Tandemly Repeated C-C-C-C-A-A Hexanucleotide of *Tetrahymena* rDNA Is Present Elsewhere in the Genome and May Be Related to the Alteration of the Somatic Genome

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ABSTRACT The ribosomal RNA genes of the *Tetrahymena* macronucleus exist as extrachromosomal, linear molecules. The termini of these molecules have been shown to contain the tandemly repeated hexanucleotide $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$. In this study the same or related sequences were found in other locations of the genome. Using the depurination method, we showed that macronuclear DNA contained this sequence even after rDNA had been removed. The sequence was found mainly in the repetitive fraction of the DNA. The presence of this sequence in both the macronucleus and the micronucleus was also shown by Southern hybridization using C-C-C-C-A-A repeat as a probe. Comparison between the hybridization patterns of macronuclei and micronuclei reveals interesting differences. Whereas the two nuclei share the same genetic origin, the majority of the restriction enzyme digestion sites flanking the C-C-C-C-A-A repeat appear to be different. Such a difference was found to be specific for this sequence, because it was not detected when other sequences were used for hybridization. These results suggest that some kind of alteration has occurred in the genome during the formation of the macronucleus, and that the C-C-C-C-A-A repeat may be related to this process.

The genes coding for ribosomal RNA in the ciliated protozoan *Tetrahymena thermophila* have been well characterized (reviewed in reference 1). They exist as extrachromosomal linear molecules in the macronucleus of this organism (2, 3). Each molecule contains a pair of rRNA genes arranged in reverse orientation (4, 5). There are ~20,000 rDNA molecules present in an average macronucleus, which contains ~45 times the DNA of a haploid genome. Like the extrachromosomal rDNA in amphibian oocytes, the extrachromosomal rDNA in *Tetrahymena* macronuclei is also the product of gene amplification (6).

The macronucleus of *Tetrahymena* is a vegetative nucleus, which is formed from a preexisting micronucleus during conjugation. The micronucleus is a germinal nucleus. It undergoes meiosis, fusion, and mitosis during conjugation, giving rise to the precursors of both the macronucleus and the micronucleus of the subsequent sexual generation. The ribosomal RNA genes of the micronucleus have also been studied. They exist as single-copy sequences in the chromosome (7). Amplification of rDNA apparently occurs during the formation of the new macronucleus, and has been detected by *in situ* hybridization (1).

The nucleotide sequences located near the two termini of the extrachromosomal rDNA have been determined (8). They were found to contain tandem repetitions of the hexanucleotide C-C-C-C-A-A. The repetitive sequences cover a region between 200 and 400 base-pairs long. The function of the C-C-C-C-A-A sequence is not known. Recently, it was found that similar sequences may be present in other locations of the genome (1). In this report we present sequencing and hybridization data in support of this argument. We have found that C-C-C-C-A-A repeats exist widely in both the macronucleus and the micronucleus. Moreover, we are able to demonstrate that the genomes of the macronucleus and micronucleus are organized differently, and such differences are probably associated specifically with the C-C-C-C-A-A repeats. The formation of the somatic genome of *Tetrahymena* apparently involves alteration processes beside gene amplification, and the C-C-C-C-A-A repeats may play an important role in these processes.

MATERIALS AND METHODS

Cells and Culture Conditions

Tetrahymena thermophila inbreeding strain B was obtained from P. Bruns (Cornell University, Ithaca, N. Y.) and used for most of these studies. *T. pigmentosa*, strain 6I, was obtained from E. Simon (University of Illinois, Chicago, Ill.). *T. pyriformis* was obtained from M. Gorovsky (University of Rochester, Roch-

ester, N. Y.). Cells were maintained and grown in axenic medium as described previously (9).

