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A micronucleus-specific sequence exists in the 5′-upstream region of calmodulin gene in *Tetrahymena thermophila*

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ABSTRACT

***Tetrahymena thermophila* possesses a transcriptionally inactive micronucleus and an active macronucleus. Both nuclei are developed from micronucleus-derived germ nuclei during conjugation. Extensive DNA rearrangement and transcriptional activation are known to be involved in macronuclear development, but little has been known about these processes in a particular functional gene. Therefore the micro- and macronuclear genomic DNAs for calmodulin gene were analyzed. A 1,384 bp micronucleus-specific sequence located about 3.5 kb upstream of calmodulin gene has been found, suggesting DNA rearrangement during macronuclear development. The micronucleus-specific sequence had 85% A + T, no extensive ORF, ATTAs at both ends, and two palindromic structures just outside of both ends. Interestingly, the micronucleus-specific sequence included a T-rich tract, $T_{16}CT_5$, in the middle, and a nearly complementary A-rich tract, $A_5TA_{10}GA_5$, existed 7 bp upstream from the initiation codon. In addition, there was a 20 bp repetitive sequence TAAT(TAAC)$_4$ about 100 bp upstream of the micronucleus-specific sequence and also in the promoter region of calmodulin gene. Although the functional significance of the micronucleus-specific sequence remains unclear, $T_{16}CT_5$ and TAAT(TAAC)$_4$ elements might exert an influence on transcription of the calmodulin gene. Stringent Southern hybridization revealed that this micronucleus-specific sequence or very similar sequence(s) were abundant in the *Tetrahymena* micronuclear genome.**

INTRODUCTION

Programmed DNA rearrangement is known to occur in a wide variety of organisms (1–6). The rearrangement often leads to the activation or the diversification of a certain gene (7). Extensive genome rearrangement is also known to occur in ciliates during macronuclear development (8, 9). Although this process is likely to have a major role in the development, little is known about its precise mechanism and function.

The ciliated protozoan *Tetrahymena thermophila* has two different nuclei: a micronucleus and a macronucleus. The micronucleus serves as the germ-line nucleus, but it is widely accepted that it remains transcriptionally inactive during vegetative growth. On the other hand, a macronucleus is transcriptionally active and sustains vegetative growth of the cell. During the sexual process of conjugation, a new macronucleus develops from one of the micronucleus-derived germ nuclei, while the old macronucleus is degraded. In the process of new macronuclear development, extensive genome rearrangement including partial elimination and rejoining of micronuclear DNA, and amplification of the rearranged macronuclear genome to the level of 45°C are known to occur (10). In *Tetrahymena*, it has been estimated from the renaturation kinetics of the micro- and macronuclear DNAs that 10 to 20% of the micronuclear DNA is eliminated (11). Some of the sequences scattered in the micronuclear genome appear to be completely eliminated during macronuclear development, so that the developed macronucleus does not include such sequences (12, 13).

However, little is known about the genome rearrangement in functional genes of *Tetrahymena*, except for rRNA genes (14, 15) and an α-tubulin gene (16). The rearrangement in rDNA is associated with fragmentation of the micronuclear genome into subchromosomal fragments to which the telomeric repeat is added. The rearrangement in the flanking regions of α-tubulin gene involves elimination by breakage and rejoining of the flanking sequences, namely interstitial deletion, but it has not been analyzed in detail.

We have investigated the genomic DNA rearrangement in *Tetrahymena* calmodulin gene including its flanking regions, as an example of a functional gene. In this report, the existence and the nucleotide sequence of a 1.4 kb micronucleus-specific (mic-specific) sequence localized 3.5 kb upstream from the initiation codon of calmodulin gene is described.

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MATERIALS AND METHODS

Cell culture

Cultivation of *Tetrahymena thermophila* (strain B1868-III or -VII) was performed as previously described (17).

Southern and Northern hybridizations

Genomic DNAs were prepared separately from *T.thermophila* micronuclei and macronuclei which were isolated by the method of Mita *et al.* (18). Total RNA from *T.thermophila* was prepared by the guanidine thiocyanate method as described by Chirgwin *et al.* (19). Three μg of micro- and macronuclear DNAs which were digested with restriction endonucleases, or ten μg of total RNA, were electrophoresed on a 0.8% agarose gel and transferred to a nylon membrane (Gene Screen Plus: Du Pont-New England Nuclear). Southern and Northern hybridizations were carried out under the same conditions as described by Takemasa, *et al.* (20).

