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A Family of DNA Sequences is Reproducibly Rearranged in the Somatic Nucleus of *Tetrahymena*

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A family of DNA sequences is reproducibly rearranged in the somatic nucleus of *Tetrahymena*

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ABSTRACT

A small family of DNA sequences is rearranged during the development of the somatic nucleus in Tetrahymena. The family is defined by 266 bp of highly conserved sequence which restriction mapping, hybridization and sequence analysis have shown is shared by a cloned micronuclear fragment and three sequences which constitute the macronuclear family. Genomic Southern hybridization experiments indicate there are five members of the family in micronuclear DNA. All of the family members are present in whole genome homozygotes and are therefore nonallelic. The three macronuclear sequences are all present in clonal cell lines and are reproducibly generated in every developing macronucleus. The rearrangement event begins 14 hours after conjugation is initiated and is nearly completed by 16 hours.

INTRODUCTION

The ciliate Tetrahymena thermophila, has two nuclei. The germ-line nucleus or micronucleus (mic), has a diploid DNA content and is, for the most part, transcriptionally inactive. The somatic nucleus or macronucleus (mac), has 45 times the haploid DNA amount (1) and is responsible for the phenotype of the cell. Though the nuclei differ in structure and function, they share a common origin. During sexual reproduction the mac is degraded and a new one develops from a mitotic product of the zygotic mic. (For a detailed account of the events of conjugation see Martindale et al., 2.)

The developing macronucleus (macronuclear anlagen) undergoes a number of changes: polyploidization of the genome, fragmentation of the chromosomes to an average size of 600 kb, elimination of about 10-20% of the micronuclear DNA sequences, amplification of the rDNA, and extensive DNA rearrangement (3,4,5,6,7,8,9,10,11). At the level of restriction fragments sizes, many DNA rearrangements are tightly regulated and highly reproducible in clonal cell lines. Other DNA rearrangements are variable. That is, a mic sequence may be rearranged in more than one way. Such variability has been reported in studies of rearranged sequences in caryonides (the four cells from a mating pair) (12,13).

DNA rearrangement occurs between 12-16 hours after the initiation of conjugation, when the developing macronuclear anlagen is between 4C and 8C (13,14). Since there are four copies of each DNA sequence at this time, there is potential for variability in the processes of elimination and rearrangement within an individual macronuclear anlagen. Thus, a newly developed mac may contain more than one form of the same sequence as a result of variable rearrangement. However, following vegetative cell division, clonal cell lines contain only one form. It has been suggested that this process is the physical basis of the genetic phenomenon of phenotypic assortment (12).

Most of the DNA which is eliminated in Tetrahymena is moderately repetitive. The DNA which is retained in the mac is, for the most part, single copy DNA. The only DNA families that have been reported in the macronucleus are the 5S RNA genes (15) the tRNA genes (16), the heat shock genes and their cognates (Findly, personal communication) and the pC6 conjugation-induced gene family (17). This is not the case in another ciliate, Oxytricha fallax, where there is a high frequency of DNA families in the mac genome (18). We have identified a small family of DNA sequences which is rearranged during mac development in Tetrahymena. The inheritance, relatedness and reproducible rearrangement of the family have been studied.

MATERIALS AND METHODS

T. thermophila strain B1868 (VII), the mic defective strain A*, strain CU399 [ChxA2/ChxA2 (cycloheximide sensitive, VI)] a functional heterokaryon, and nullisomic strains were kindly provided by P. Bruns.

All cells were grown at 29°C with swirling at 90 rpm in 2% PPYS (2.0% proteose peptone (Difco), 0.1% yeast extract, and 0.003% sequestrine (Ciba-Geigy)) prepared as described by Gorovsky et al. (19). Stocks were maintained in 1% PPYS as described by Karrer (20).

Genomic exclusion (21) of CU399 and A* was performed as described by Karrer et al. (22).

Nuclear DNA was isolated as described by Howard and Blackburn (12) with the following modifications. Cells were grown in 2-500 ml of PPYS at 29°C in Fernbach flasks to a density of $2.5\text{-}5.0 \times 10^5$ cells/ml. Solution A contained 8% acacia gum and the filtration and CsCl gradient steps were omitted. After phenol:chloroform extraction and ethanol precipitation, the DNA pellet was extracted 5-6 times with ether. The dried pellet was dissolved in TE (10 mM Tris, 1 mM EDTA), pH 8.0, and precipitated with ethanol in the presence of 2.5M NH_4Ac. Mic DNA prepared in this way was routinely 80-85% free of mac DNA

and the yield was 50-100%. Mac DNA contained less than 1% mic DNA as judged by microscopic examination of the nuclei.

Anlagen DNA was prepared as described by Harrison et al. (23). The nuclei were stained with methyl green, and the proportion of anlagen was estimated by counting nuclei under both phase-contrast and bright-field optics. The percentage of anlagen DNA was estimated using values for DNA content of the nuclei as determined by Allis and Dennison (24). The 10, 12, 14 and 16 hour preparations contained 75%, 92%, 86% and 92% anlagen DNA, respectively.

Small scale plasmid isolations were performed using the boiling method essentially as described by Maniatis et al. (25). The alkali lysis procedure (26) as described by Maniatis et al. (25) was followed for large scale plasmid preparations and the DNA purified by centrifugation to equilibrium in CsCl-ethidium bromide density gradients (25).

Restriction enzymes were purchased from New England Biolabs or Boehringer-Mannheim and were used according to manufacturer's instructions.