Nuclei Isolation and DNA Extraction

Macronuclei and micronuclei were isolated according to the method described by Gorovsky et al. (9). Contamination of micronuclei by macronuclei was generally <1 in 200. DNA was prepared from whole cells or macronuclei by phenol extraction and from micronuclei by sedimentation in CsCl density gradients as described previously (2, 10). rDNA was isolated from purified whole cell DNA by sedimentation in a CsCl gradient containing Hoechst 33258 dye as described by Wild and Gall (11).

Radioactive Labeling of DNA

For in vivo labeling of DNA, the cells were grown in defined medium with $H_3{}^{32}PO_4$ (New England Nuclear, Boston, Mass.) as described by Blackburn and Gall (8). The specific activity of the DNA was $>3 \times 10^5$ cpm/μg. In vitro labeling of DNA was accomplished by nick translation (12).

Preparation of Labeled C-C-C-C-A-A Repeats

Purified rDNA from *Tetrahymena thermophila* was incubated with DNA polymerase I from *Escherichia coli* (Boehringer, grade I) in 50 mM Tris-HCl pH 7.6, 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 50 μg/ml bovine serum albumin, 10 μM dCTP, and 2 μM dATP, while either one or both triphosphates were labeled with α-^{32}P. The incubation was carried out for 75 min at 15°C. The reaction was stopped by addition of EDTA, the aqueous phase extracted with phenol, and the DNA precipitated twice with ethanol in the presence of carrier tRNA from *E. coli*. It has previously been shown that, by using this procedure, label incorporated into the rDNA is nearly all found in the terminal restriction fragment of the rDNA, and the rDNA sequence that becomes labeled is a tandemly repeated sequence, $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$, where n is between 20 and 70 (8). The labeled DNA was then digested with excess restriction nuclease Alu I for 2 h at 37°C to cut the labeled repeating sequence away from other rDNA sequences before use in Southern hybridization experiments.

To analyze the rDNA sequence labeled, an aliquot of labeled and digested rDNA was fractionated by electrophoresis in a 1% agarose gel. Virtually all of the label was found in a fragment previously identified as containing the termini of the palindromic rDNA molecule (8). The labeled sequences were identified by depurination analysis (Fig. 1). The major products labeled were the pyrimidine tract C_4 and inorganic phosphate. This is consistent with preferential labeling of the tandemly repeated hexanucleotide C-C-C-C-A-A.

Reannealing and Fractionation of ^{32}P Main-peak DNA

The ^{32}P main-peak DNA (12.5 μg/ml in 0.12 M PB; PB was sodium phosphate at pH 6.8) was sheared by 20 passages through a 26-gauge needle. The DNA was then denatured by heating to 100°C for 5 min, and transferred to a water bath at 70°C. This temperature was selected for reannealing because it is 26° below the calculated T_m of repeated C-C-C-C-A-A DNA. At times corresponding to C_0t (C_0, initial concentration; t, time) values of 0.1 and 1.0, reannealed DNA was separated batchwise from single-stranded DNA by addition of 200 μl of packed hydroxyapatite in 0.12 M PB. The clear supernate (containing single-stranded DNA) was removed, and the hydroxyapatite was washed once with 2 ml of 0.12 M PB to remove trapped single-stranded DNA. Adsorbed DNA was released by addition of 0.6 ml of 0.5 M PB and heating to 100°C for 5 min. Yields of the highly repetitive (fraction B: $C_0t \leq 0.1$), middle repetitive (fraction C: $0.1 < C_0t \leq 1.0$), and unique sequence fractions obtained by this procedure were estimated by counting aliquots in Aquasol scintillation cocktail. In the experiment shown here, 4% of the DNA was found in the highly repetitive fraction, and 6.3% in the middle repetitive fraction. These three fractions, along with a sample of unfractionated main-peak DNA (fraction A), were dialyzed overnight against distilled water and ethanol-precipitated in the presence of cold carrier RNA for further analysis.

