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Sequence characterization of *Tetrahymena* macronuclear DNA ends

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ABSTRACT

Tetrahymena is a ciliated protozoan which has two nuclei: a micronucleus, which maintains the genetic continuity of the cell, and the macronucleus which is derived from the micronucleus after sexual conjugation. A macronuclear DNA library was constructed to contain DNA ends. A probe containing C_4A_2 repeats which are known to be present at macronuclear DNA ends (1) was used to screen the library. Three clones were characterized by sequencing, restriction enzyme mapping and Bal 31 digestion. The data indicate that these three clones represent macronuclear DNA ends which were generated by DNA fragmentation during macronuclear formation. The sequencing data at the C_4A_2 repeat junction show a conserved sequence of five nucleotides, TTATT. Sequences further away show no obvious homologies except that they are highly enriched in AT. This structure is quite different from the subtelomeric sequences of other organisms.

INTRODUCTION

Telomeres, the ends of eucaryotic chromosomes, are specialized structures which play an important role in the completion of replication of linear DNA molecules and providing stability to the chromosome. The termini of several lower eucaryotes have been found to be similar to each other and consist of simple tandem repeats, e.g., C_4A_2, C_4A_4, C_3TA_2, C_3TA_n, $C_{1-8}T$, and $C_{1-3}A$ in the holotrichous, hypotrichous ciliates, trypanosomes, Physarum rDNA (ribosomal RNA gene), Dictyostelium rDNA and yeast, respectively (2).

Tetrahymena, a ciliated protozoan, is a good model system for the study of telomeres because of the abundant chromosome ends in the somatic nucleus (i.e., macronucleus). The macronuclear DNA makes up 95% of the nuclear DNA, divides amitotically, and is responsible for the phenotype of the cell. The macronucleus is derived from the micronucleus after each sexual conjugation. During macronuclear formation, the five micronuclear chromosome pairs are fragmented into several hundred subchromosomal-sized pieces, ranging in size from 21 kb (the rDNA molecule) to greater than 1100 kb (3). As judged by sensitivity to the double-stranded exonuclease Bal 31, many and perhaps all,

of these subchromosomal DNAs contain C_4A_2 repeats or similar sequences at their ends (1). Although the lack of chromosome condensation in the macronucleus has precluded the cytological analysis of the chromosomal structure, we shall refer to the natural duplex ends in the DNA as chromosome ends or telomeres.

Formation of the rRNA gene in the Tetrahymena macronucleus involves both fragmentation and telomere addition. During macronuclear formation, the single copy present in the micronucleus is excised, leaving free ends on both sides and forms a head-to-head dimeric palindromic copy (4-9). Although 20-70 tandemly repeated copies of C_4A_2 are present at each end of the macronuclear rDNA molecules (10), there is only one C_4A_2 at the 3' end of the micronuclear copy (11). Nearly 2 kb of the 5' junction of the micronuclear rDNA has also been sequenced, and not a single C_4A_2 is found (9) . Therefore, addition of C_4A_2 repeats to newly formed ends must be an integral part of the macronuclear development.

So far, the molecular characterization of Tetrahymena macronuclear DNA ends has been limited to the rDNA molecule. Since the rDNA is a very specialized molecule, being the most amplified and the smallest molecule, it might not be representative of other Tetrahymena telomeres. In order to better understand general telomeric characteristics, we have cloned Tetrahymena DNA segments adjacent to macronuclear chromosome ends. Three of the recombinants, chosen for the presence of C_4A_2 repeats, were analyzed. The data show that all three clones contain perfect repeats of C_4A_2, and share a five nucleotide sequence homology in the region immediately adjacent to it.

MATERIALS AND METHODS

Cell Cultures and DNA Isolation

Tetrahymena thermophila inbreeding strain BIV was grown in axenic medium and nuclei were prepared as described by Gorovsky et al. (12). The nuclear DNA was isolated by phenol extraction as described previously (13).