Restriction fragments from recombinant clones were isolated from preparative agarose gels (Seakem ME) with NA45 DEAE membrane from Schleicher and Schuell membranes using the provided procedure. Hind III digested mac DNA was made 20 mM EDTA and 10% glycerol and separated on a preparative 0.8% agarose gel in 10 mM Tris-base, pH 7.8; 16 mM NaAc; and 1 mM EDTA. DNA of the desired size was electroeluted into a well cut from the gel containing 50% glycerol in running buffer. The DNA was then concentrated on a Schleicher and Schuell Elutip-d column according to the manufacturer's instructions. Southern hybridization of the fractionated DNA confirmed that the desired DNA fragments were present. This DNA was ligated to Hind III digested, alkaline phosphatase treated pBR322 (New England Biolabs) and used to transform E. coli strain HB101. The recombinant DNA was screened by replicating colonies onto nitrocellulose according to Procedure II of Maniatis et al. (25) and binding the liberated DNA by Procedure I of Maniatis et al. (25). Subcloning was carried out using the pUC 18 plasmid as the vector and JM 83 for the bacterial host (27). pUC 18 was a gift from D.T. Rogers.

DNA was blotted to BA85 nitrocellulose (Schleicher and Schuell) according to the method of Southern (28) except that a piece of Whatman 3MM paper saturated with 20X SSC (SSC is 0.15 M NaCl; 0.015 Na citrate. pH7.0) replaced the reservior of 20X SSC. Blotting was for 2.5-6 hours. Hybridizations were done using dextran-sulfate (29) in 7X SSC and 40% formamide at 33°C. Hybridized filters were washed as previously described

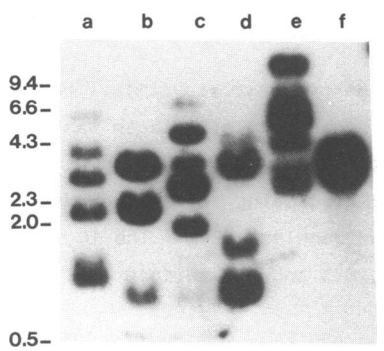

Figure 1. Mac restriction fragments with homology to the mic 1.4 kb Hind III fragment are rearranged. 4 ug of mic and mac DNA digested with Hind III (a & b), Eco RI (c & d) and Bgl II (e & f) were electrophoresesed on a 0.7% agarose gel, blotted and hybridized with nick-translated mic 1.4 kb clone. Lanes a, c, and e contain mic DNA, lanes b, d, and f mac DNA. The sizes (in kb) indicated at the left are from Hind III digested lambda DNA.

(20). Probes were labelled to a specific activity of 0.5-1x10^8 cpm/ug (30).

The dideoxy method as described by Sanger et al. (31) was used to sequence supercoiled DNA plasmids (32) using the direct and reverse M13 primers from Boehringer-Mannheim.

RESULTS

DNA rearrangement is a regular developmental event.

A cloned mic 1.4 kb Hind III fragment from a pBR322 plasmid recombinant DNA library of micronuclear DNA was used to probe Hind III digested mic and mac DNA (Fig. 1a and b). A small family of sequences was detected in both. The sizes of the mic fragments were 6.0, 4.0, 3.2, 2.2, 1.6, and 1.4 kb, the latter being the size of the cloned fragment. The mac fragment sizes were 3.5, 2.0 and 1.0 kb. The difference in the mic and mac patterns suggested that this family of sequences was rearranged during mac development.

This was confirmed using several different restriction enzymes. Mic and mac DNA digested with Eco RI, and Bgl II (Fig. 1, c-f) were hybridized with the mic 1.4 kb probe. In most cases the mobilities of the fragments differed between the two nuclear DNAs. It was also noted that there were 5 or 6 mic fragments in DNA digested with several enzymes and that the number of mic fragments was always greater than the number of mac fragments, raising the possibility that some of the mic copies were eliminated or that the mic family members were fused during mac development.

The developmental stage during which the rearrangements occurred could not be determined from the previous experiments because the DNAs were isolated from cells that had been grown vegetatively for many years. The rearrangements could have occurred during mac development or during subsequent vegetative propagation of the cells. To determine which of these

possibilities was correct, a mating was performed and the hybridization patterns obtained from DNA isolated from the newly developed mac of the progeny was examined.

A genomic exclusion mating (21) was done in order to create whole genome homozygotes which allows one to distinguish between allelic forms in the mic. A* is a mutant cell line with a hypoploid mic. When these cells are mated with micronucleate cells, the micronucleate partner transfers a haploid nucleus but the mic of the A* cells is degraded. The cells separate and each mic undergoes endoreduplication and becomes diploid (round I). The cells can pair again and successfully mate (round II). In this experiment CU399 cells were used as the micronucleate cells. CU399 cells are heterokaryons, the mic contains a gene which confers cycloheximide resistance and the mac has the wild type allele. Since the mac is responsible for the phenotype of the cell, the parental cells die in the presence of the drug. However, their progeny make a new mac from the mic containing the drug resistant gene, and can survive cycloheximide treatment.