Hydroxyapatite (HTP hydroxyapatite, DNA grade, fine mesh; Bio-Rad Laboratories, Inc., Richmond, Calif.) was pretreated before use by three incubations at 100°C for 10 min in fresh 0.5 M PB (30 ml of PB/1 ml of hydroxyapatite). PB was decanted after each heating step. This treatment was then repeated using 0.12 M PB, in which the hydroxyapatite was then stored at 4°C.

Depurination Analysis of [^{32}P]DNA

[^{32}P]DNA, labeled in vivo or in vitro as described above, was analyzed by depurination and two-dimensional fingerprinting of pyrimidine tracts as described (13).

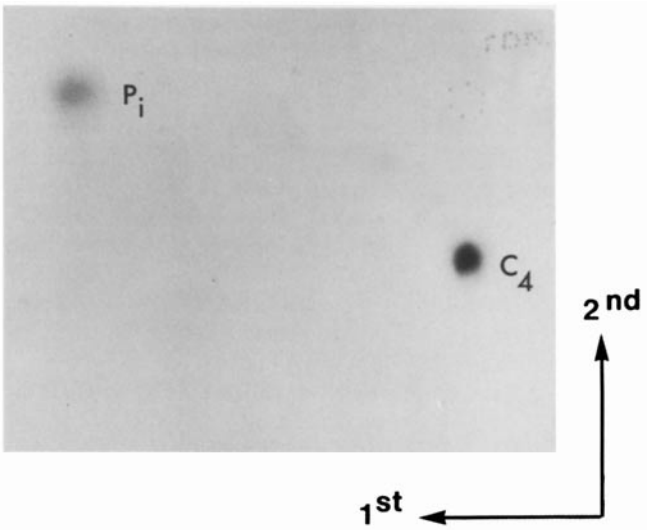

FIGURE 1 Depurination fingerprint of rDNA labeled in vitro by DNA polymerase I in the presence of [α-^{32}P]dATP and [α-^{32}P]dCTP. rDNA was incubated with DNA polymerase I from *E. coli* to label the repeating hexanucleotide sequences C-C-C-C-A-A at the termini of the rDNA, as described in Materials and Methods. The labeled pyrimidine tract C_4 was identified from its position relative to the other, weakly labeled pyrimidine tracts in the fingerprint. C_4 and inorganic phosphate are indicated. The first dimension fractionation was done by electrophoresis in 7 M urea, pH 3.5, on cellulose acetate, followed by transfer to a DEAE cellulose thin-layer plate, and the second dimension was done by homochromatography at 60°C, in 7 M urea containing 3% RNA that had been hydrolyzed in 0.1 M KOH for 30 min at 25°C and neutralized before addition of urea. P_i, inorganic phosphate.

Gel Electrophoresis, Blotting, and Hybridization

Restriction endonucleases EcoR1, Hind III, Bam H1, and Hae III were either prepared in the laboratory or purchased from New England Biolabs. Digestions of the DNA were done under standard conditions as specified by the company. Normally, twofold excess of the enzyme was used to ensure complete digestion of the DNA. Digested DNA was analyzed in an agarose slab gel using the conditions described by Helling et al. (14). Hind III digested phage λ DNA was used as a size marker for electrophoresis (15). DNA was blotted from the gel onto nitrocellulose filter according to the method of Southern (16). Hybridizations of Southern blots were carried out in 40% formamide, 4 × SSC (SSC contained 0.15 M NaCl and 0.015 M sodium citrate at pH 7.0), 0.1 M Tris-HCl, pH 7.4, 0.5% SDS, and Denhardt solution (17) for overnight at 37°C. After hybridization, the filter was washed extensively in 2 × SSC at room temperature before autoradiography.