DNA Cloning

The construction of a library which contains macronuclear DNA ends is illustrated in Figure 1. High molecular weight Tetrahymena DNA was obtained as described previously (13). After Proteinase K treatment, the DNA was sedimented on a 15-30% continuous sucrose gradient by centrifugation at 24,000 rpm for 17 hours in a Spinco SW27 rotor at 4°C. Fractions from the gradients were analyzed by electrophoresis in 0.5% gels using Type V agarose (Sigma) and those fractions containing high molecular weight DNA were pooled

and used for cloning. This DNA was gently treated with S1 nuclease to provide blunt ends as described by Pickup et al. (14) except that incubation was done at 37°C rather than 55°C. An EcoRI linker was produced by kinasing the Sma I-hexamer (dCCCGGG) and annealing it with the EcoRI-Sma I adaptor d(AATTCCCGGG) as described by Bahl et al. (15). The phenol extracted Tetrahymena DNA was ligated to the linker DNA. The linker-Tetrahymena DNA was then partially digested with Bgl II to aim for the clonable size range of 7-20 kb, and precipitated with 5% PEG as described in Lis (16). The DNA was then kinased and ligated with Charon 30 (17) arms, which had been purified from Charon 30 DNA by sedimentation on a 5-20% NaCl gradient after EcoRI and BamHI digestion. The ligated DNA was in vitro packaged into phage particles by a modified version of the method of Hohn (18) and plated on the host E. coli strain Q358. The phage were screened for the presence of C_4A_2 repeats by using band d of pTt2512 (19) for plaque hybridization as described by Benton and Davis (20).

Gel Electrophoresis and Hybridizations

Agarose gel electrophoresis of DNA, blotting of the DNA onto nitrocellulose filters and hybridization of the filters were carried out as described previously (21,22). Hind III-digested λ DNA was included in the gels as size markers. Restriction enzymes and Bal 31 were purchased from Bethesda Research Laboratories and New England Biolabs, Inc. Digestion with Bal 31 was carried out as described earlier (8). The method of Rigby et al. (23) was used for nick translation.

Hybridization was generally carried out at 65°C for 16-18 hours in 4XSSC (SSC contains 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7), 0.1 M Tris HCl, pH 7.4, 0.5% SDS and 5X Denhardt solution (24). The hybridized filters were washed extensively at room temperature with 2XSSC, unless otherwise noted, and then at least 1 hour at 65°C prior to autoradiography. When searching for cross homology between the λ clones, the hybridization conditions were less stringent, using 60°C rather than 65°C.

A C_4A_2 probe was prepared for colony hybridization by kinasing the oligomer $(C_4A_2)_3$ with γ-^{32}P ATP (25). Hybridizations were carried out at 30°C for 16-18 hours in 2XSSC, 0.1 M Tris HCl, pH 7.4, 0.5% SDS and 5X Denhardt solution. After hybridization, the filters were washed extensively with 2XSSC at room temperature.

Restriction Mapping and DNA Sequencing

Restriction maps of the cloned DNA was obtained primarily from single and double restriction enzyme digests. Various DNA fragments were subcloned

into the vector pEMBL 8+ (26).

Nucleotide sequences of DNA were determined by the chemical method of Maxam and Gilbert (27) and/or by the dideoxy chain termination method of Sanger et al. (28). When using the chemical method, DNA fragments were labelled by filling in the 3' end, using the large fragment of E. coli DNA polymerase.

Subclones for sequencing the C_4A_2 junctions were obtained by Eco RI digestion of each of the λ clones and treating the DNA with the exonuclease Bal 31 for various lengths of time. The DNA was then ligated with Eco RI linkers and digested with Eco RI and Hind III. The DNA was subcloned into the vector pEMBL 9+.

RESULTS

Cloning of macronuclear DNA ends

In order to study macronuclear telomeric sequences, it was necessary to use a cloning strategy which would include real ends. This was accomplished as shown diagramatically in Figure 1 and described in the Materials and