Cloning and sequencing of a macronuclear 4.2 kb fragment which corresponds to a micronuclear 5.6 kb fragment including a mic-specific sequence

Macronuclear DNA was digested with *Eco*RI and fractionated by electrophoresis on a 0.8% agarose gel. *Eco*RI-digested 4.2 kb fragments, including the sequence which hybridized with the calmodulin cDNA probe, were ligated with pUC18. The constructed subgenomic library was screened by colony hybridization with ^{32}P-labeled *T.thermophila* calmodulin cDNA (21) as a probe, using the same conditions as for Southern hybridization. Dual-directional deletions of each insert were done using exonuclease III and mung bean nuclease, and nucleotide sequences of the necessary parts of the macronuclear 4.2 kb fragment were determined by the dideoxy chain termination method (22, 23).

Cloning and sequencing of a micronuclear 2.2 kb fragment including mic-specific sequence

A micronuclear 2.2 kb fragment was amplified by the polymerase chain reaction (PCR) method using micronuclear genomic DNA as the template and synthesized oligonucleotide primers corresponding to nucleotide sequences of both termini of a macronuclear 0.8 kb *Eco*RI/*Eco*RV fragment derived from the macronuclear 4.2 kb fragment. This PCR product was ligated into pCR1000 (Invitrogen) and then subcloned into pUC18 digested with *Sma*I. The entire nucleotide sequence of the micronuclear 2.2 kb fragment was determined by the method as described above.

Determination of transcription starting sites by primer extension method

Poly(A)$^+$ RNA was prepared from the total *T.thermophila* RNA by oligotex dT30. The 27-mer oligonucleotide complementary to nucleotides from −3 to +24 of calmodulin cDNA was synthesized. Primer extension method was performed as described by McKnight *et al.* (24) with the following modifications. The primer was labeled with T4 polynucleotide kinase. The labeled primer (2×10^5 cpm) was added to 5 μg of poly(A)$^+$ RNA. The mixture was hybridized at 65°C for 2 hours and allowed to cool down slowly to 37°C. Reverse transcription was carried out with 100 units of Moloney Murine Leukemia Virus reverse transcriptase, and the primer extension products were subjected to electrophoresis using a 6% sequencing gel.

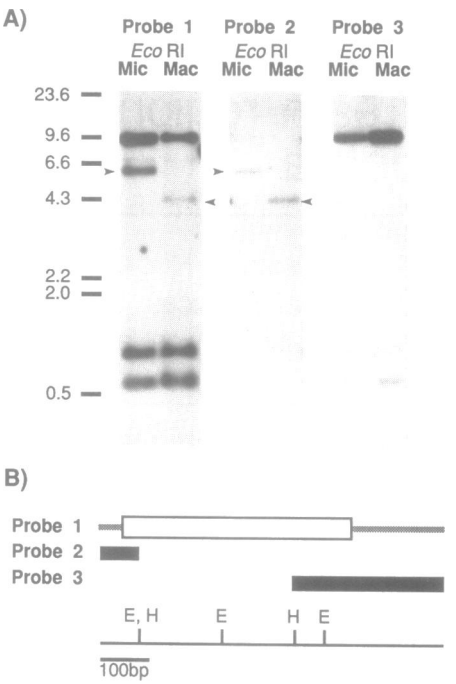

Figure 1. Comparison between micro- and macronuclear DNAs by Southern hybridization using calmodulin cDNA and its 5′- and 3′-fragments as probes. **(A)** Micro- and macronuclear DNAs were digested with *Eco*RI, electrophoresed, and blotted for hybridization. Probe 1 is the full length calmodulin cDNA. Probes 2 and 3 were 5′- and 3′-*Hin*dIII fragments of the calmodulin cDNA, respectively. The kb-markers are represented in the left using *Hin*dIII-digested λ DNA. Mic, micronuclear genomic DNA; Mac, macronuclear genomic DNA. Note the difference between the micronuclear 5.6 kb fragment and the macronuclear 4.2 kb fragment (arrowheads). **(B)** Restriction maps of the calmodulin cDNA and the three probes used for Southern hybridizations in (A). In probe 1, the protein coding region is indicated by an open box and untranslated regions are indicated by stippled bars. Two solid bars (probes 2 and 3) indicate 5′- and 3′-*Hin*dIII fragments of the calmodulin cDNA, respectively. E, *Eco*RI site; H, *Hin*dIII site.