Cloning of the Micronuclear DNA

Purified micronuclear DNA was digested with both EcoR1 and Bam H1 and ligated with the plasmid vector pBR322. The vector had previously been digested with the same enzymes, and the small DNA fragment had been removed by gel electrophoresis. The recombinant DNA was used to transform the *E. coli* strain HB101. Ampicillin-resistant transformants were isolated and tested for tetracycline sensitivity. About 50% of the ampicillin-resistant strains were sensitive to tetracycline. These clones were collected and hybridized with ^{32}P-labeled total micronuclear DNA according to the method of Grunstein and Hogness (18). Three clones showed preferential hybridization, and one of them, pTt2837, was found to contain a sequence not present in the macronucleus (19). The second clone, pTt2140, also seemed to contain micronuclear specific sequences. The third clone, pTt2152, contained sequences found in both nuclei and was used in this study.

RESULTS

Depurination Study of Macronuclear DNA

The fractions of *Tetrahymena* main-peak DNA containing the highly repetitive, middle repetitive, and unique sequences

were analyzed by depurination and fingerprinting of the pyrimidine tracts obtained. Fig. 2 shows fingerprints of depurinated total main-peak DNA after removal of rDNA (*A*), highly repetitive (*B*), middle repetitive (*C*), and unique sequence (*D*) fractions. Both of the repetitive sequence fractions were enriched for the pyrimidine tract C_4, which was present in amounts in excess of that expected on a random basis. No enrichment over the amount of C_4 expected on a random basis was found in the unique sequence fraction. T_4 endonuclease IV digestion studies of the various C_0t fractions (8) suggested that the extra C_4 detected was actually derived from the repeated sequence C-C-C-C-A-A (data not shown).

It has been found that the amplified rDNA contains the repeated hexanucleotide sequence at its termini. This sequence accounts for 1–2% of the total rDNA. rDNA was removed in good yield from the main-peak DNA in these experiments. Therefore, it is not likely that rDNA contamination alone can account for the enrichment of the C_4 observed in the repetitive sequence fractions of main peak DNA.

The Southern hybridization experiments described below confirmed that the tandemly repeated hexanucleotide is a component of chromosomal DNA as well as of amplified rDNA.

Southern Hybridization of C-C-C-C-A-A Sequence

Equal amounts of macronuclear and micronuclear DNA were digested with the restriction endonucleases EcoRI, Hind III, and Hae III, and analyzed by electrophoresis in an agarose gel. The DNAs were blotted onto a nitrocellulose filter and hybridized with labeled C-C-C-C-A-A repeats prepared as described in Materials and Methods. The autoradiographs of the hybridization are shown in Fig. 3. The hybridization patterns were complex but reproducible. In addition to the band corresponding to the terminal rDNA fragments, numerous bands were detected in both nuclear DNAs. Because a single hexanucleotide is probably too short to form a stable hybrid under the condition used, these results suggest that C-C-C-C-A-A or a similar sequence is present in the genome as tandem repeats. The complicated hybridization patterns suggest that the repeats occurred in numerous clusters in both genomes.

When macronuclear and micronuclear DNAs were compared, the hybridization patterns were found to be very different. This was true for all three enzymes tested, and was most apparent in the case of Hind III. Although analysis of every individual band was not possible, it was clear that the majority of the bands were different between the two nuclei. Because none of the restriction enzymes used recognized C-C-C-C-A-A repeats, the differences observed must be attributable to differences in the restriction sites flanking the C-C-C-C-A-A repeats. Methylation of these sites is probably not the molecular mechanism. Although it has been found in other eukaryotes, methyl cytosine has not been detected in either nucleus of *Tetrahymena* (20; Rae and M.-C. Yao, unpublished observation). Methyl adenine, on the other hand, accounts for ~0.8% of the adenine in the macronucleus and has not been detected in the micronucleus (20). Difference in methyl adenine alone is not sufficient to explain our observations. This is most apparent in the case of Hae III digestion because adenine is not part of the recognition sequence of this enzyme.