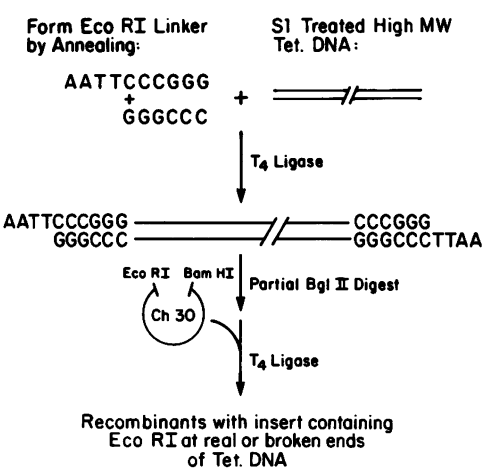

Figure 1. Cloning of macronuclear DNA ends. A linker was made by annealing a kinased hexamer (dCCCGGG) with a decamer d(AATTCCCGGG). This will provide the desired 5'-AATT-3' protruding end, making Eco RI digestion unnecessary. This linker was blunt-end ligated to high molecular weight Tetrahymena DNA which had been gently treated with S1 nuclease. The linker-Tetrahymena DNA was partially digested with Bgl II and ligated to Charon 30 which had been digested to completion with Eco RI and Bam HI. This protocol should produce clones which have an Eco RI site immediately adjacent to a DNA end, either real (and therefore containing a telomeric sequence) or artificially generated during DNA isolation.

Methods section. It is important to note that the only products which could be cloned presumably are those that have an Eco RI linker attached to an end, either real or generated by DNA isolation procedures. Also, the vast majority of the clonable pieces should consist of macronuclear DNA since the high molecular weight DNA is made up of 95% macronuclear DNA and 5% micronuclear DNA.

Band d of pTt 2512 is known to hybridize to C_4A_2 repeats which are found at or near macronuclear ends and to internal micronuclear C_4A_2 repeats (8, 19). The band d was used as a hybridization probe to screen approximately 5000 plaques. 85 positive clones were obtained. Our analysis will be confined to three of those which showed C_4A_2 repeats at or near the EcoRI site.

Hybridization Characteristics of the Cloned DNA

Restriction mapping and Bal 31 sensitivity were used to verify that the three clones were derived from macronuclear ends. To map the genomic DNA in relation to each clone, micronuclear, macronuclear and cloned DNA were digested with various restriction enzymes. The digests were fractionated by gel electrophoresis, blotted onto a nitrocellulose filter, and then hybridized with a portion of the cloned DNA. Probe A, located fairly close to the C_4A_2 repeats, from each clone (see Figure 3) was used for the hybridizations.

Figure 2 shows the results of some of these hybridizations. Regardless of the restriction enzymes used or the clone hybridized, the results are consistent in two respects. First, the hybridizing micronuclear band is always larger than the macronuclear band. This is consistent with the view that the macronuclear DNA free end is produced by fragmentation, which will be discussed in more detail later. Second, the macronuclear DNA fragments are similar in size to the cloned DNA fragments. This result indicates that we have indeed cloned a macronuclear and not a micronuclear DNA fragment.

It should be noted that the macronuclear DNA bands are broader and fuzzier when compared to micronuclear DNA bands, indicating heterogeneity typical of DNA ends in Tetrahymena (8,10). Also, heterogeneity of all three clones has been a common characteristic, as can clearly be seen as multiple bands in clones cTt 1169 and 1175 (see Figures 2d-h). As we will see in the next section, sequencing results show that this heterogeneity is due to differences in the numbers of C_4A_2 repeats.

Figure 3 summarizes the hybridization data of Figure 2 by lining up the restriction sites of the micro-, macronuclear and cloned DNA (see also