CU399 cells were mixed with A* cells after starvation to induce pair formation. Round I pairs were isolated and put into PPYS to multiply. These cell populations were starved a second time and allowed to undergo round II mating at which time new macronuclei were formed. The cells were treated with cycloheximide to kill any cells which had not successfully mated. After twenty-five vegetative generations, mic and mac DNAs were isolated from six clonal lines derived from independent pairs of mating cells, and digested with Hind III. A Southern blot analysis of the DNA showed that the number and sizes of restriction fragments with homology to the family were identical in each cell line to the CU399 parent (Fig. 2a). Therefore, the DNA rearrangements occur as a regular developmental event during macronuclear development. (The apparent difference in the intensity of the macronuclear 1.0 kb fragments was due to differential loading of the DNA in the various lanes.) The identical pattern of micronuclear restriction fragments in the parents and progeny indicates that the six micronuclear fragments are non allelic and that these sequences are stable in the germline.

The three macronuclear fragments could have been the result of variable rearrangements of one of the micronuclear sequences in individual cells of the population as have been previously reported (10,12). In Tetrahymena, genes which are allelic in the macronucleus undergo a process known as phenotypic assortment (33) leaving only one form in the mac. Single cell clones from one of the mating pairs were isolated more than two years after the mating. The

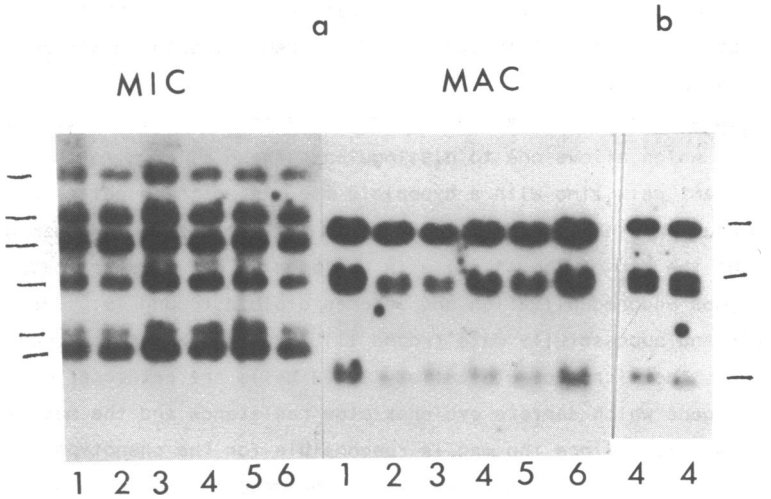

Figure 2. Reproducibly rearranged mac restriction fragments.
(a) Hind III digested mic and mac DNA were isolated from the progeny of six different genomic exclusion matings at twenty five vegetative generations after conjugation. The DNAs were electrophoresed on a 0.8% agarose gel, blotted and hybridized with nick-translated mic 1.4 kb clone. DNA in mic lane 1 and mac lane 1 were isolated from the same cells, etc. (b) Hind III digested mac DNA from two clonal cell lines generated from single cells of line 4 shown in (a). Mic fragments are indicated by lines on the left (6.0, 4.0, 3.2, 2.2, 1,6 and 1.4 kb), mac fragments are indicated on the right (3.5, 2.0, 1.0 kb).

hybridization pattern of mac DNA from these cells was the same as the parental cells (Fig. 2b). Thus, the same rearrangements occurred reproducibly in each cell and were maintained through phenotypic assortment, suggesting that they are non allelic in the mac.

In Figure 2b the 2.0 kb mac DNA fragment ran as a doublet in the DNAs from both clonal cell lines. Although the doublet has been observed in the DNA from a variety of cell lines, its basis is not understood. It was not seen in the DNA from strain CU399, the micronucleate parent of the cell lines in Figure 6a. Nor was it present in DNA from the progeny of any of the exconjugant pairs at twenty five fissions. It did, however appear in the DNAs of the two clonal cell lines derived from the pair 4 culture. This might suggest it was the result of a DNA modification or a somatic event which occurred late in some cell lines. On the other hand, the doublet was also found in anlagen DNA from cells at 16 hours of conjugation. Thus it can be generated during mac anlagen development, before the first cell division of the exconjugants. Since the doublet was also present in mac DNA from the

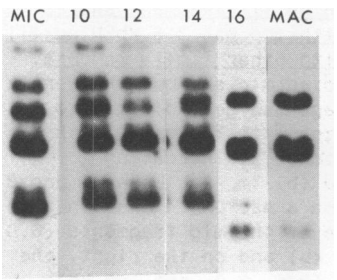

Figure 3. DNA rearrangement occurs during mac anlagen development.
4 ug of mac anlagen DNA isolated from cells at 10, 12, 14 and 16 hours after the initiation of conjugation, were digested with Hind III, separated on a 0.8% agarose gel and hybridized with nick-translated mic 1.4 kb clone. Mic and mac DNA were included for comparison. The 16 hour DNA was electrophoresed on a separate gel.

older cell line BVII (Figure 8f), the two forms do not undergo phenotypic assortment and thus are apparently nonallelic in the mac.

The DNA family is rearranged early in mac development.

Anlagen DNA was isolated from CU399 and CU401 mating cells at 10, 12, 14 and 16 hours of conjugation. These preparations contained 75%, 92%, 86% and 92% anlagen DNA respectively, the majority of the contamination being mac DNA. There was less than 1% mic DNA in these preparations. A Southern blot of these DNAs digested with Hind III, was probed with the mic 1.4 kb clone (Fig. 3). At 10 and 12 hours the hybridization pattern appeared to be the same as mic DNA. At 14 hours a band appeared just above the mic 3.2 kb fragment that comigrated with the macronuclear 3.5 kb fragment. Since no mac size fragments were visible in the 10 hour DNA preparation which contained 24% macronuclear DNA, the mac size fragment in the 14 hour DNA preparation which had only 13% mac DNA, can not be accounted for by mac DNA contamination. All three mac fragments were present by 16 hours. In an over-exposure of the 16 hour anlagen blot, faint bands were seen at 1.4 and 1.6 kb, the size of mic fragments. Thus, the rearrangements are initiated by 14 and are almost complete by 16 hours of conjugation.