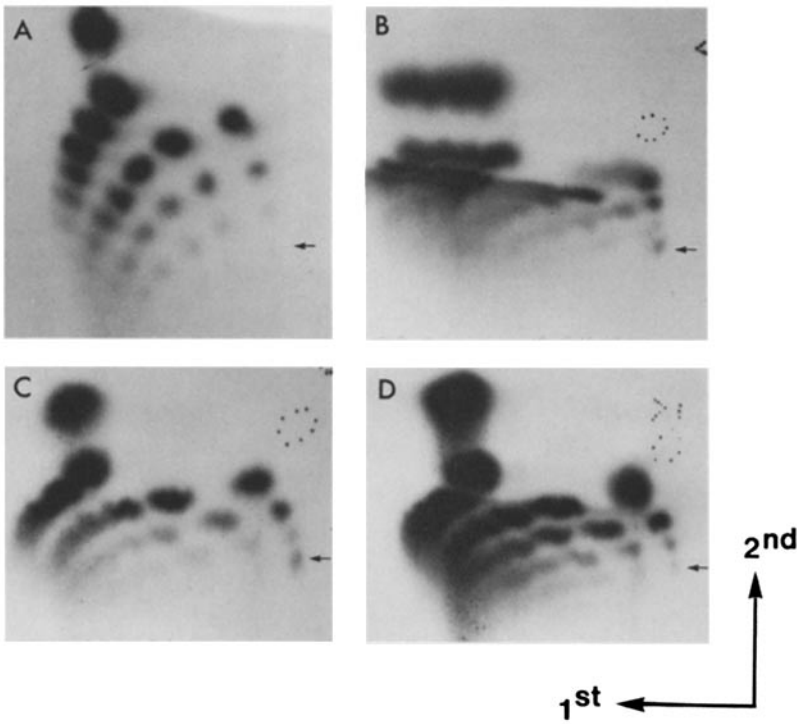

FIGURE 2 Two-dimensional fractionation of pyrimidine tracts of *Tetrahymena* DNA. Main-peak DNA from *Tetrahymena*, labeled in vivo with ^{32}P and separated from rDNA, was fractionated into highly repetitive (*B*), middle repetitive (*C*) and unique (*D*) sequence fractions. Each fraction, and total main-peak DNA (*A*), was depurinated and fingerprinted. Fractionation in two dimensions was done as described in the legend to Fig. 1. The dotted circles indicate the position of the xylene cyanole FF dye marker. The arrows indicate the position of the pyrimidine tract C_4. Note that repetitive DNAs (*B* and *C*) contain more than random amounts of C_4.

