micronuclear sequence is shown for B1868 (14).

Tetrahymena is not necessarily precise at the nucleotide level.
Addition of Telomeric Repeats

Greider and Blackburn (16,17) have demonstrated that an activity present in Tetrahymena cell extracts synthesizes long tracts of $(T_2G_4)_n$ de novo, given an appropriate telomeric sequence DNA oligonucleotide primer. Since the telomeric C_4A_2 : T_2G_4 repeats are added de novo to DNA ends lacking telomeric repeats following chromosome breakage (15,19,20), the sequences immediately adjacent to those repeats in our macronuclear end clones must have at some time been substrates for the addition of telomeric repeats. We tested the telomere-adjacent sequence from pTtMe3 for its ability to function as a substrate for synthesis of telomeric repeats in vitro, using crude cell extracts prepared from a mating culture of Tetrahymena at approximately nine hours after mating was initiated. At this time the early stages of macronuclear development take place.

Two 20-base oligomers corresponding to each strand of the sequence adjacent to the telomeric repeats in pTtMe3 were tested, as either the single-stranded oligodeoxynucleotides 5'-AACTTTTTTTAGGAATAAAA-3' and 5'-TTTTATTCCTAAAAAAGTT-3', or as a duplex between the two oligomers. A DNA oligomer consisting of $(T_2G_4)_4$ was a positive control. The assay was carried out essentially as described by Greider and Blackburn (16). As shown in Figure 6, no telomere elongation activity was detected with any of the test oligomers at any concentration tested in this assay, although $(T_2G_4)_4$ was an active substrate for elongation under the same conditions.

DISCUSSION

Analysis of the DNA sequences of five cloned macronuclear telomeres revealed no significant sequence homology other than the telomeric C₄A₂:T₂G₄ repeats, and no obvious conserved secondary structure. The 5 base sequence TTATT is present within 10 bases of the telomeric repeats in 6 of the total of 8 macronuclear telomeres that have been characterized in this work and previously (21). The 7-nucleotide sequence 5'-TTATTTT-3' found within a few base pairs of the telomeric repeats in two of the three macronuclear end clones characterized previously (21) was present within 50 bases of the telomeric repeats in only one of the five clones that we examined. As the macronuclear telomere-adjacent sequences analyzed so far are 80-95% A+T over that distance from the telomeric repeats, the appearance of these sequences near the repeats is not statistically significant.

The macronuclear telomeric regions that we have characterized are all present as only one (pTtMel_{2,4}) or a few (pTtMe₃) copies in the micronucleus. The simplest interpretation of the genomic mapping data is that each is processed to generate a single species of macronuclear DNA molecule. In contrast, in Paramecium alternative macronuclear telomere addition sites are found to be located several kb apart (39). The presence of widely separated alternative breakage sites suggests that there may be several independent signals identifying different sites for chromosome fragmentation, and that any one of those sites can be used during macronuclear development. It will be necessary to look at more examples of Tetrahymena macronuclear ends to determine whether there is any potential for large-scale variability in the sites of chromosome breakage during Tetrahymena macronuclear development.

The high A+T content of the sequences immediately adjacent to the telomeric repeats is the only evident characteristic shared by all of the macronuclear ends that have been examined. Thus it seems unlikely that the sequences destined to become macronuclear ends provide cis-acting signals specifying the sites of micronuclear chromosome breakage. Also, since the (C₄A₂:T₂G₄) repeats are added de novo during macronuclear

development, and even a single repeat is generally not present a regions destined to form macronuclear telomeres (15,19), these repeats cannot play an essential role in chromosome fragmentation. These facts lead to the conclusion that cis-acting signals for chromosome breakage lie between the precursors to macronuclear DNA molecules, in DNA regions that are eliminated during macronuclear development. Recently Yao et al. (15) identified a 15-nucleotide sequence (Cbs) which is associated with chromosome breakage sites in the micronucleus, and which is eliminated during macronuclear development. Although there is no direct evidence that the Cbs sequence is involved in chromosome fragmentation, its preferential association with chromosome breakage sites is convincing circumstantial evidence for its functional importance. It is interesting that no corresponding conserved sequence is found near telomere addition sites in either the holotrichous ciliate Paramecium (39) or the hypotrichous ciliate Oxytricha (L. Klobutcher, pers. comm.). This suggests that the types of recognition sites specifying chromosome breakage, and their relation to telomere addition sites, may differ between ciliates.

To explore further the process of telomere addition, we examined two developmentally independent examples of a particular macronuclear end. The finding that the site of rDNA telomere addition in a homozygous inbred strain varied by 5 bp could reflect imprecision in the choice of chromosome breakage site, or could reflect the opportunity for exonucleotic degradation of the DNA after breakage and before telomere addition. Microheterogeneity of the position of telomeric repeat addition has also been found in homozygous Paramecium (39).

To examine the requirements for addition of telomeric repeats to a newly created DNA end, we assayed in vitro addition of telomeric repeats to the sequence found immediately adjacent to the telomeric repeats in macronuclear end clone pTtMe3, in the form of single or double stranded synthetic DNA oligonucleotides. However, this sequence was not a substrate for addition of telomeric repeats in vitro, under conditions where a

telomeric sequence oligonucleotide was active as a substrate. One possible explanation for this result is that addition of telomeric repeats to a non-telomeric-sequence substrate may be mechanistically coupled to chromosome breakage. Since the ends of broken chromosomes are extremely unstable in vivo in the absence of telomere addition (40,41,42), it would seem to be advantageous for telomeric repeats to be added as rapidly as possible following breakage.

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