Relationship among the family members.

Several approaches were taken to determine the degree of homology shared among the family members. A series of identical Hind III digested mic and mac DNA blots were made, hybridized with the 1.4 kb Hind III mic fragment and washed at increasing temperatures. Figure 4 shows that all of the fragments were still present at 10°C below the Tm of bulk Tetrahymena DNA, which means that these fragments share a region of homology with the probe which is >95% conserved (34). There was a preferential loss of the probe from all of the fragments except from the cloned fragment itself and from the 3.5 kb mac fragment at Tm -2°C. Therefore, it appeared that the probe shared more

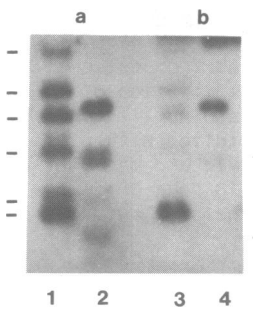

Figure 4. The mic 1.4 kb Hind III fragment shares more homology with the mac 3.5 kb Hind III fragment than with other family members. Aliquotes of Hind III digested mic and mac DNA were electrophoresed on a 0.7% agarose gel, blotted to nitrocellulose and hybridized with the mic 1.4 kb clone. The filters were washed at increasing stringency: (a) Tm -10 and (b) Tm -2. Lanes 1 and 3, contain mic DNA; lanes 2 and 4 mac DNA. The lines on the left indicate the sizes of the mic fragments (6.0, 4.0, 3.2, 2.2, 1.6 and 1.4 kb) and on the right, the sizes of the mac fragments (3,5. 2.0, and 1.0 kb).

homology with the largest mac fragment than with any of the other mic or mac fragments.

Genomic restriction mapping experiments were done to estimate the extent of homology between the mic 1.4 kb clone and the mac 3.5 kb Hind III fragment. A restriction map of the mic 1.4 kb clone (Fig. 5) shows an Eco RI site 0.2 kb from the left Hind III site. A blot of mac DNA digested with both Hind III and Eco RI showed bands of 3.3 and 0.2 kb, suggesting that the 3.5 kb Hind III mac fragment also had an Eco RI site 0.2 kb from one end (Fig. 6c & d). Since the 0.2 kb fragment was the only other fragment seen, the 2.0 and 1.0 kb mac Hind III fragments must also have been cleaved. It was postulated that both of these fragments also contained Eco RI sites 0.2 kb from one end and it must be only the region between the Hind III and Eco RI sites that shared homology

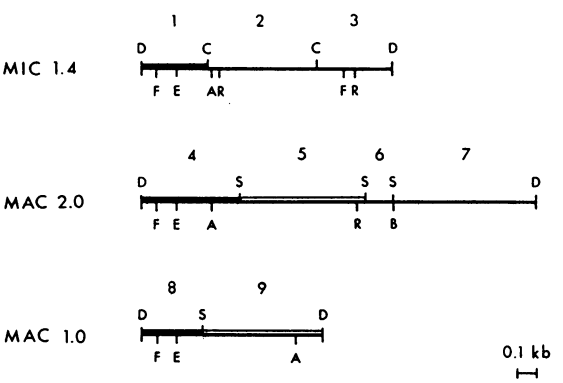

Figure 5. Restriction maps of clones.
Restriction maps of the mic 1.4, mac 2.0 and mac 1.0 kb clones are aligned at the Hind III, Hinf I and Eco RI sites. The numbers and letters above the maps indicate the subclone numbers and the restriction sites used for subcloning. D = Hind III, F = Hinf I, E = Eco RI, C = Hind II, A = Alu I, R = RSA I, S = Sau 3A I and B = Bgl II.

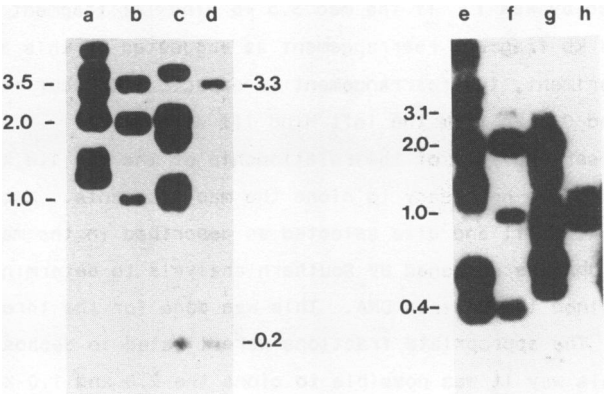

Figure 6. Genomic restriction mapping of mic and mac DNA indicates that mac the 3.5 kb Hind III fragment shares 0.4 - 0.5 kb of homology with the mic 1.4 kb fragment.

