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Germ Line-Specific DNA Sequences Are Present on All Five Micronuclear Chromosomes in *Tetrahymena thermophila*

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The development of the macronucleus from the zygotic micronucleus in the ciliated protozoan *Tetrahymena* spp. involves the elimination of specific DNA sequences (M. C. Yao and M. Gorovsky, *Chromosoma* **48**:1-18 1974). The present study demonstrates that micronucleus-specific DNA is present on all five of the micronuclear chromosomes. Fragments of micronuclear DNA from *Tetrahymena thermophila* were cloned in the plasmid vector pBR322. A procedure was developed to examine the organization of the cloned sequences in micro- and macronuclear DNA without nick translating each individual probe. Twenty-three percent of randomly selected DNA sequences examined by this method were micronucleus (germ line) specific. They were all members of families of repeated sequences. Hybridization of six micronucleus-specific DNA sequences to micronuclear DNA from nullisomic strains of *T. thermophila*, which are lacking one or more pairs of chromosomes in the micronucleus, suggested that these sequences are present on several chromosomes. One micronucleus-specific sequence was shown by in situ hybridization to be present on all five of the micronuclear chromosomes.

The ciliated protozoa of the genus *Tetrahymena* contain a diploid germ line micronucleus and a polyploid macronucleus, which is responsible for most, if not all, of the transcriptional activity in a vegetative cell. During sexual reproduction (conjugation), the macronucleus is destroyed and is replaced by a new macronucleus which develops from one of the mitotic products of the zygotic micronucleus. The development of the new macronucleus involves extensive genome reorganization, including polyploidization of the bulk of the genome to the level of 45c, amplification of the genes which code for 17S and 25S rRNA, and the possible fragmentation of the chromosomes into subchromosomal elements (for a review, see reference 12). Renaturation kinetics of micro- and macronuclear DNA suggest that 10 to 20% of the micronuclear DNA is eliminated during the formation of the macronucleus and that most of these sequences are repeated on the order of 100 times in the micronuclear genome (34).

Several micronucleus-specific (mic-specific) DNA sequences have been isolated on the basis of linkage in the micronuclear genome to the sequence C-C-C-C-A-A. This hexanucleotide occurs in blocks of tandem repeats which have very different arrangements in the micro- and macronuclear genomes (31). C₄A₂ repeats occur predominantly at the ends of DNA molecules in the macronucleus (6, 36). The C₄A₂ repeats

which have been isolated from micronuclear DNA thus far are all flanked by mic-specific DNA sequences. At least one single-copy mic-specific sequence and several families of repeated mic-specific sequences have been identified by this approach (6, 30).

Very little is known about the mechanisms whereby some DNA sequences are eliminated from the developing macronucleus and others are retained. One possible model would invoke underreplication and subsequent dilution of the mic-specific sequences in the macronuclei of dividing cells. However, Yokoyama and Yao (37) have presented evidence that mic-specific DNA is replicated a few times early in the formation of the macronuclear anlagen and then is eliminated, presumably by specific degradation, before any nuclear division occurs.

The localization of mic-specific DNA sequences on the five micronuclear chromosomes of *Tetrahymena* spp. places constraints on the mechanism of chromatin diminution in this organism. If all of the mic-specific DNA sequences, and only mic-specific DNA, are present on one of the five micronuclear chromosomes, that chromosome can be completely eliminated from the macronuclear genome. Alternatively, if mic-specific DNA sequences were present on several of the micronuclear chromosomes, then some fragmentation of the genome must occur during the development of the macronucleus to sepa-

rate DNA sequences which are eliminated from those which are retained.

Two kinds of evidence support the latter model. First, there is a single copy of the 17S and 25S RNA-coding sequences (rDNA) in the micronucleus of *Tetrahymena* spp. It is excised from the genome (32) and amplified several hundredfold in the macronucleus (35), where it is present in the form of 21-kilobase (kb) extrachromosomal inverted repeats (9, 10, 16). Excision of the rDNA from the micronuclear chromosome involves chromosome breakage with the elimination of 2.8 kb of flanking DNA and the generation of a free chromosome end in the macronucleus (29). Thus, one mic-specific sequence lies between the only DNA sequence which has been shown to be highly amplified in the macronuclear DNA and a sequence which will be converted to a telomere. Second, Yao (30) hybridized a mic-specific sequence to micronuclear DNA from a strain which lacks three of the five micronuclear chromosomes. The probe hybridized to a subset of the bands which appear in the micronuclear DNA from a wild-type cell, suggesting that some, but not all, copies of that sequence were present on one or more of the chromosomes 2, 3, and 5.