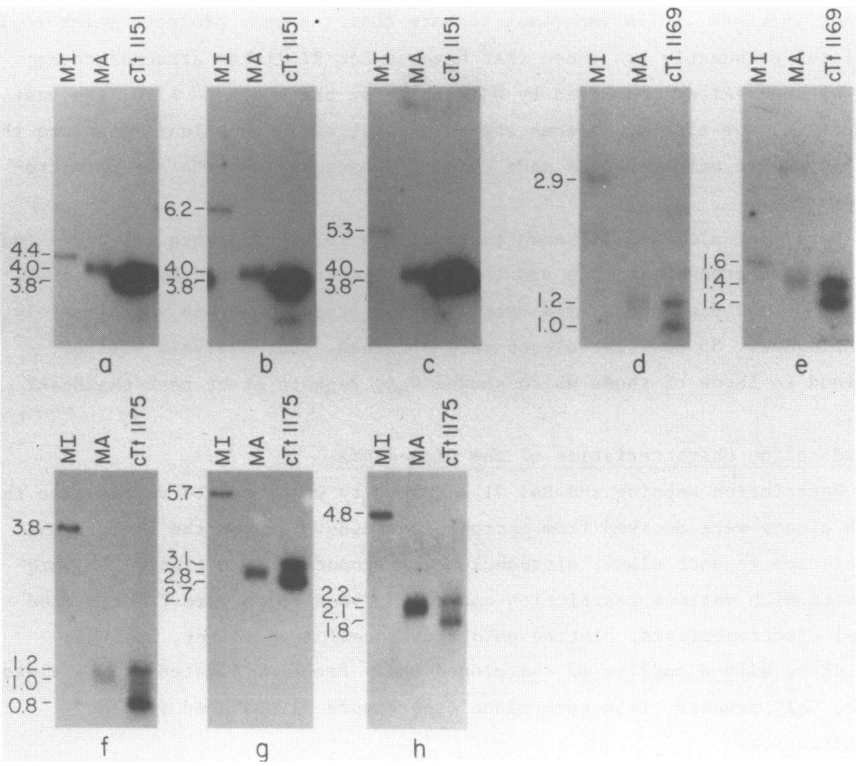

Figure 2. Southern hybridization of nuclear and cloned DNA using probe A from each clone. Approximately 1.5 μg each of micronuclear and macronuclear DNA and between 2.5 and 25 ng of cloned DNAs were digested with various enzymes, separated on 1% agarose gels and blotted. Probe A from cTt 1151, 1169 and 1175 (see Figure 3) were hybridized to filters shown as sets a-c, d-e, and f-h, respectively. The DNAs in each set were digested with: a, d and f, Hae III; b and g, Hha I; e, Rsa I; c and h, Hpa II. The cloned DNAs were also digested with Eco RI. The sizes (in kilobases) of the hybridizing bands are indicated to the left of each set.

Figure 4). It should be noted that this map is derived from hybridization data only. The black box shown in each clone represents the variable C_4A_2 region. It can be seen that the macronuclear and cloned DNA are basically the same in the restriction sites examined, including the alignment between the Eco RI linker and the macronuclear DNA terminus. Alignment of the restriction sites of the larger micronuclear DNA segment with the other two suggests that the smaller macronuclear fragment is produced from the micronuclear DNA by fragmentation.

The exonuclease Bal 31 digests DNA free ends (29). Therefore, when high molecular DNA is treated with Bal 31 and then subjected to restriction

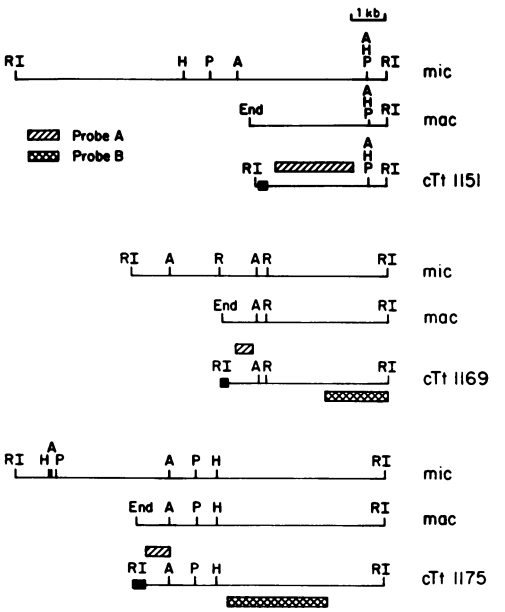

Figure 3. Restriction maps of the genomic DNAs detectable by hybridization with probe A of each respective clone. This diagram summarizes the hybridization data shown in Figures 2 and 4. Because probe A from cTt 1169 and 1175 did not hybridize to the Bal 31-treated macronuclear DNA, the filters shown in Figures 4b and c were hybridized to the more proximal probe B. However, probe A and B gave the identical Eco RI restriction map of micro- and macronuclear DNA. The hatched box above each clone designates the subcloned fragment used as probe A. This map is derived from hybridization data only and therefore only detects restriction enzyme sites on either side of this probe A. The cross-hatched box below clones cTt 1169 and 1175 designates probe B which was used for Figures 4b and c, respectively. The black box at the end of each clone designates the variable C_4A_2 repeat region. A: Hae III; H: Hha I; P: Hpa II; R: Rsa I; RI: Eco RI; End: chromosomal end.

enzyme digestion, a shortening of the fragment of interest is an indication of a free end. This method has been applied successfully to the analysis of Tetrahymena DNA (1,8,9), and it is known that the internal clusters of the C_4A_2 repeats in the micronuclear genome are not sensitive to Bal 31 digestion, whereas the terminal clusters in the macronucleus are (1). Micronuclear and macronuclear DNA with and without Bal 31 treatment, and cloned DNA were digested with Eco RI, separated by size using gel electrophoresis and blotted onto a nitrocellulose filter. Probe A of cTt 1151 and probe B from cTt 1169 and 1175 (see Figure 3) were hybridized to the filters. Figure 4 shows that the Bal 31-treated macronuclear DNAs were definitely shortened.