Mic and mac DNA were digested with Hind III (a & b), Hind III and Eco RII (c & d), Hind III and Hind II (e & f) and Hind III and RSA I (g & h) separated on a 0.7% agarose gels, blotted and hybridized with the mic 1.4 kb clone. The mic DNA lanes are a, c, e and g, the mac DNA lanes b, d, f and h. Sizes (kb) refer to fragments in lanes b, d and f.

with the probe. This was subsequently verified by restriction mapping and subclone cross-hybridization (next section). These results are consistent with the thermal stability experiment which indicated that the 3.5 kb Hind III mac fragment shared more homology with the mic 1.4 kb fragment than the other mac fragments. The homology of the mac 3.5 kb fragment extends beyond the conserved Eco RI site that is present in both the other mac fragments, as evidenced by the 3.3 kb Hind III-Eco RI band in this experiment. The 0.2 kb Hind III-Eco RI fragment contains the region of homology which defines the family.

The cloned 1.4 kb mic fragment has a Hind III site 0.4 kb and and RSA I site 0.5 from the left Hind III site (Fig. 5). In mac DNA digested with Hind III and Hind II, fragments were observed at 3.1, 2.0, 1.0 kb and 0.4 kb (Fig. 6B, lane f) suggesting that the 3.5 kb mac Hind III fragment had a Hind II site 0.4 kb from one end. Neither the mac 2.0 nor 1.0 kb Hind III fragment was cleaved with Hind II. In mac DNA digested with Hind III and RSA I, fragments were present at 1.2 and 1.0 kb (Fig. 6h). Since there was an 0.4 kb Hind III-Hind II fragment but no 0.5 kb Hind III-RSA I fragment in the digested mac DNA, the 3.5 kb Hind III mac band must share between 0.4 and 0.5 kb of homology with the mic 1.4 kb mic probe. The 2.0 kb mac fragment must

also be cleaved by RSA I. If the mac 3.5 kb Hind III fragment is the product of the mic 1.4 kb fragment rearrangement as suggested by this and the thermal stability experiment, the rearrangement is expected to occur in the region between 0.4 and 0.5 kb from the left Hind III site.

For a closer analysis of the relationship of the mic 1.4 kb probe to the mac fragments it was necessary to clone the mac fragments. Mac DNA was digested with Hind III and size selected as described in the materials and methods. The DNA was screened by Southern analysis to determine which eluted fraction contained the desired DNA. This was done for the three different size classes. The appropriate fractions were ligated to dephosphorylated pBR322. In this way it was possible to clone the 2.0 and 1.0 kb Hind III mac fragments. Repeated attempts to clone the mac 3.5 kb Hind III fragment were unsuccessful.

Cross-hybridization experiments using subclones of the cloned mic and mac fragments were performed to determine the extent of homology more exactly. Restriction maps of the mac 2.0 kb and 1.0 kb clones are aligned with the restriction map of the mic clone in Fig. 5. Both cloned mac DNAs have an Eco RI site 0.2 kb from the Hind III sites, as suggested from the Southern analysis of genomic DNA (Fig. 6d). All three fragments were subcloned as indicated (Fig. 5). Three identical filters were made of the various subcloned DNAs digested with the restriction enzymes necessary to release the inserts. One filter was hybridized with the entire mic 1.4 kb clone, one with the mac 2.0 clone and one with the mac 1.0 kb clone. Similar filters were hybridized with subclones 2, 5 and 9. Results from this cross-hybridization experiment (Table 1) showed that all three Hind III fragments shared a sequence block of homology which extends slightly beyond the Eco RI site. An interesting finding was that the both subclones of the mac 1.0 clone shared homology with the first 1.2 kb of the mac 2.0 kb clone though from a comparision of their restriction maps it can be seen that the two are not identical. The subclones which share extensive homology are indicated in Figure 5.

In order to further characterize the rearrangement, we did experiments to test whether the cloned mac fragments were uniquely related to any of the mic Hind III fragments. Filters of Hind III digested mic and mac DNA were hybridized with each of the subclones (summarized in Table 2). Mic subclone 1 is an 0.4 kb Hind III-Hind II fragment which contains the 0.2 kb sequence that we have shown shares homology with the mac fragments. Its hybridization pattern was the same as with the entire mic 1.4 kb clone except for the

Table 1. Homology shared between the cloned micronuclear and macronuclear fragments and the subclones

	Subclones								
Probe	mic 1.4			mac 2.0				mac 1.0	
	1	2	3	4	5	6	7	8	9
mic 1.4	+	+	+	+	−	−	−	+	−
mac 2.0	+	+/−	−	+	+	+	+	+	+
mac 1.0	+	+/−	−	+	+	−	−	+	+
subclone 2								−	+/−
subclone 5	−	−	−	−	+	−	−	−	+
subclone 9	−	+/−	−	−	+	−	−	−	+

Table 2. Homology of subclones to micronuclear and macronuclear family members

	Subclones								
Hind III family members	mic 1.4			mac 2.0				mac 1.0	
	1	2	3	4	5	6	7	8	9
MIC 6.0	+	+	−	+	+	+	+	+	+
4.0	+	+	+	+	+	+	+	+	+
3.2	+	+	+	+	+	+	+	+	+
2.2	+	+	+	+	+	+	+	+	+
1.6	−	+	+	+	−	−	−	−	−
1.4	+	+	+	+	−	−	−	+	−
MAC 3.5	+	+/−	−	+	+	+	+	+	+
2.0	+	−	−	+	+	+	+	+	+
1.0	+	−	−	+	+	−	−	+	+

absence of the 1.6 kb fragment. Since this subclone contains the 0.2 kb Hind III-Eco RI fragment that defines the family, it was considered likely that all of the mic fragments except the 1.6 kb fragment have homology to the common sequence block and are, by definition, the five sequences which constitute the mic family. This was confirmed by hybridization of mic DNA digested with HindIII with the 0.2 kb HindIII/EcoRI fragment (data not shown).

