Internal micronuclear DNA regions which include sequences homologous to macronuclear telomeres are deleted during development in *Tetrahymena*

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ABSTRACT

C₄A₂ repeats are present in multiple clusters in both the macronucleus and micronucleus of *Tetrahymena*. Although the macronucleus is generated from the micronucleus after sexual conjugation, the repeats are telomeric sequences in the macronucleus but are internally located in the micronucleus (1). This study investigates the fate of the sequences adjacent to the micronuclear C₄A₂ repeats. Southern blot analyses of 21 C₄A₂-containing micronuclear clones show that extensive elimination of the adjacent sequences occurs during the formation of the macronucleus. Comparison of one C₄A₂-containing micronuclear clone with its derived macronuclear segment indicates that approximately 4.5 kb of DNA, which includes the C₄A₂ repeats and adjacent sequences on both sides is deleted from the macronucleus. The two regions adjoining the deletion are joined together to form a contiguous segment in the macronucleus. This excision of C₄A₂ repeats and surrounding sequences and the rejoining of the retained segments is probably the mechanism by which all or most of the other C₄A₂ adjacent sequences are eliminated.

INTRODUCTION

Tetrahymena thermophila is a ciliated protozoan which contains two nuclei: a micronucleus and a macronucleus. The macronucleus has approximately 45 times the DNA of a haploid genome and is responsible for the phenotype of the cell. During sexual conjugation, haploid meiotic products of the micronuclei are exchanged between mating partners. The new macronuclei and micronuclei are formed from the zygotic nuclei. The original macronuclei are degraded (2). Therefore, the micronucleus is solely responsible for the genetic continuity of the cell. Under such a dichotomy of nuclear roles, amplification and elimination of certain DNA sequences can and does occur during macronuclear development without affecting the germline of the organism. For instance, 10-20% of micronuclear DNA is absent from or underreplicated in the macronucleus (3). One specific gene, the ribosomal RNA gene, is amplified from a single integrated chromosomal copy in the micronucleus to about 9,000

extrachromosomal palindromic copies in the macronucleus (4).

A third type of reorganization, besides elimination and amplification, appears to involve tandemly repeating units of the hexanucleotide CCCCAG. C₄A₂ repeats were originally discovered to be the terminal sequence of the macronuclear extrachromosomal rDNA molecules (5). Subsequently, similar terminal sequences have been found in other organisms (6-12) and, furthermore, the *Tetrahymena* C₄A₂ repeats and *Oxytricha* C₄A₄ repeats have been used in yeast to produce a functional telomere (13,14,15). These findings suggest that this sequence is biologically important.

Using C₄A₂ repeats to hybridize to Southern blots of *Tetrahymena* micro- and macronuclear genomic DNAs digested with various restriction enzymes, it was found that this repeating sequence is prevalent in both nuclei. Moreover, the banding patterns of the restriction fragments containing this sequence were completely different between the two nuclei (16, 17). This difference is now known to be due to the C₄A₂ repeats being present at various internal regions in the micronucleus but probably exclusively at free ends in the macronucleus (1). One simple interpretation of the difference in C₄A₂ repeat organization is that site specific DNA fragmentation might have occurred during macronuclear development at or near the micronuclear C₄A₂ repeats to produce fragmented chromosomes with C₄A₂ repeats at their ends (18). To date, the evidence pertaining to this fragmentation model has been negative (19,20,21).

However, is it still possible that some of the micronuclear C₄A₂ repeats are sites of fragmentation and subsequent macronuclear telomeric formation? If not, what are the roles of these internal micronuclear C₄A₂ repeats? With these questions in mind, we isolated 21 micronuclear C₄A₂-containing clones. Since C₄A₂ repeats are repetitive in both nuclei, we present data on the fate of C₄A₂ adjacent sequences. Southern blot hybridization showed that there is extensive elimination of the C₄A₂ adjacent sequences. In addition, none of these micronuclear C₄A₂ repeats are found to be sites for the formation of macronuclear free ends. In one particular case we are able to compare one micronuclear clone to the derived macronuclear cloned segment, and show that a region of approximately 4.5 kb, which includes the C₄A₂ repeats and adjacent sequences, was deleted from the macronucleus. The extensive elimination which has been observed in all micronuclear C₄A₂-containing segments can be explained by a similar deletion mechanism.