The work described here extended that observation by a systematic analysis of six different mic-specific sequences selected at random. They were hybridized to micronuclear DNA from seven nullisomic strains of *Tetrahymena thermophila* which, taken together, cover the entire genome. The results indicated that none of these sequences, which are each repeated in the genome, was located exclusively on any one chromosome. These results were confirmed by in situ hybridization, which showed that at least one of these sequences is present on all five chromosomes.

MATERIALS AND METHODS

Cell culture. For DNA extractions, *T. thermophila* wild-type strain BVII and nullisomic strains CU371 (nulli 1L, 2R), CU373 (nulli 1R), CU359 (nulli 2, 3, 5), CU361 (nulli 3), CU358 (nulli 3, 4, 5), CU357 (nulli 4), and CU359 (nulli 5) were grown in 2% protease peptone-0.1% yeast extract-0.003% sequestrine (PPYS), prepared as described by Gorovsky et al. (14). Cultures in 500 ml of medium were maintained at 29°C in 11 diphtheria toxin bottles and were aerated with a gas dispersion tube. For mating, strains CU399:Chx/Chx (cy-s, VI) and CU401:Mpr/Mpr (6mp-s, VII) were grown in 500-ml Erlenmeyer flasks containing 100 ml of PPYS in a shaking incubator at 125 rpm, 29°C.

Subcellular fractionation. Micronuclei were separated from macronuclei by the procedure of Gorovsky et al. (14). Mitochondria were isolated by the method of Schwab-Stey et al. (27).

DNA isolation. Macronuclear DNA was extracted from the first macronuclear pellet prepared by the

procedure of Gorovsky et al. (14). The pelleted nuclei were lysed in 1% sodium dodecyl sulfate (SDS) in 0.1× SSC (standard saline citrate; 1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0). The lysate was incubated for 2 to 4 h at 37°C in 100 µg of pronase per ml and was phenol extracted, and nucleic acids were precipitated from the aqueous layer by adding 2 volumes of cold 95% ethanol. The precipitate was redissolved in 2× SSC and was digested with 100 µg of pancreatic RNase A per ml-40 U of T1 RNase per ml at 37°C for 1 h. Pronase was added to a concentration of 100 µg/ml, and digestion was continued for an additional hour. The solution was phenol extracted, and the DNA was precipitated from ethanol. DNA was isolated from mitochondria by the same procedure used for macronuclear DNA.

DNA was isolated from micronuclei by a simplified procedure in which the pelleted micronuclei were lysed in 1% SDS in 0.1× SSC. The lysate was brought to 100 µg of pronase per ml, incubated at 37°C for 1 h, and phenol extracted. The DNA was precipitated by the addition of NaCl to 0.1 M and 2 volumes of cold 95% ethanol.

Construction of the plasmid bank and isolation of plasmid DNA. DNA from a preparation of micronuclei containing less than 0.3% contaminating macronuclei (6% macronuclear DNA) was digested with the restriction enzyme *Hind*III. The fragments were ligated to the plasmid vector pBR322, which had been digested with *Hind*III and treated with calf intestine alkaline phosphatase. Transformants resistant to 20 µg of ampicillin per ml and susceptible to 20 µg of tetracycline per ml were stored in microtiter plates as described by Gergen et al. (11).

Plasmid DNA was prepared by the method of Meagher et al. (20), except that the ether extraction was omitted. Precipitated DNA was redissolved in 270 µl of 5 mM Tris-0.25 mM EDTA (pH 7.9) and transferred to a microfuge tube. Thirty microliters of 0.5 M magnesium acetate-2.5 M lithium acetate was added, and the DNA was precipitated a second time by the addition of 3 volumes of 95% ethanol. Precipitated DNA was stored at -20°C.