Subclone 2, which contains the adjacent 0.6 kb Hind II sequence hybridized to all of the micronuclear fragments and faintly to the 3.5 kb Hind III fragment in mac DNA. Since we know from the genomic Southern (Fig. 6) that the mic 1.4 clone shares less than 0.10 kb of homology beyond the Hind II site, this clone must contain about 0.50 kb of mic specific sequence. The last 0.4 kb Hind II-Hind III fragment, subclone 3, is also a mic specific sequence; it hybridized to all of the micronuclear fragments with the exception of the 6.0 kb but to none of the macronuclear fragments. Thus, approximately 0.9 kb of the mic 1.4 kb fragment was eliminated during the rearrangement event.

When the mac 2.0 clone was used to probe Hind III digested mic and mac DNA, a pattern similar to that seen with the mic 1.4 clone was observed. There were slight differences in the intensity of hybridization, but all the same size mic fragments appeared as well as a new band at 1.0 kb. Since subclone 4 contains the region of homology which defines the family, its hybridization to all of the family members was expected. The pattern of hybridization obtained with subclones 5, 6 and 7 were different from the whole mac 2.0 kb clones as expected from the subclone cross-hybridization experiment. None of them hybridized to the cloned 1.4 kb mic fragment but they did hybridize to the four largest mic fragments. Subclone 7 also hybridized to the new 1.0 kb mic fragment seen with the whole mac 2.0 kb clone. Subclone 5 hybridized to all of the mac fragments and subclones 6 and 7 to the 3.5 and 2.0 kb mac fragments as predicted from the cross hybridization of the subclones. The 1.0 kb mac band seen with subclone 7 can not be the cloned fragment since the cloned 1.0 kb mac fragment does not hybridize to it (Table 1). This new 1.0 kb fragment must migrate to the same position as the cloned 1.0 kb fragment in the filter probed with 2.0 kb mac fragment. This sequence may not be rearranged, since there is also a 1.0 kb fragment in the mic DNA hybridized with both the mac 2.0 kb fragment and subclone 7.

The mac 1.0 clone hybridized to all of the same size mic and mac fragments as the mic 1.4 clone. This was expected since it contains the 0.2

kb region which defines the family. It also hybridized to additional mic fragments of 2.5 and 1.0 kb. Hybridization with subclones 8 and 9 looked much like that with mac 1.0, again varying in the intensity of a particular fragment. Subclone 9 did not hybridize to the mic 4.0 kb fragment. There was a relatively intense band at about 1.4 kb in the mic DNA probed with the mac 1.0 clone and subclone 9. Since in the subclone experiment 2 and 9 cross hybridize only weakly (Table 1), subclone 9 must detect a new mic fragment of about 1.4 kb.

The conclusions which can be drawn from these experiments are: 1) some portion of the 0.2 kb Hind III-Eco RI fragment that was present in all of the mac fragments, was also present in all except the 1.6 kb mic fragments seen with the mic 1.4 clone; 2) the mac 2.0 and 1.0 clones contain sequences which were common to several mic family members that were not shared with the cloned mic 1.4 kb fragment; 3) all of the mac 2.0 subclones hybridized to the mac 3.5 Hind III fragment. Therefore, a similar relationship may exist between the mac 3.5 and mac 2.0 as does between the mac 2.0 and 1.0 fragments where both of the subclones of the 1.0 kb fragment share homology with the mac 2.0 kb fragment (Table 1). 4) There were no mac fragment subclones which were uniquely related to any of the mic family members.

The common region of the mic and mac clones were sequenced to determine if the mac members of the family were the rearranged products of one progenitor sequence through alternate processing or the products different progenitor sequences. If the two cloned mac fragments are not identical, then we would predict that they were the products of different mic precursor sequences. Mic 1.4 subclone 1, mac 2.0 subclone 4 and mac 1.0 subclone 8 were further subcloned by digesting with Hind III and Eco RI to isolate the common sequence block and sequenced by the dideoxy method (31). Each clone was sequenced three times and some in both directions. The sequences are compared in Figure 7. The results of this analysis showed that all three differed from each other in sequence by 3-4%, confirming the results of the thermal stability experiment. All of the sequence differences in this region were single base substitutions. Since both mac clones differed from the cloned mic sequence we concluded that this mic fragment was not the precursor of either cloned mac fragment. And since the mac sequences differ from each other as well, we believe that they were derived from two different mic precursors. A very striking finding was a stretch of 122 bp (bases 110-232) that was perfectly conserved in all three cloned fragments. We also sequenced approximately 200 bp adjacent to each of the common sequence blocks. The