RESULTS

Comparison of micro- and macronuclear calmodulin genes by Southern hybridization

Micronuclear and macronuclear DNAs were digested with several restriction enzymes and analyzed by Southern hybridization using calmodulin cDNA as a probe. When both DNAs were digested with *Eco*RI, four fragments were detected in each nuclear DNA (Fig. 1A, probe 1). One of micronuclear fragments was 5.6 kb long and it was 1.4 kb longer than its macronuclear counterpart (4.2 kb). The remaining three fragments did not show any difference in mobilities between respective micro- and macronuclear bands. The simplest interpretation of this result is that a 1.4 kb long sequence is present in the micronuclear calmodulin gene or its flanking regions.

Location of the putative 1.4 kb sequence

To investigate the location of the 1.4 kb sequence, *Eco*RI-digested micro- and macronuclear DNAs were analyzed using 5′- or 3′-regions of calmodulin cDNA as probes (Fig. 1B). As shown in the experiments using probes 2 and 3 in Fig. 1A, only 5′-region probe hybridized to the fragments which had different mobilities between micro- and macronuclear DNAs (Fig. 1A, probe 2). This suggests that the possible mic-specific sequence

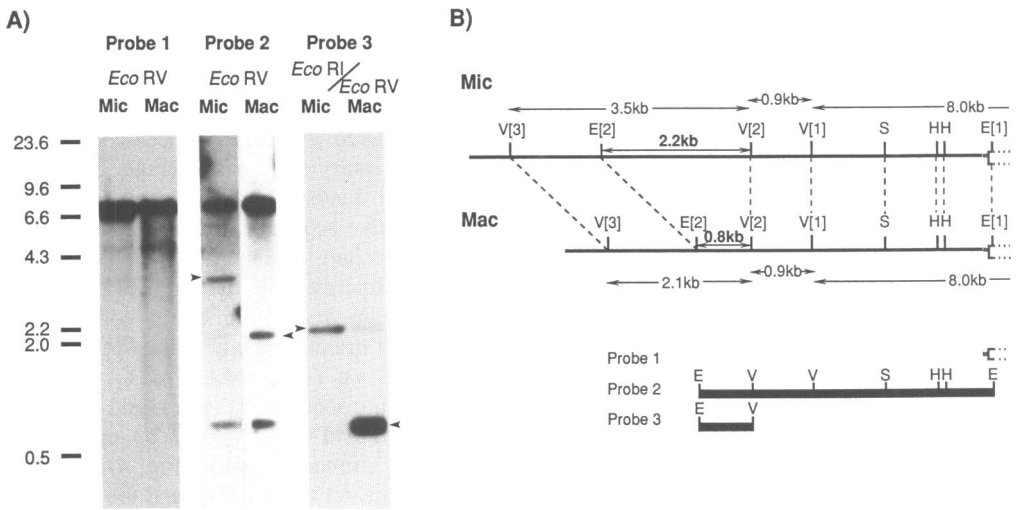

Figure 2. Southern hybridization patterns and restriction maps of the 5′-upstream regions of micro- and macronuclear genomic DNAs for calmodulin gene. **(A)** Southern hybridizations using calmodulin cDNA (probe 1), macronuclear 4.2 kb (probe 2) and 0.8 kb fragments (probe 3) as probes (see B). Micronuclear genomic DNA (Mic) and macronuclear genomic DNA (Mac) were digested with *Eco*RV or *Eco*RI/*Eco*RV, as indicated, electrophoresed and blotted for hybridization. The Kb-markers are represented in the left. Note that 1.4 kb-long differences between micronuclear and macronuclear fragments (3.5 kb and 2.1 kb, 2.2 kb and 0.8 kb) are seen in the middle and left lanes, respectively (arrowheads). **(B)** Restriction maps of Mic and Mac of 5′-upstream region of calmodulin gene. They are based on the restriction map of the macronuclear 4.2 kb fragment and results obtained in Figs. 1A and 2A. Macronuclear 4.2 kb (probe 2) and 0.8 kb fragments (probe 3) used as probes are indicated by solid bars and a part of calmodulin cDNA (probe 1) is also indicated. E[number], EcoRI site; V[number], *Eco*RV site; H, *Hin*dIII site; S, *Sac*I site. Restriction sites are numbered (in brackets) for convenience.