MATERIALS AND METHODS

Cell cultures and DNA isolation

Tetrahymena thermophila inbreeding strain BIV was grown in axenic medium as described by Gorovsky et al. (22). Micro- and macronuclei were isolated as described previously (22). The nuclear DNA was obtained by phenol extraction as described earlier (23).

Cloning, screening and isolation of phage DNA

The micronuclear library used was constructed previously (24). Total micronuclear DNA was partially digested with EcoRI and ligated into the phage λ vector Charon 4A. Clones containing tandem repeats of C₄A₂ were screened and purified by three sequential rounds of plaque hybridization (25). The probe used for the first and third hybridization was fragment d of pTt2512 (19) and the second was rDNA, both of which are known to contain C₄A₂ tandem repeats. 21 of the positive clones were selected for this study. The recombinant phages were propagated in the E. coli strain DP50 Sup F. Most of the samples were purified in CsCl gradient before DNA isolation. However, some of the samples were isolated directly from the agarose plate lysates by precipitation with polyethylene glycol, and phenol extraction.

The macronuclear DNA clones used in this study were constructed by partial Mbo I digestion of macronuclear DNA and ligating into the Bam HI sites of the phage λ vector L47.1 (26). The 3.5 kb EcoRI-Hha I fragment of the micronuclear clone cTt606 (see Figure 3), which hybridizes to a unique micro- and macronuclear band, was used as a hybridization probe to screen for the corresponding macronuclear DNA clone. Approximately 4×10^4 plaques were screened. Five positive clones were obtained. The recombinant phages were propagated in the E. coli strain Q358.

Gel electrophoresis and blotting

Restriction enzymes and the exonuclease Bal 31 were purchased from New England Biolabs or from Bethesda Research Laboratories. Digestion with Bal 31 was as described (1). EcoRI digested pBR322 was added to macronuclear DNA and then the two DNAs were digested with Bal 31. An aliquot of the DNAs was separated by size using gel electrophoresis, stained with ethidium bromide and photographed under UV illumination. The extent of exonuclease digestion was monitored by the shortening of the pBR322 fragment. Agarose gel electrophoresis was carried out in a horizontal slab gel apparatus following the method of Helling et al. (27). The method of Southern (28) was followed, with slight modifications, to transfer the DNA from the gel

onto a nitrocellulose filter.

Low temperature agarose was used when removal of DNA from a gel was desired. The gel segment was cut out, melted at 65°C and agarose removed by phenol extraction.

DNA labelling, hybridization and autoradiography

All DNAs were labelled in vitro with ^{32}P by nick translation (29), except the C_4A_2 repeats of rDNA, which were labelled by a modified method of nick translation described earlier (5). The radioisotopes used were ^{32}P dATP and dCTP (Amersham, 400 Ci/mmol or $\sim 3,000$ Ci/mmol). Hybridization was normally carried out at 37°C for 16-18 hours in 40% formamide, 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 Denhardt solution (30). The hybridization filters were washed extensively with 2XSSC at room temperature and then at 65°C for at least 1 hour before autoradiography. For genomic blots when more stringent conditions were required, washing was done at 65°C with 0.2XSSC. Autoradiography was detected by using Kodak XAR-5 X-ray film with a Dupont intensifying screen.

RESULTS

Micronuclear clones containing C_4A_2 repeats were isolated from a Charon 4A library by plaque hybridization procedures (25). The library had been previously constructed by ligating partially EcoRI digested micronuclear DNA into the vector Charon 4A (24). Approximately 300 positive clones hybridized in a complete micronuclear library. 21 of these micronuclear clones are included in this analysis.

Since C_4A_2 repeats are prevalent in both micro- and macronuclear DNA, the only way to determine their specific involvement in rearrangement is to follow the fate of the individual adjacent sequences. Analysis of the adjacent sequences was conducted by one of the two following methods.