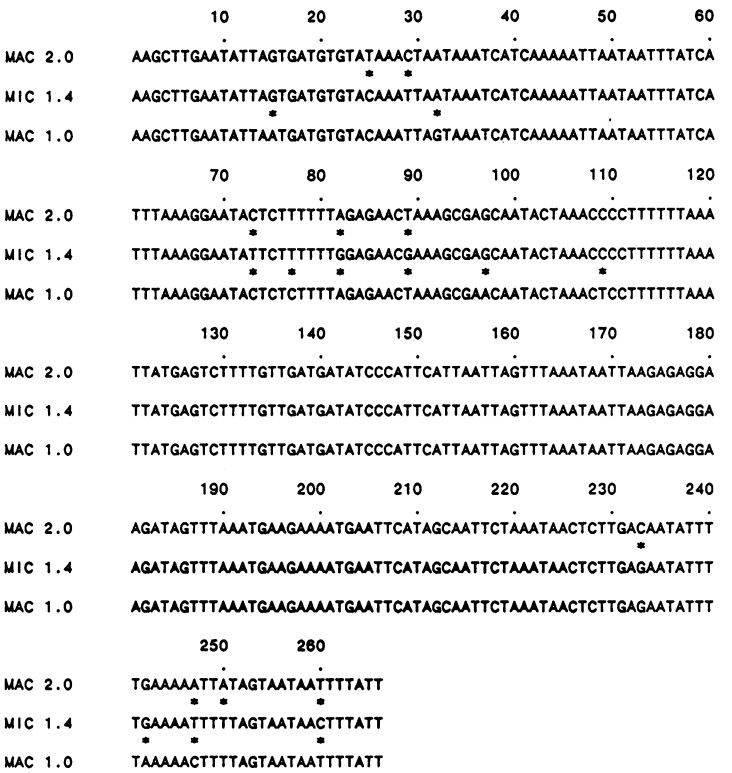

Figure 7. The conserved regions of the mic 1.4, mac 2.0 and mac 1.0 clones are 97% homologous.

The DNA sequences of starting from the Hind III site to just beyond the Eco RI site was determined for each of the clones using the dideoxy sequencing procedure. The * between the lines indicate the bases which differ. The region from base 110-232 is perfectly conserved. Note the Eco RI site (GAATTC) at bases 203-208.

first 57 bp were also highly conserved. The sequence diversity then increased to 34-46%, with the two mac fragments being slightly more homologous to each other than they were to the mic fragment. All of the sequences were >80% A/T suggesting that these regions do not code for protein since the A/T content of five protein-coding Tetrahymena genes which have been sequenced was 51-57% (35).

Chromosomal location of the micronuclear family members.

Nullisomic T. thermophila strains which lack one or more pairs of chromosomes in their mic have been constructed by P. Bruns and his colleagues (36). These cell lines are viable because the transcription which occurs

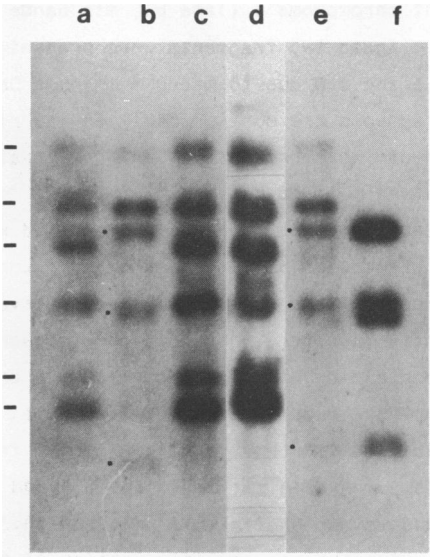

Figure 8. The two largest members of the mic family are on chromosome 1 and the other four are on chromosome 5.

Mic DNA isolated from nullisomic strains of Tetrahymena were digested with Hind III, electrophoresed on a 0.8% agarose gel, blotted and hybridized with nick-translated mic 1.4 clone. Lane (a) contains DNA from strain CU399, (b) CU414, nullI 2,3,4,5, (c) CU361, nullI 3, (d) CU357, nullI 4, (e) X019-2, nullI 3L,4,5 and (f) mac DNA. CU414 (lane b) and X019-2 (lane e) DNAs have bands the size of mac fragments as a result of mac DNA contamination ( ). The lines to the left indicate the mic fragments.

during the vegetative stage of the life cycle takes place in the mac, which is intact. Using a combination of nullisomic strains, it is possible to assign genes or DNA fragments to specific chromosomes (36). Mic DNA was isolated from strains CU414 (nullisomic for chromosomes 2, 3, 4, 5), CU361 (nullisomic for chromosome 3), CU357 (nullisomic for chromosome 4) and X019-2 (nullisomic for chromosomes 3L, 4, 5) by the same procedure used for wild type cells.

There is a greater problem of mac DNA contamination in mic DNA from nullisomic strains than in mic DNA from wild type cells. This is especially true in nullisomic strains missing three or four chromosomes, since the amount of DNA in the mic is reduced by the number of chromosomes missing. The mic DNA preparations from strains CU414 and X019-2 were contaminated with more than 33% mac DNA, the other preparations contained less than 15% mac DNA. A Southern blot of Hind III digested mic DNA from the nullisomics was hybridized with the mic 1.4 kb clone (Fig. 8). In the lane containing mic DNA from the

strain missing all but chromosome 1 (lane b), mic bands were seen at 6.0 and 4.0 kb suggesting that these two fragments were present on chromosome 1. Mac bands were seen at 3.5 and 2.0 due to mac DNA in this DNA preparation. The 6.0 and 4.0 kb mic fragments are most probably on the right arm of chromosome 1 since all the fragments were present in DNA from strain CU372, which is nullisomic for the left arm (data not shown).