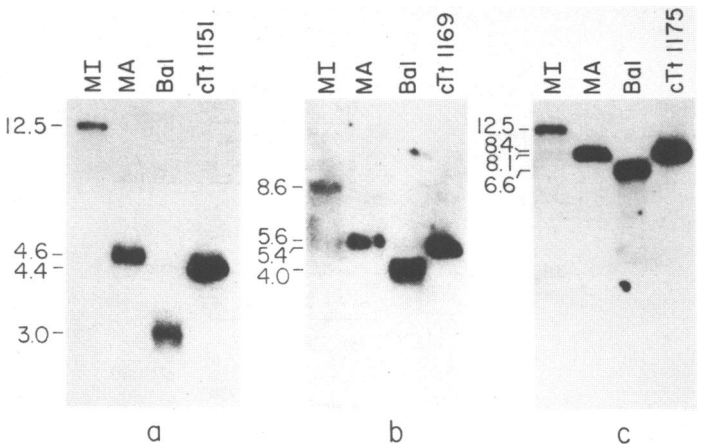

Figure 4. Southern hybridization of macronuclear DNA with and without Bal 31 digestion and micronuclear and cloned DNA. Lanes designated Bal contain macronuclear DNA which had been digested with Bal 31 prior to Eco RI digestion. Approximately 1.5 μg of micronuclear DNA, macronuclear DNA with and without Bal 31 digestion and about 2.5 ng of cloned DNAs were digested with Eco RI, separated on 1% agarose gels and blotted. Probe A of cTt 1151 was hybridized to set a, probe B of cTt 1169 and 1175 (see Figure 3) were hybridized to sets b and c, respectively. Sets a and c were washed in 2XSSC at 65°C while set b was washed in 0.5XSSC at 65°C. The sizes of the hybridizing bands are given in kilobases at the left of each set.

Taken together, the results shown above indicate that the three λ clones contain true macronuclear DNA ends. It is interesting to see if these three macronuclear ends have anything in common with each other besides the C_4A_2 repeats. In order to search for homologous regions, various subclones were used to hybridize to filters containing 40 ng of the λ clone from which they were derived and 2 ug of the other two λ clones (50X more DNA) after Eco RI or Hind III digestion. The subclones consisted of non-C_4A_2 adjacent to the two Eco RI sites and portions from the central region. The hybridizations were carried out at a lower stringency (4-6XSSC, 0.1 M Tris, 0.5% SDS, 5X Denhardt at 60°C). No cross-hybridization with the other clones was detected (data not shown). Therefore, there does not seem to be any obvious regions of homology shared between the clones.

Sequence of the C_4A_2 Repeats and Adjacent Regions

The three clones, cTt 1151, 1169 and 1175 were sequenced from the Eco RI site adjacent to the C_4A_2 repeats using the method of Maxam and Gilbert (27). All three clones contained long stretches of C_4A_2 repeats. Within each of these clones, different numbers of C_4A_2 repeats were detected. Two different sizes of C_4A_2-containing bands were isolated from both cTt 1169