is included in the 5′ side or 5′-flanking region of the micronuclear calmodulin gene.

To obtain more details about the localization of the mic-specific sequence, we isolated the *Eco*RI-digested 4.2 kb macronuclear fragment which has a different mobility from that of its micronuclear counterpart (5.6 kb). A subgenomic library was constructed with the 4.2 kb *Eco*RI fragments of macronuclear DNA, which was screened by colony hybridization using calmodulin cDNA as a probe. Of about 8,000 recombinant clones in the library, one positive clone was isolated. The restriction map of this clone was constructed (Fig. 2B, probe 2: 4.2 kb macronuclear fragment in which two *Eco*RV sites exist).

Then the 4.2 kb macronuclear fragment was used as a probe to hybridize to *Eco*RV-digested micro- and macronuclear DNAs (Fig. 2A, probe 2). Three fragments were detected in each nuclear DNA. The longest fragment (8.0 kb) corresponded to the fragment including the coding region shown in Fig. 2B, because the fragment hybridized with the calmodulin cDNA probe (Fig. 2A, probe 1). The shortest fragment was the 0.9 kb *Eco*RV fragment (*Eco*RV[2]/*Eco*RV[1]) which was situated immediately at the 5′ side of the 8.0 kb fragment in Fig. 2B. The mobilities of these bands from micro- and macronuclear DNA were identical, namely, downstream from *Eco*RV[2] site, no difference between micro- and macronuclear DNA was detected (Fig. 2B). The 3.5 kb and 2.1 kb fragments (micro- and macronuclear ones, respectively) corresponded to the most 5′-side *Eco*RV fragments (*Eco*RV[3]/*Eco*RV[2]) in Fig. 2B and the difference of 1.4 kb length detected in Fig. 1A was shared. Moreover, when we used the *Eco*RI[2]/*Eco*RV[2] fragment (0.8 kb) of macronuclear DNA as a probe (Fig. 2A, probe 3) to hybridize with *Eco*RI/*Eco*RV-double digested micro- and macronuclear DNAs, the hybridized micro- and macronuclear fragments were 2.2 kb and 0.8 kb in length, respectively, also the 1.4 kb difference. Therefore, the mic-specific sequence appeared to be included in the micronuclear 2.2 kb fragment between *Eco*RI[2] and *Eco*RV[2] sites.

Existence of the 1.4 kb mic-specific sequence

In order to substantiate the existence of mic-specific sequence, we sequenced the macronuclear 0.8 kb and micronuclear 2.2 kb *Eco*RI[2]/*Eco*RV[2] fragments. First the 0.8 kb and 2.2 kb *Eco*RI[2]/*Eco*RV[2] fragments were subcloned, then sequenced. As shown in Fig. 3, comparison between the 0.8 kb macronuclear DNA sequence and the corresponding 2.2 kb micronuclear DNA sequence indicated that the only difference was a mic-specific sequence of 1,384 bp existing 3.5 kb upstream from the initiation codon. The micronuclear nucleotide sequence other than the mic-specific sequence was exactly the same as the macronuclear nucleotide sequence. This strongly suggests that the macronuclear genomic DNA is rejoined after eliminating the 1,384 bp mic-specific sequence from the micronuclear genomic DNA.