It can be deduced that the 3.2, 2.2, 1.6 and 1.4 kb mic Hind III fragments all map to chromosome 5. In lanes c and d containing DNA from strains missing chromosome 3 and chromosome 4 respectively, all the mic fragments were present. Therefore, none of these sequences are on chromosomes 3 or 4. In lane e, containing DNA from a null 3L, 4 and 5 strain, there was hybridization to only the 6.0 and 4.0 kb mic fragments. Since chromosome 2 is present in this strain, the four smaller mic Hind III fragments can be assigned to chromosome 5. Hybridization to the 3.5 and 2.0 kb mac fragments in lane e was again due to mac DNA in this preparation. When CU414 and X019-2 DNAs were hybridized using the mic specific portion of the mic 1.4 clone (subclone 3,) the mac fragments are absent (data not shown). These results show that two of the mic fragments are unlinked to the other four.

DISCUSSION

It has been estimated that there are on the order of 5,000 sites in the *T. thermophila* genome which undergo rearrangement events during the development of the mac from the mic (10). In this study an isolated mic Hind III fragment hybridizes to a family of DNA sequences that is rearranged in the mac. The rearrangement event has begun by 14 hours after conjugation is initiated and is nearly completed by 16 hours. A small amount of the 1.4 and 1.6 kb mic Hind III fragments are still detectable at 16 hours. It was calculated that the DNA in this preparation was 92% anlagen DNA with the remainder being mostly mac DNA from unpaired cells. Thus, it seems unlikely that the mic fragments are from contaminating mic DNA. Fourteen to 16 hours was the time of rearrangement for another sequence (IIC7) studied in this laboratory using the same DNA preparations. The time of initiation of these rearrangements is in agreement with the study of the micronuclear specific X-H sequences by Brunk and Conover (14). Another sequence studied by Yao et al. (10) is rearranged between 12 and 14 hours of conjugation. The differences in the time of completion of the rearrangements in these studies probably reflect slight differences in the conditions of cell culture and conjugation between the laboratories.

In the Southern blots of Hind III digested mic and mac DNA, the number of family members was found to be reduced from five in the mic to three in the mac, indicating that three of the common block sequences are eliminated or fused. Some DNA rearrangements in ciliates have been characterized as deletions (10,37). It is possible that a similar mechanism is occurring in this DNA family. The mac 3.5 kb Hind III molecule, by genomic restriction mapping and thermal stability experiments, appears most likely to be the rearranged product of the mic 1.4 kb molecule. We have shown that 0.9 kb of the mic 1.4 kb fragment is eliminated from the mac. Since this deletion extends beyond the region covered by the 1.4 kb Hind III mic fragment, we can not detect the distal portion of the sequence which is retained in the mac.

Genomic Southern blot analysis indicated that an intricate series of relationships exists among this family of repeated sequences. Since five or six fragments are generated in mic DNA digested with several enzymes, it is likely that the family members are fairly far (>10 kb) apart from one another. Three of the five family members are on chromosome five and two are on chromosome one. If some of these sequences are clustered in pairs, each pair may be joined by a deletion event to form one contiguous molecule in the mac. Alternatively, some mic fragments may be eliminated entirely, and others rearranged to generate the mac members of the family. A third possibility is that since these sequences are on more than one chromosome, some of the rearrangements could result from interchromosomal recombination, though there is no precedent for this kind of rearrangement in the developing mac of Tetrahymena. Any of these events or a combination of them would explain a reduction in the number and a change in the size of fragments containing the common sequence block during mac development.

Although this is the first small family of rearranged mac sequences to be described in detail in Tetrahymena, such families are common in the hypotrichous ciliate, Oxytrich.. Cartinhour and Herrick (18) observed that one half of the mac gene-size pieces of DNA belong to sequence families. The family described in this study is similar to one discussed in the Q. fallax report in that both families contain a conserved sequence block which exists in several contexts.

Another similarity with the sequence family of Q. fallax is that at the level of restriction fragment size, the T. thermophila family is generated precisely every time a new mac develops. It is also stably inherited during vegetative growth. This is not the case for all of the rearranged sequences studied in T. thermophila. Austerberry et al. (13) and Howard Blackburn (12)

have shown that some rearranged sequences display alternate processing in different caryonides. One case has been observed where two different rearrangements are present in a clonal cell line, and only one or the other is maintained after a number of vegetative fissions as is expected for a sequence undergoing phenotypic assortment.

The manner in which the family is generated is also unknown. Cartinhour and Herrick (18) have suggested that in O. fallax the DNA family could be generated by alternate processing of a single micronuclear precursor. It is possible that a similar mechanism could generate the Tetrahymena family from a single micronuclear precursor and that other mic family members are eliminated. Alternatively, each of the mac family members we describe here could be the product of different mic precursors generated by one or a combination of mechanisms described previously.

Based on the data presented, the latter possibility is more, likely. Klobutcher et al. (37) have found that a 3-5% difference in sequence is indicative of different versions of family members in Oxytricha and that one cloned mac sequence was identical to its mic counterpart. The common sequence blocks of the two mac fragments we studied differ from each other and from the mic 1.4 kb fragment by 3-4%. Therefore, unless the rate of somatic mutation is much higher in Tetrahymena than in Oxytricha, our data suggests that neither mac fragment is the rearranged product of the mic 1.4 kb fragment and the mac fragments must each have different mic precursors. More conclusive evidence awaits the isolation and sequencing of other members of the mic family.

Note added in proof: The 3.5 kb Hind III fragment with homology to IE2 has been isolated from strain BI3840. The first 266 bp were sequenced by K. Thorne and are 100% homologous to the corresponding sequence in the 1.4 kb mic fragment.

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