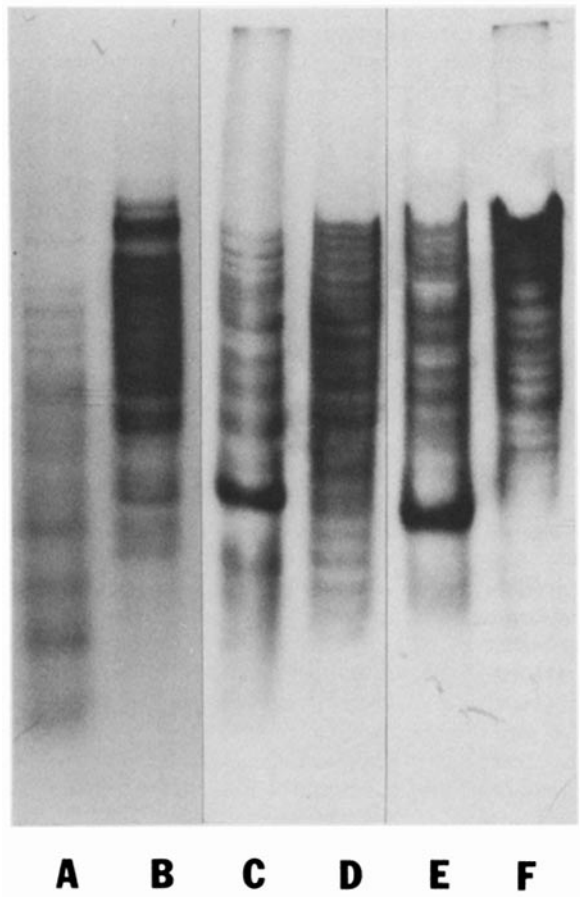

FIGURE 3 Southern hybridization of C-C-C-C-A-A repeats. Roughly equal amounts of micronuclear and macronuclear DNAs were digested with excess amounts of restriction endonucleases and analyzed in a 0.7% agarose gel. The DNA was then blotted according to the method of Southern (16) and hybridized with ^{32}P-labeled C-C-C-C-A-A repeats prepared from rDNA. Lanes *A*, *C*, and *E* contained macronuclear DNA, lanes *B*, *D*, and *F* micronuclear DNA. The DNA was digested with Hind III in lanes *A* and *B*, EcoR1 in lanes *C* and *D*, and Hae III in lanes *E* and *F*. Both macronuclear and micronuclear DNA hybridized with the probe in numerous bands. However, the banding patterns were quite different between the two DNAs. The rDNA fragment that contained C-C-C-C-A-A repeats hybridized extensively with the probe in the macronucleus in lanes *C* and *E*. This fragment was too small to be detected after Hind III digestion (lane *A*).

Southern Hybridization of Non-C-C-C-C-C-A-A Sequence

The difference in the hybridization observed between the two nuclear DNAs seems to be specific to C-C-C-C-A-A repeats. To illustrate this point other repetitive sequences were used for similar hybridization experiments. We have cloned micronuclear DNA fragments in the bacterial plasmid PBR322 after digestion with both EcoRI and Bam HI. Clones that might contain repetitive sequences were selected by colony hybridization, with total micronuclear DNA as a probe. One clone, pTt2152, was selected by this method for this analysis. Fig. 4 shows the Southern hybridization patterns of macronuclear and micronuclear DNAs with pTt2152. The hybridization occurred in multiple bands. The banding patterns were complicated, but appeared to be rather similar between the two nuclear DNAs. Although minor differences could be seen, the majority of the bands seemed to be present in both nuclei. It suggested that the difference between the two nuclear DNAs detected using C-C-C-C-A-A repeats was not a general feature of all repetitive sequences, and was probably specific to the hexanucleotide repeat.

Further support of this argument was obtained from studies of 5S DNA. Kimmel and Gorovsky (21) have shown that the 5S RNA gene is organized in multiple clusters of tandem repeats in both the macronucleus and the micronucleus. Using 5S RNA as a probe, they were able to show the similarity in 5S DNA organization between these two nuclear DNAs by Southern hybridization. Fig. 4 shows a similar experiment using cloned 5S DNA provided by Kimmel as a probe. Although minor differences could be detected, the majority of the bands in these two nuclear DNAs were quite similar. This result illustrated once more the similarity of the macronuclear and the micronuclear genome.

Conservation of C-C-C-C-A-A Repeats

The organization of C-C-C-C-A-A repeats in other species of *Tetrahymena* has also been examined. Whole-cell DNA,