Characterization of the mic-specific sequence and its flanking regions

The mic-specific sequence and its flanking regions were very rich in A+T (85% and 83%, respectively). The junction parts of both ends contained the 4 bp sequence 5′-ATTA-3′ (Fig. 3, small boxes). Two kinds of palindromic structures were found just outside of both junctions: A 20 bp palindrome laid adjacent to the 5′-junction, and an unrelated palindrome of 23 bp laid adjacent to the 3′-junction (bidirectional arrows in Fig. 3). Three ARS consensus sequences were found in the mic-specific sequence (dashed lines in Fig. 3). This might reflect the phase difference in DNA replication between micro- and macronucleus.

No extensive ORF was detected in the mic-specific sequence. Homology search using the EMBL data bank failed to detect any highly homologous sequence with the mic-specific sequence. Moreover, when the total *T. thermophila* RNA was analyzed by Northern hybridization using the mic-specific sequence as a probe, no hybridizing transcript was detected (data not shown). These results show that there is no protein-coding region in the mic-specific sequence.

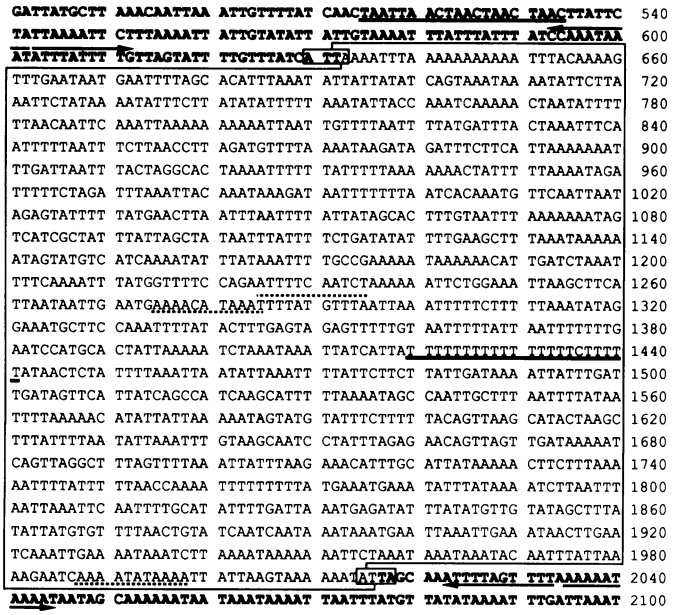

Figure 3. Nucleotide sequence of mic-specific sequence and its flanking regions. The main nucleotide sequence of the 2.2 kb (*Eco*RI[2]/*Eco*RV[2]) micronuclear fragment (see legend of Fig. 2B) is shown, numbered from the 5′-end. The block of sequence enclosed by lines is mic-specific sequence (1,384 bp). Micronuclear DNA sequence depicted with bold letters is shown as the sequence which completely coincides with the sequence of the macronuclear 0.8 kb fragment (see legend of Fig. 2B). Open boxes which enclose ATTA represent a possible junction consensus motif. Palindromic structures are underlined with bidirectional arrows. The 20 bp repetitive motif, TAAT(TAAC)$_4$, and T-rich tract, $T_{16}CT_5$, are underlined in bold. Dashed lines represent ARS consensus sequences. As the nucleotide sequences of micro- and macronuclear DNA fragments from no.1−480 and no.2101−2157 were completely identical, these sequences are omitted from the Figure (refer to the EMBL data bank).

The possibility that the mic-specific sequence might have some steric effects on the conformation of 5′-promoter region of calmodulin gene was considered. The nucleotide sequence of the 5′-promoter region was determined as shown in Fig. 4A and it was compared with the mic-specific sequence to look for possible complementary sequence(s) (Figs. 3 and 4A). As a result, we found two kinds of possible candidate sequences. One is a 20 bp repetitive sequence TAAT(TAAC)$_4$. This sequence existed 98 bp upstream from the 5′-junction of the mic-specific sequence (Fig. 3). Interestingly, the same 20 bp sequence also existed 116 bp upstream from the initiation codon of the calmodulin gene in a direct orientation but on the opposite strand (Fig. 4A). The other one is a T-rich tract, $T_{16}CT_5$, in the middle region of the mic-specific sequence (Fig. 3), and a nearly complementary A-rich tract, $A_5TA_{10}GA_5$, at 7 bp upstream from the initiation codon of calmodulin gene (Fig. 4A).