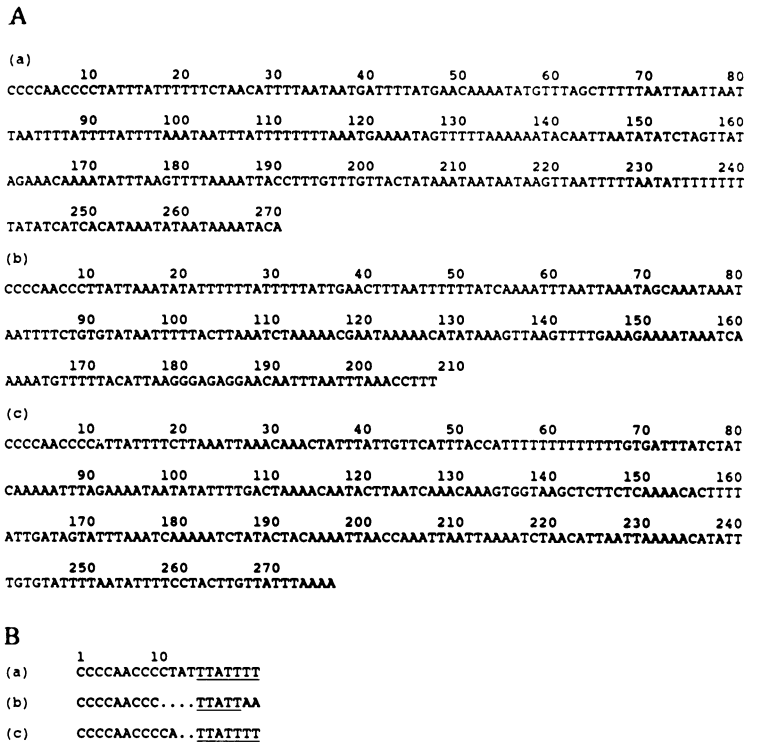

Figure 5. The C_4A_2 junction sequence of the macronuclear telomeric clones (a) cTt 1151, (b) cTt 1169 and (c) cTt 1175. Heterogeneous lengths of perfect C_4A_2 repeats (see text) were located prior to the sequence shown here. Position 1 starts with the last full C_4A_2 and is written 5'-3'. Panel A shows the sequence obtained for cTt 1151 (a), 1169 (b) and 1175 (c). The A+T composition ranges from 83-90%. Panel B shows the first few nucleotides of each clone aligned to show the consensus sequence of TTATT at the junction. The interrupting dots are inserted to show the best alignment of the sequences.

and 1175. Sequencing showed that in both cTt 1169 and 1175, the lower bands contained 50 repeats while the higher bands had more than 63 C_4A_2 repeats. cTt 1151 had repeat lengths of 8 and 25 but unexpectedly had 170 non-C_4A_2 nucleotides between the Eco RI site and the C_4A_2 repeats. Further work using probes from either side of the C_4A_2 repeats to hybridize to genomic DNA showed that this 170 bases and 2 adjacent Eco RI fragments on the one side were unrelated to the C_4A_2 repeats and its adjacent sequence on the other side (data not shown). The simplest interpretation of the data is that the real end was ligated with an artificially created end. Since the C_4A_2 repeats and the sequences on the one side of the clone seemed to be a

true representative of a macronuclear telomere, the analysis of this clone was limited to this 4.4 kb Eco RI fragment (shown in Figure 3).

It was necessary to subclone the λ clones with shortened C_4A_2 repeats to sequence the junction because of the long length of the C_4A_2 repeats and the lack of useful restriction enzyme sites close to the C_4A_2 repeats. This was accomplished by treating Eco RI digested cloned DNA with Bal 31 for various lengths of time. The DNA from the time points which had ends shortened by 100-400 bp were pooled and ligated to Eco RI linkers. The DNA was then double digested with Eco RI and Hind III and ligated into the vector pEMBL 9+. White colonies were screened for presence of C_4A_2 repeats by hybridization to the oligonucleotide $(C_4A_2)_3$.

Since the heterogeneity of the original λ clones raises doubts as to the authenticity of the sequences obtained, it is of major importance that the sequences of the junctions are confirmed by independent subclones (as in the case of cTt 1151 and 1175) or by two different size of bands of the same subclone (as in the case of cTt 1169). In all cases, only the number of C_4A_2 repeats differs. The junction sequences remain the same.

The C_4A_2-adjacent sequences for the three λ clones are shown in Figure 5A. The major characteristic of the junction sequences of all three clones is the AT-richness, ranging from 83-90% A+T. But in the region approximately 1 kb away from the junction (data not shown), the A+T content becomes somewhat less than even the typical Tetrahymena A+T composition of 75% (30). The other interesting point is the seven nucleotide consensus sequence shared by cTt 1151 and 1175 (TTATTTT), the first five of which are also shared by cTt 1169. Figure 5B aligns these regions of the three clones. The shared consensus sequence is underscored.