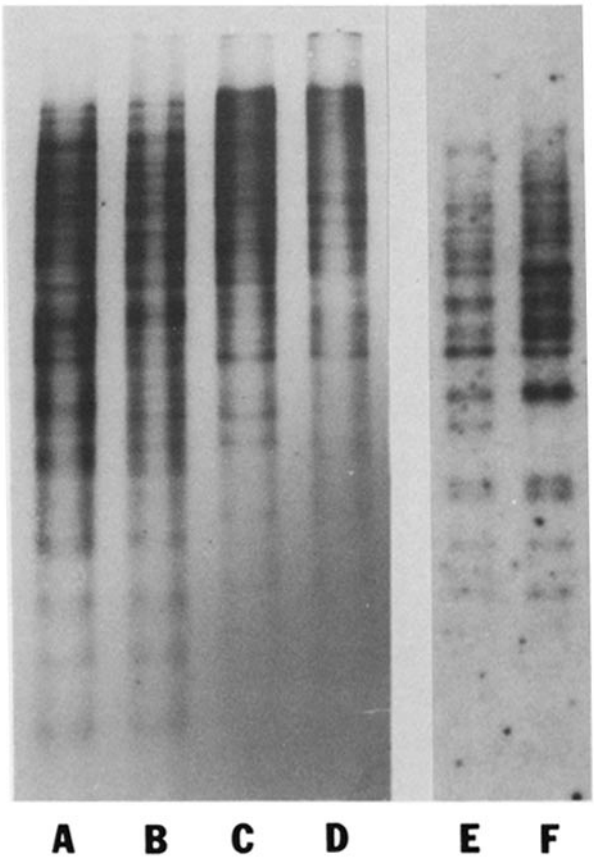

FIGURE 4 Southern hybridization with non-C-C-C-C-A-A sequences. Macronuclear and micronuclear DNAs were digested with either Hind III (lanes *A* and *B*) or EcoRI (lanes *C* and *D*) and hybridized with DNA from clone pTt2152 by use of the method described in Fig. 3. pTt2152 is a randomly selected fragment of micronuclear DNA cloned in pBR322. It hybridized equally well with both nuclear DNAs and in a similar pattern, although minor differences did exist. Lanes *E* and *F* show a similar experiment using 5S DNA as a probe and EcoR1 to digest the nuclear DNA. Again, similar patterns were detected between the two nuclear DNAs, although some bands were clearly different. Micronuclear DNA was contained in lanes *A*, *C*, and *E*, and macronuclear DNA in lanes *B*, *D*, and *F*.

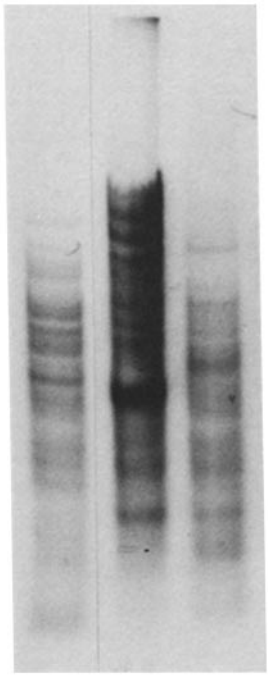

FIGURE 5 Hybridization of C-C-C-C-A-A repeats to other species of *Tetrahymena*. Whole cell DNA was prepared from three species of *Tetrahymena*, digested with Hind III, and hybridized with C-C-C-C-A-A repeats by use of the method described in Fig. 3. Lane *A* contained *T. pyriformis* DNA. Lane *B* contained *T. pigmentosa* strain 6I (formally syngen 6) DNA. Lane *C* contained *T. thermophila* DNA. All strains showed extensive hybridization, indicated that the repeated C-C-C-C-A-A sequence was present in all these species of *Tetrahymena*.

which is composed mainly of macronuclear DNA, was isolated from the species *T. pigmentosa* and *T. pyriformis* as well as *T. thermophila*. The DNA were analyzed by Southern hybridization and the results are shown in Fig. 5. All strains compared showed complicated hybridization patterns. The three species studied are not known to be related to each other in any particular manner. It is therefore not surprising that their hybridization patterns are quite different. The results indicated that all three species studied contained C-C-C-C-A-A repeats or other similar sequences in their macronucleus. It is likely that the C-C-C-C-A-A repeats are present in most species of *Tetrahymena*.