Since the ATTA(ATTG)$_4$ and the $A_5TA_{10}GA_5$ existed 116 bp and 7 bp upstream from the initiation codon, respectively, we then investigated the transcription starting site(s) and the position of putative TATA box(es). The transcription starting sites of calmodulin gene were determined by the primer extension method. As shown in Fig. 4B, there were four strong starting sites (−88, -61, −58, −52 bp upstream from ATG, arrowheads in Fig. 4A and B), and several weaker ones within the region between −49 and −88 bp (Fig. 4B). In addition, about ten putative TATA boxes (sequences differing in no more than one nucleotide from the TATA box consensus sequences (25), $TATA^{T}/_{A}A^{T}/_{A}$) were found in −49 to −300 bp upstream from ATG (dashed lines in Fig. 4A). The complementary sequence of TAAT(TAAC)$_4$ overlapped with one of the putative TATA box clusters, and the A-rich tract existed downstream from the transcription starting sites.

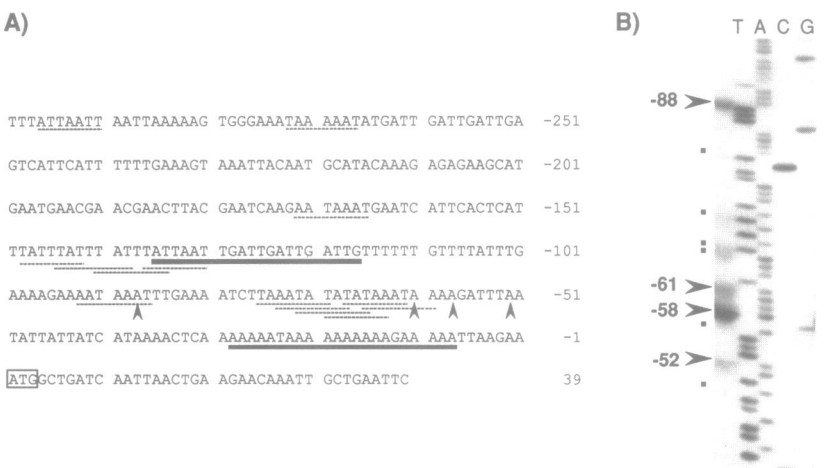

Figure 4. Nucleotide sequence of the 5′-flanking region of calmodulin gene. **(A)** Using the macronuclear 4.2 kb fragment, the nucleotide sequence of the 1.5 kb *Sac*I/*Eco*RI[1] fragment was determined (see Fig. 2B). The translation initiation codon (ATG) is boxed, and designated as +1. Here, nucleotide sequence from no. 39 up to no. −300 is shown (For the sequence of the remaining upstream region, refer to the EMBL data bank). The 20 bp repetitive motif, ATTA(ATTG)$_4$, and A-rich tract, $A_5TA_{10}GA_5$, are underlined in bold. Arrowheads indicate strong transcription starting sites identified by primer extension (see B). The sequences of the nearest match to the TATA consensus are indicated by dashed underlines. **(B)** Transcription starting sites of the calmodulin gene were detected by primer extension as described in 'Materials and Methods'. Primer extension products are shown in the left most lane. The strong transcription starting sites are shown with both arrowheads and nucleotide numbers, and weak starting sites are shown with dots. For reference, sequencing ladders (T,A,C,G) are represented by dideoxy sequencing using the same ^{32}P-labeled primer (right 4 lanes).

Analysis of micro- and macronuclear DNAs by Southern hybridization using mic-specific sequence as a probe

Micro- and macronuclear DNAs were analyzed by Southern hybridization using the mic-specific sequence and macronuclear fragment (control) as probes (Fig. 5) to look for sequence(s) homologous to the mic-specific sequence. Southern hybridization was carried out under the same conditions, namely high stringency conditions. The *Eco*RI- or *Eco*RI/*Eco*RV-digested micronuclear DNA fragments gave rise to many positive bands which hybridized with the mic-specific sequence. This result indicates that many sequences homologous to the mic-specific sequence are scattered in the micronuclear genome. In contrast, no significant hybridizing band was detected in the macronuclear DNA under the same conditions.