DISCUSSION

We have cloned and characterized three macronuclear DNA ends. Hybridization of these macronuclear and cloned DNA cleaved with various restriction enzymes showed that 1) the hybridizing micronuclear band was larger than the macronuclear band and 2) the macronuclear and cloned DNA had similar sized bands. Both restriction mapping and Bal 31 sensitivity studies indicate that the cloned DNA represents macronuclear ends which were produced by micronuclear chromosome fragmentation.

All three of the macronuclear end clones showed multiple perfect repeats of C_4A_2. However, there was heterogeneity within each clone which was shown to be due to different numbers of C_4A_2 repeats. In our cloning, it seems most probable that the unstable repeat sequences undergo deletion.

Comparison of each of the sequenced DNA clones with its corresponding macronuclear DNA fragment indicates that there are approximately 70-115 C_4A_2 repeats at each of the three genomic telomeres.

The macronuclear rDNA molecule has 20-70 copies of C_4A_2 repeats added onto the single CCCCAA present in the micronuclear rRNA gene when it is made into an extrachromosomal macronuclear molecule (10,11). To determine whether a consensus sequence may be involved as a signal for C_4A_2 addition, we subcloned the macronuclear ends and sequenced the region immediately adjacent to the C_4A_2 repeats. Within three nucleotides of the C_4A_2 junction, there is a short homology, 5'-TTATTTT-3', between two of the macronuclear clones, the first five bases of which are also present in the third clone. The 5'-TTATTT-3' consensus sequence occurs 27 nucleotides from the C_4A_2 repeats in the macronuclear rDNA molecule (31). This is the complementary sequence found 14-20 nucleotides prior to the mRNA poly A addition site (AAUAAA) (32). Whether the presence of such a sequence is fully coincidental is not yet clear. It is possible that such a structure is important for C_4A_2 addition during development, or telomere function during cellular growth.

The striking characteristics of the C_4A_2-adjacent region is its 83-90% A+T content. Other Tetrahymena AT-rich regions which have been characterized are located near the center and the termini of Tetrahymena rDNA molecules. These two regions allow autonomous replication of plasmids in yeast (31). Chromatin studies of the AT-rich central region of the Tetrahymena rDNA molecule suggest that the AT-richness may be more important for general structural properties rather than the sequences per se (33).

Other than the short sequence homology at the critical junction region, we found no other homology in the subtelomeric region, either between or within the clones. Each telomeric clone contains essentially unique DNA sequence. Probes from both extremes of the cloned DNA hybridize to only one micro- and macronuclear band (see Figures 2 and 4). Consistent with this result is the finding that the three clones do not cross-hybridize with each other. This is certainly unlike the telomere-associated sequences which hybridize to numerous telomeric locations, such as those in yeast (34), rye grass (35,36), and Drosophila (37,38).

In addition, our subtelomeric regions showed no repeats which are commonly found in other organisms. For instance, the Physarum and Dictyostelium rDNA molecules, trypanosomes and yeast carry several tandemly

repeated telomere-associated sequences of unit lengths of 140, 29, 29 and 36 bp respectively (39-42).

The present analyses show some similarities to the hypotrichs which also contain unique sequence associated with the macronuclear DNA termini (43,44). The macronuclear DNA in hypotrichs consist of very short, gene-sized pieces (45), all of which contain a short, specific terminal repeat of C_4A_4 (46-48). In addition, Euplotes aediculatus has been shown to contain a common sequence of five bp (TTGAA) which appears 17 nucleotides from the C_4A_4 junction (47). However, the much larger macronuclear DNA fragments of Tetrahymena have heterogeneous and long lengths of C_4A_2 repeats. Importantly, our telomeric clones showed that they have different starting points for the C_4A_2 repeats. For the sake of convenience, we have been using the terminology of C_4A_2 repeats. However, as a repeat sequence it could also consist of any of the six permutations such as CCAACC or CAACCC, etc. Our three clones show that the repeat has no fixed starting point. It is 5'-CCCCAA-3' in rDNA (31), but in the present macronuclear ends, it is 5'-AACCCC-3', CAACCC and ACCCCA (see Figure 5B). The only consistency is each clone's uniqueness. At present the biological significance of the different C_4A_2 composition is not known. The exact rule of initiation and addition of the telomeric sequences remains to be determined.

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