Analyses of two clones using purified fragments

Two clones, cTt608 and 614, were digested with EcoRI, fractionated by electrophoresis and transferred to nitrocellulose filters. The filters were hybridized to a probe containing C_4A_2 repeats. cTt608 contained only one EcoRI Tetrahymena DNA insert, while cTt614 contained five EcoRI fragments. The analysis of cTt614 was confined to the C_4A_2 -containing EcoRI Fragment 4, which was approximately 3.9 kb. This precaution was taken to eliminate any complications arising from unrelated EcoRI fragments being ligated into the same clone. The EcoRI fragments containing C_4A_2 repeats were isolated,

FIGURE 1. Southern hybridization of micro- and macronuclear DNA with fragments of micronuclear clones. The nuclear DNAs were digested with EcoRI, separated by electrophoresis in a 1.0% agarose gel and blotted. The micronuclear lanes (MI) contain about 1 μ g of DNA. The macronuclear lanes (MA) contain about 5 μ g of DNA in sets a and b and approximately 1 μ g of DNA in the remaining sets. Sets a, b, c, and d were hybridized with Hha I fragments of sizes 6, 2.3, 1.7 and 1 kb, respectively, of micronuclear clone cTt608. Sets e and f were hybridized with Hha I fragments of sizes 2.3 and 0.5 kb, respectively, of micronuclear clone cTt614. Only set a shows any hybridization to the macronuclear DNA. The black bars indicate the positions of the molecular weight markers using Hind III digested λ DNA in these same gels: 23.7, 9.5, 6.8, 4.3, 2.3, and 2.0 kb. Sets a and b are in one gel and sets c-f in another.

digested with HhaI, separated by gel electrophoresis, and the fragments which contain C₄A₂ repeats were again determined by hybridization. The non-C₄A₂-containing HhaI fragments are 6, 2.3, 1.7, and 1 kb for cTt608 and 2.3 and .5 kb for cTt614. These fragments were isolated, radioactively labelled and hybridized to both micro- and macronuclear genomic DNA. In all cases, the micronuclear hybridization showed numerous bands (see Figure 1). In contrast, five of these fragments showed no macronuclear hybridization. Only the 6 kb fragment of cTt608, H8-1, showed any macronuclear hybridization. As shown in Figure 1a, there is hybridization to one macronuclear

band which is probably a doublet.

The hybridizing macronuclear band must be derived from not more than two of the micronuclear fragments, either in an unaltered or a rearranged form. The other hybridizing micronuclear fragments must be eliminated during macronuclear formation. Since the cloned micronuclear EcoRI segment is 11.7 kb, and the hybridized macronuclear fragments are 3.2 kb, either the micronuclear clone is unrelated to the macronuclear band or the retained sequence has been rearranged.

We further investigated this problem by using a subfragment of H8-1 as a probe. This 3.9 kb probe reduces the number of hybridizing bands of the micronuclear DNA to 5. In three different restriction enzyme digestions we found that 2 of the 5 bands hybridized in the micronuclear DNA are identical in size to the two bands found in the macronuclear DNA. The simplest interpretation is that the two macronuclear sequences are derived from the micronuclear genome without rearrangement, and thus are unrelated to the 11.7 kb fragment of cTt608. We have subsequently cloned one of the two macronuclear DNA fragments, and found that the restriction map appears to be unrelated to cTt608. Thus, the sequence in H8-1 is most likely also eliminated from the macronucleus.

In summary, our data suggest that all of the cloned sequences bordering the restriction fragments containing two different clusters of C₄A₂ repeats are eliminated during macronuclear formation. Published information on three other micronuclear C₄A₂-containing clones also show complete elimination of adjacent sequences (19,20). Thus, elimination of C₄A₂ adjacent sequences appears to be a rather general phenomenon. In order to find out whether the elimination of these sequences is associated with chromosome fragmentation, and thus the formation of free ends, it is necessary to examine the structure of the retained portions of adjacent sequences in the macronucleus. Thus, more clones which contain C₄A₂ repeats were examined with the hope that some might contain adjacent sequences which are retained in the macronucleus. In order to screen a larger number of clones, we have taken the approach described below which eliminates the need for mapping the clones and isolating subfragments for use as probes.