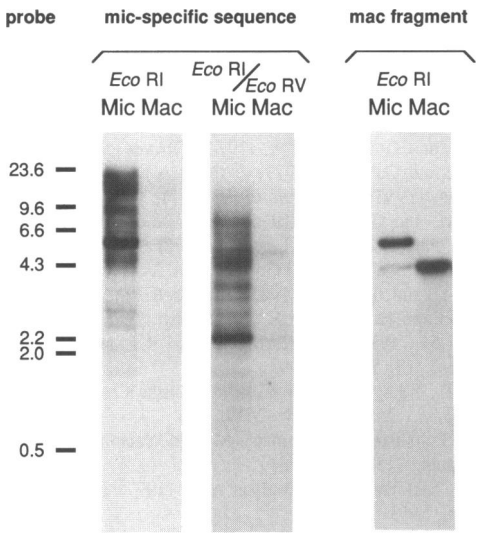

Figure 5. Southern hybridization of micro- and macronuclear DNAs probed with the mic-specific sequence. Almost all of the mic-specific sequence (1,359 bp, nucleotide no.647 to 2006 in Fig. 3) was synthesized by PCR and used as a probe. As a control probe a macronuclear 2.7 kb *Eco*RI/*Sac*I fragment (see Fig. 2) was used. The kb-markers are represented in the left. Micronuclear (Mic) and macronuclear (Mac) genomic DNAs were digested with *Eco*RI or *Eco*RI/*Eco*RV and Southern hybridization in each lane was carried out under the ordinary high stringency condition. Note that many positive bands are seen in Mic lanes but no significant band is seen in Mac lanes in the case of using mic-specific sequence, while the micronuclear 5.6 kb *Eco*RI fragment and the macronuclear 4.2 kb *Eco*RI fragment strongly hybridized with the 2.7 kb mac fragment. Weak hybridizations are seen with the Mic 4.2 kb and Mac 5.6 kb bands, owing to the contamination of macronuclei in the micronuclear fraction and that of micronuclei in the macronuclear fraction, respectively.

DISCUSSION

From the present experimental results (Figs. 1−3), we reached the conclusion that DNA rearrangement should occur at the 5′-flanking region of the calmodulin gene, since the 1,384 bp mic-specific sequence exists about 3.5kb upstream of the micronuclear calmodulin gene but does not in the macronuclear one.

To know sequence motifs common to mic-specific sequences in *Tetrahymena*, the mic-specific sequence described here was compared with the two mic-specific sequences previously described by Yao *et al.* (M region and R region)(26, 27). All three mic-specific sequences and their flanking sequences are very rich in A+T. Therefore, it seems unlikely that the elimination of mic-specific sequence leads to create or destroy protein-coding regions. All junctions of three mic-specific sequences contain the direct repeats. Short repeats, such as, 5′-ATTA-3′, 5′-TAATT-3′, 5′-TAAACA-3′ and 5′-AATAATTG-3′ appear to be one of features typical of the DNA deletion junctions in *T.thermophila*. However, these three mic-specific sequences only share some common structures. The R region and the mic-specific sequence described here have different palindromic structures near each of the junctions, but the M region does not. An unusual polypurine tract (5′-A_5G_5-3′) required for the splicing of the M region (28) is found only in the M flanking region. On the other hand, the 20 bp repetitive sequence, $TAAT(TAAC)_4$ and the T-rich tract, $T_{16}CT_5$, described here are not found in or near the