DISCUSSION

Using sequencing and hybridization techniques, we have shown that the simple repeated hexanucleotide $(C\text{-}C\text{-}C\text{-}C\text{-}A\text{-}A)_n$, which was found originally in the termini of rDNA, is also present in other locations of the *Tetrahymena* genome. The hybridization data suggested that the hexanucleotide sequence exists as clusters of tandem repeats in the genome. The total number of clusters is probably rather high as suggested by the complex banding pattern of the Southern hybridization experiments. The precise number of clusters cannot be determined from these data alone. However, in a recent attempt to isolate C-C-C-C-A-A sequence by cloning, it was found that, in a recombinant DNA library that contained one haploid genome equivalent of micronuclear DNA, ~200 recombinant clones hybridized with the C-C-C-C-A-A sequence (R. Yokoyama and M.-C. Yao, unpublished observation). Although this number should not be taken too seriously, it nonetheless gives some indication of the total number of clusters of C-C-C-C-A-A repeats in the *Tetrahymena* genome.

It is interesting that the C-C-C-C-A-A repeats hybridize rather differently with macronuclear and micronuclear DNAs. Although the complexity of the banding patterns did not permit a detailed comparison of each individual band, it was apparent that the majority of the bands were different between these two nuclei. The data strongly suggested that the C-C-C-C-A-A repeats were surrounded by different restriction enzyme digestion sites in the macronucleus and the micronucleus. The exact molecular basis for this difference is not known. As mentioned earlier, base modification alone is not sufficient to explain this kind of difference. The difference more likely results from certain kinds of changes in the sequence organization of the genome, such as gene rearrangement or chromsosome fragmentation, which might have occurred during the formation of the macronucleus.

Although drastic differences were observed between macronuclear and micronuclear DNA by hybridization with C-C-C-C-A-A repeats, the two nuclear DNAs are rather similar in other aspects. Earlier studies on renaturation kinetics have shown that the macronucleus contains ~80–90% of the sequences present in the micronucleus (10). The size of the macronuclear DNA was also known to be quite large, although there were conflicting reports on whether it was actually the same size as the micronuclear chromosome (22, 23). Our hybridization results from the two unrelated repetitive sequences, pTt2152 and 5S DNA, also suggested that the sequence organizations of the two nuclear DNA were rather similar, at least in the regions adjacent to these sequences. Hence the difference detected with the C-C-C-C-A-A repeats is not likely a common feature of all sequences in the genome and may be associated specifically with these hexanucleotide repeats.

That the C-C-C-C-A-A repeats also exist in the termini of rDNA may not be a mere coincidence. It has been found recently that the amplification of rDNA in *Tetrahymena* is accompanied by chromosome breakage. The breakage probably occurs near the C-C-C-C-A-A repeats of the rDNA in the chromosome, presumably as a process to generate the extrachromosomal rDNA for amplification (24). It is conceivable that similar kinds of breakage could also occur near other C-C-C-C-A-A repeats in the chromosome. This process would explain the differences in the C-C-C-C-A-A hybridization patterns observed. In fact, chromosome breakage has been observed in another group of ciliates, *Oxytricha*, where the macronuclear DNA is broken down into gene-sized fragments (25). Clearly, more studies are needed to support this argument. For instance, it is crucial now to find out whether macronuclear DNA is indeed smaller than the chromosome, and whether C-C-C-C-A-A repeats are often associated with the ends of the chromosome fragments in the macronucleus. Cloning of the C-C-C-C-A-A repeats from the micronucleus is under way. Study of the flanking sequences in the macronucleus and micronucleus might reveal the nature of the alteration process. One such clone has recently been characterized. It was found that the flanking sequences are repetitive in the micronucleus and are eliminated from the macronucleus during development (M.-C. Yao, manuscript in preparation). Although the significance of this finding is not entirely clear, it does suggest a possible relationship between the C-C-C-C-A-A repeats and the process of DNA elimination. Moreover, it was found

recently that the C-C-C-C-A-A repeats were present in the ends of the macronuclear DNA of the related tetrahymenid (Katzen, A. L., G. Cann, and E. H. Blackburn, manuscript submitted for publication). It is not inconceivable that the repeated hexanucleotide has an important role in the reorganization of the *Tetrahymena* genome during differentiation.

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