Use of whole micronuclear clones for macronuclear hybridization

As indicated in the previous section, macronuclear hybridization was rarely seen when C₄A₂ adjacent sequences were used as probes. However, hybridization to the whole micronuclear cloned DNA would complicate analysis because of the repetitive C₄A₂ banding pattern. To avoid this problem, we

made use of the exonuclease Bal 31 which is known to digest DNA free ends (31). Since C₄A₂ repeats are contained at the ends of macronuclear DNA (1), Bal 31 digestion of macronuclear DNA can eliminate hybridization of the macronuclear DNA to the cloned C₄A₂ repeats. Here we have taken advantage of this property in order to detect any possible hybridization of sequences in the C₄A₂-containing clone to the macronuclear genome.

For 19 C₄A₂-containing micronuclear clones, each whole cloned DNA was nick translated and hybridized to macronuclear DNA, with and without Bal 31 digestion. 14 of the 19 clones showed no detectable hybridization to the Bal 31 digested macronuclear DNA. Five examples are shown in Figure 2A. An amount of the cloned micronuclear DNA corresponding to one haploid genome equivalent was included in one macronuclear DNA lane so that the sensitivity of hybridization could be assessed. Based on this result, it is concluded that at least the majority, and perhaps all, of the non-C₄A₂ sequences in the cloned micronuclear DNA are eliminated in 14 of the 19 cases.

The remaining five clones show hybridization to the macronuclear DNA. Four examples are shown in Figure 2B. In all cases the macronuclear banding patterns are fairly simple. However, the only clone which showed a unique band was clone cTt606. In order to establish an unambiguous relationship between a macronuclear DNA fragment and the micronuclear DNA clone isolated, it would be simplest if the cloned DNA, or part of it, is a unique sequence in both the macro- and the micronuclear genome. For this reason, clone cTt606 was chosen to further study the relationship between the micronuclear segment and its macronuclear counterpart.

Comparison of a micronuclear segment with its derived macronuclear segment

The micronuclear clone cTt606 was characterized in more detail. The C₄A₂ repeat was localized to a 1.5 kb Hha I fragment contained within a 5.6 kb EcoRI fragment. A map of this clone is shown in Figure 3. Various restriction fragments were used for hybridization to the two nuclear DNAs. A 3.5 kb Hha I/EcoRI segment (U3.5) was found to hybridize to a unique band in both nuclear DNAs. As shown in Figure 4a, the hybridizing segments are different in size, being 5.6 and 3.3 kb in the EcoRI digested micro- and macronuclear DNA, respectively. A weaker, second micronuclear band is also detected, but is removed under more stringent washing conditions.

Since there is only one homologous segment in each of the nuclear DNAs, it is unequivocal that they are related. Moreover, when different restriction enzymes are used, we always get a macronuclear segment which is smaller than the micronuclear segment. This result suggests that this segment of

FIGURE 2. Hybridization of whole micronuclear DNA clones to macronuclear DNA with and without Bal 31 digestion. Panels A and B show examples of no hybridization and hybridization, respectively, to macronuclear DNA which has been digested with Bal 31 to remove 600–800 bp from the ends. This extent of digestion removes most of the terminal C₄A₂ repeats (1). Lanes designated MA/c (Panel A) contain approximately 1 μ g of macronuclear DNA and an amount equivalent to one copy per haploid genome of the cloned micronuclear DNA. Lanes designated Bal and MA contain approximately 2 μ g of macronuclear DNA with and without Bal 31 digestion, respectively. The DNAs

are digested with Eco RI, separated on 1% agarose gels and blotted for hybridization. The set designated 606 was hybridized to the 5.6 kb Eco RI gel purified fragment of cTt606 (see Figure 3). The remaining sets were hybridized to each respective whole cloned DNA. The open triangles shown in the MA/c lanes of sets 617 and 635 indicate obvious bands which show hybridization to the micronuclear clone. The other MA/c lanes do not contain such distinct bands, presumably because of the higher background hybridization to C_4A_2 . This assumption is supported by the extent of hybridization to the C_4A_2 -containing 2.4 kb terminal fragments of the rDNA molecules, as indicated by solid triangles. Since these sets were collected from several gels which had been electrophoresed under slightly different conditions, the average locations of λ Hind III digested DNA are indicated by black bars to the left of the group of gels which had similar marker positions.

micronuclear DNA is somehow rearranged during macronuclear development, and that the rearrangement may involve fragmentation or deletion. To study this rearrangement, we isolated the homologous macronuclear segment from a macronuclear DNA library.