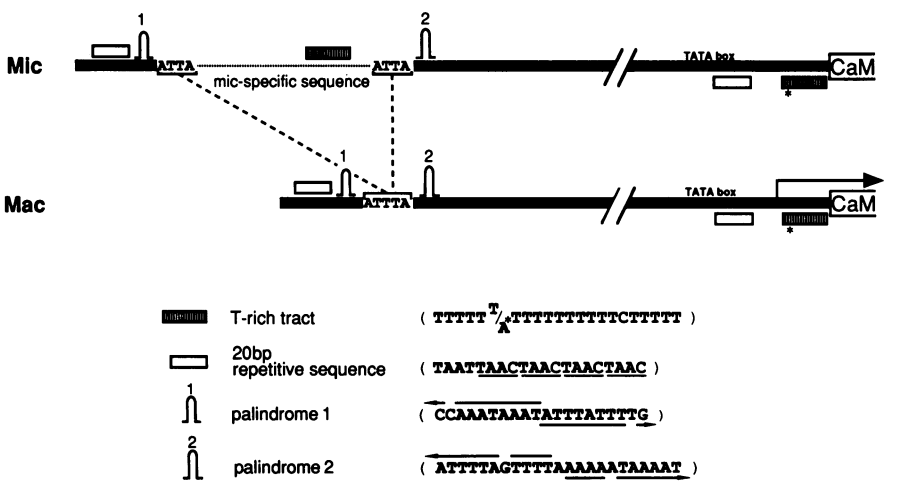

Figure 6. Schematic illustration of structures of the 5′-upstream regions of micro- and macronuclear calmodulin genes. The upper and lower solid bars depict the 5′-upstream regions of the calmodulin gene (CaM) of the micronuclear (Mic) and the macronuclear (Mac) genomes, respectively, with the mic-specific sequence indicated by a dashed line. The relative positions of two junction sequences (ATTA), the two palindromes (1 and 2), 20 bp repetitive sequence (open box), and T-rich tract (striped box) are indicated schematically. The nucleotide sequences of these structures are shown below. TATA box indicates one of the putative TATA box clusters.

two mic-specific sequences known so far (M and R regions). Until now, mic-specific sequences called internal eliminated sequences (IESs) have been sequenced and analyzed in hypotrichous ciliates, such as *Oxytricha* (29, 30). The *Tetrahymena* mic-specific sequence described here and the hypotrichous ciliates IESs appear to be dissimilar in size and the primary sequence, except that both include 2−6 bp direct repeats in general at the ends of each sequence and one copy of terminal repeat is retained in the macronuclear DNA.

As for the mic-specific sequence we found, a schematic summary of the data in Figs. 1−4 is in Fig. 6. The 20 bp repetitive sequence exists about 100 bp upstream of the mic-specific sequence and about 120 bp upstream from the initiation codon (Figs. 3 and 4A). The latter region presumably contains promoters of calmodulin gene, although we can not yet exclude a possibility that an intron might be present in the 5′ untranslated region. As a matter of fact, the region has some characters of the promoter regions of *Tetrahymena* genes previously reported by Brunk and Sadler (31). Concerning the TATA box of calmodulin gene, we could not determine authentic TATA boxes from many TATA box consensus sequences. But, the 20 bp repetitive sequence, $TAAT(TAAC)_4$, overlaps with one of the putative TATA box clusters (Figs. 4A and 6). Another complementary sequence was a T-rich tract, $T_{16}CT_5$, in the middle region of the mic-specific sequence and in the untranslated region of calmodulin gene (Figs. 3, 4, and 6). Although we have not yet ascertained whether these complementary sequences are able to interact, we are tempted to speculate that these sequences might play important roles in the regulation of transcriptional activity of the calmodulin gene, in other words, presence and absence of these sequences might be responsible for the different transcriptional activities observed in micro- and macronucleus.

The hybridization patterns of the mic-specific sequence with restriction enzyme-digested micro- and macronuclear DNA fragments are very interesting. The mic-specific sequence found in the calmodulin gene hybridizes with many DNA fragments from micronuclear genome, but not with any fragment from macronuclear genome (Fig. 5). In contrast, a control probe which has a high A+T content like the mic-specific sequence (a macronuclear 2.7 kb fragment, mac fragment in Fig. 2) hybridizes with single fragments in micro- and macronuclear genomes. A high A+T content in a fragments used as a probe does not bring about an unusual hybridization pattern. Therefore, we can conclude that the mic-specific sequence found in the present study is not unique to the calmodulin gene, but the sequence itself or very similar sequence(s) are scattered in many parts of the micronuclear genome of *T. thermophila*. Since these sequences appear to be eliminated from the micronuclear genome in the process of DNA rearrangement during macronuclear development, they might affect transcription of other functional genes which include such sequences in their flanking regions.

In order to determine the significance of the mic-specific sequence, we are now attempting further functional characterization of this sequence using an *in vitro* transcription assay system.

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