The library had been constructed previously by ligating partially Mbo I digested macronuclear DNA into the Bam HI sites of L47.1 (26). Five independent macronuclear clones were isolated which hybridized to the U3.5 probe of cTt606. Four of the five contained both a 1.2 and a 3.3 kb fragment in addition to other EcoRI fragments. One of the four, cTt1152, was chosen as the macronuclear clone to compare with cTt606.

Restriction enzyme mapping and Southern blot hybridization were used to determine the regions of similarity and dissimilarity between cTt606 and 1152. The 3.3 kb EcoRI segment of the macronuclear clone showed homology to the 5.6 and 1.4 kb EcoRI fragments of cTt606. As shown in the map on Figure 3, the homologous regions are located at the right side and extreme left side of cTt606. However, there is a deletion of approximately 4.5 kb from the intermediate region which includes the HhaI 1.5 kb fragment containing the C_4A_2 repeats and regions on either side of this 1.5 kb HhaI fragment. Thus the C_4A_2 repeats cannot be at the deletion junction. The two adjacent regions of the deletion must be joined together to form a contiguous segment in the macronucleus.

It should be emphasized that the differences between the macronuclear and the micronuclear clone are not a cloning artifact. We have used several different restriction enzymes to digest the genomic DNAs and their corresponding clones, cTt606 and 1152, and hybridized with nick translated U3.5. In all cases, we found that the clones are representative of their respective genomic DNAs (Figure 4). Therefore, the deletion must be part of the differentiation process in the formation of the macronucleus.

FIGURE 3. Comparison of the micronuclear DNA clone cTt606 and macronuclear DNA clone cTt1152. The maps were constructed by single and double enzyme digestions and by hybridization with isolated fragments. The open bar indicates the region deleted from the macronucleus. This includes the 1.5 kb Hha I fragment which contains the C_4A_2 tandem repeats (denoted by *). The hatched bars with straight lines represent the regions in which the deletion junctions must lie. The black bars indicate regions of homology in the two clones. The solid lines in cTt1152 indicate cloned DNA which lie outside of the cloned micronuclear regions. The brackets at the ends of cTt606 and 1152 indicate the vector arms of Ch4A and L47.1 respectively. The dashed lines connecting the two clones indicate the regions of deletion and rejoining which results in a contiguous macronuclear segment. The hatched bar with diagonal lines above cTt606 (U3.5) represents the 3.5 kb EcoRI/Hha I fragment used as a probe to screen for the macronuclear clones, as well as for Southern hybridization of the genomic DNAs. A: Hae III, B: Bgl II, H: Hha I, R: Eco RI, V: Eco RV, X: Xba I.

Thus, in the only case where direct comparison of the macro- and micronuclear DNA was possible, the sequences immediately adjacent to the C_4A_2 repeats in the micronucleus are eliminated from the macronucleus by a deletion mechanism which does not lead to the formation of free ends. This finding sets stringent limitations on the possible roles of the micronuclear C_4A_2 repeats. Although it is not possible to draw a general conclusion from a single observed case, it is worthwhile to point out that, to date, after a total of 24 C_4A_2 clusters have been examined, not a single case is known to involve the formation of free ends. 16 of the 21 clones studied here contain adjacent sequences that are totally eliminated, and thus cannot be used for further analysis of the rearrangement process. Four of the other five clones which contain adjacent sequences sharing homology with some macronuclear DNA have not been further characterized because they contain elements that are repetitive in both nuclei. It is quite possible, as in the case of clone cTt608, that the hybridizing macronuclear fragments are

FIGURE 4. Comparison of micro- and macronuclear genomic DNA and their corresponding cloned DNAs by hybridization to U3.5. Between 0.25 and 2.5 ng each of cTt606 and cTt1152 DNA and approximately 1.5 μ g each of micro- and macronuclear DNAs were digested with various enzymes, separated on 1% agarose gels, and blotted. The unique sequence, U3.5 (see Figure 3) of the micronuclear clone cTt606 was used as a probe. The DNAs in each set were digested with: a, EcoRI; b, Hind III and EcoRI; c, Hae III and Eco RI; d, Bgl II and Hae III. The filter of set a was washed in 2XSSC at 65°C after hybridization. These washing conditions show a weaker second micronuclear band which can be removed by more stringent washing conditions. The remaining sets were washed in 0.2XSSC at 65°C. The sizes of the hybridizing bands are given in kilobases.

derived from locations other than the sequences represented by the micronuclear clones used to detect them. Alternatively, some of the hybridizing macronuclear fragments may be related to the cloned DNA. However, in any case they clearly are not responsible for generating a macronuclear free end because when the macronuclear DNA was digested with Bal 31 to remove 600-800 bp from the ends, the hybridizing bands did not decrease in size (see Figure 2B). This indicates that the hybridizing sequences are not adjacent to chromosomal ends. Thus, it is quite likely that most of the elimination associated with C₄A₂ repeats are caused by a deletion mechanism, as characterized with clones cTt606 and 1152, and not by fragmentation.

DISCUSSION

In this paper we present data on the fate of sequences adjacent to micronuclear C_4A_2 repeats. We report the only demonstrated case of elimination of the C_4A_2 repeats and adjacent sequences by a deletion mechanism. Comparison of one micronuclear clone with its derived macronuclear segment shows that approximately 4.5 kb of DNA, which includes the C_4A_2 repeats and sequences on both sides, is deleted from the macronucleus. The left and right regions of the sequences represented by the micronuclear clone cTt606 (see Figure 3) are joined together to form a contiguous macronuclear sequence. The deleted segment shows no homology to the macronuclear clone. This is consistent with the finding that hybridization of a unique segment from the micronuclear clone to genomic DNA always shows the macronuclear fragments to be smaller than the micronuclear ones. The deletion cannot be due to a cloning artifact since in all cases the sizes of restriction fragments in genomic DNA and the corresponding cloned DNA agree.

In addition to this one case of C_4A_2 -related DNA elimination, we surveyed 20 other C_4A_2 -containing micronuclear clones. The data indicate that there is extensive elimination of sequences surrounding the C_4A_2 repeats during formation of the macronucleus. 15 of the clones show no hybridization to macronuclear DNA which has had C_4A_2 repeats removed by Bal 31 digestion. The clones contain on the average 10-15 kb of Tetrahymena DNA. Since the C_4A_2 repeats are not likely to be immediately adjacent to the vector DNA in most cases, elimination probably occurs on both sides of the C_4A_2 repeats.

Elimination of sequences adjacent to micronuclear C_4A_2 repeats during macronuclear formation has been reported before (19,20). However, this is the most extensive survey and it is especially noteworthy that DNA elimination occurs so widely in C_4A_2 -related sequences. It seems quite likely that all of the C_4A_2 -related DNA elimination might also occur by deletion, as demonstrated by the comparison of cTt606 and 1152. Of the 15 clones which show no detectable hybridization to the macronucleus, the elimination probably included a larger area than that contained in the clone. In the remaining cases, hybridizing fragments are detected in the macronucleus, but none are located at free DNA ends (Figure 2B). Thus, If the micronuclear C_4A_2 repeats are to be utilized as telomeres, our results suggest that the mechanism cannot involve fragmentation at or near the micronuclear C_4A_2 repeats to generate free ends, or any type of deletion

event which would place the C₄A₂ repeats adjacent to a sequence to be retained.

Although it is quite striking that every C₄A₂-containing micronuclear clone had sequences that were destined to be eliminated during macronuclear formation, elimination is not solely a C₄A₂-related occurrence. In a previous study by this laboratory, it was found that seven out of ten randomly chosen, cloned micronuclear DNA fragments contain sequences which are repetitive in the micronucleus, all of which appear to be eliminated in the macronucleus (19). In addition, it seems that elimination is frequently caused by deletion (26,32). Although deletion and elimination are not uniquely associated with C₄A₂ repeats, it seems significant that the C₄A₂-containing micronuclear clones contain such large stretches of sequences that are eliminated. It remains an interesting possibility that the micronuclear C₄A₂ repeats might serve as one of the signals for DNA deletion in Tetrahymena.

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