Filter hybridization. DNA was digested with the restriction endonuclease *Hind*III (Bethesda Research Laboratories) under the conditions recommended by the supplier. DNA fragments were separated by electrophoresis through 0.7% agarose gels in 40 mM Tris base-20 mM sodium acetate-2 mM disodium EDTA-18 mM NaCl (pH 8.0) buffer for 390V · h. The DNA was transferred to nitrocellulose filters by the method of Southern (28) and was probed with DNA nick translated as described by Maniatis et al. (17).

Preliminary screening of the plasmid bank for repeated mic-specific DNA sequences was carried out by a two-step sandwich hybridization procedure. Unlabeled recombinant plasmid DNA was linearized by digestion with the restriction enzyme *Pst*I, heat denatured, and prehybridized to genomic DNA immobilized on a nitrocellulose filter in 50% formamide-5× SSC-25 mM sodium phosphate (pH 6.8)-1× Denhardt solution-200 g of sonicated, alkali-denatured salmon sperm DNA per ml for 8 to 18 h at 33°C. Excess plasmid DNA was removed by rinsing the filter in the bag with 5 ml of hybridization solution without plasmid DNA. The filters were subsequently hybridized with radioactive plasmid pBR322 under the same

conditions used for the first hybridization, except that incubation was at 42°C. The probe hybridizes to the vector portion of the recombinant plasmid DNA, which is in turn hybridized to the homologous DNA fragment on the filter. This procedure obviates the necessity of separately nick translating each cloned fragment.

After hybridization, the filters were washed four times for at least 30 min each at increasing stringency: first wash, 5× SSC, 1× Denhardt solution, and 0.1% SDS at 42°C; second wash, 6× SSC and 0.1% SDS at 55°C; third wash, 2× SSC and 0.1% SDS at 55°C; fourth wash, 2× SSC at 55°C. Putative mic-specific DNA clones were tested for homology to nick-translated mitochondrial DNA by hybridization under the same conditions as described above, at 33°C.

Cytology. Matings were done by the method of Bruns and Brussard (7). Strains CU399 (mating type VI) and CU401 (mating type VII) were grown to a density of 2.5×10^5 cells per ml in PPYS. The cells were pelleted by centrifugation at $100 \times g$ for 1.5 min, washed in 10 mM Tris (pH 7.4), gently resuspended in the same solution at a density of 10^5 cells per ml, and starved separately for 23 h. The cells were counted, and equal numbers were mixed together, diluted to 8×10^4 cells per ml, and incubated at 29°C without shaking to permit pairing. At 1.5 h after mixing, 77% of the cells were in pairs. Pairing reached 91% by 4.5 h, at which time the cells were fixed.

Mating cells were concentrated from 100 to 0.75 ml by centrifugation at $100 \times g$ for 1.5 min and were fixed for 10 min at room temperature by adding the concentrated cell suspension to 9 ml of fresh Schaudinn fixative (2 parts of saturated HgCl₂, 1 part of absolute ethanol, 1.5% glacial acetic acid). The cells were washed three times for 5 min in 70% ethanol. Fixed cells can be stored in 70% ethanol at 4°C for several days. Longer storage decreases cell spreading and chromosome dispersion when the slides are prepared.

Cells were postfixed by transferring 10 drops of the cell suspension in a pipette into 4 ml of fresh absolute ethanol-glacial acetic acid (3:1). After 5 min to 1 h, a drop of the cell suspension was allowed to fall from a height of 30 cm onto a clean microscope slide and was air dried.

Slides were treated before hybridization essentially by the method of Yokoyama and Yao (37). They were washed for 10 min each in 70% ethanol, and in two washes of 0.01% I₂ in 70% ethanol (to remove excess HgCl₂), dehydrated in 70, 95, and 100% ethanol, and air dried. The slides were then incubated for 30 min at 70°C in 2× SSC (3) and rinsed three times for 10 min in 0.1 M triethanolamine (pH 8.0). Acetic anhydride was added to the third triethanolamine rinse, with stirring (15), and the slides were dehydrated in ethanol as above. Immediately before hybridization, the slides were immersed in 0.07 N NaOH–0.3 M NaCl for 2.5 min to denature the DNA and then were dehydrated by passage through ethanol.

Hybridizations were done in dextran sulfate by the method of Yokoyama and Yao (37) except that salmon sperm DNA at a final concentration of 200 µg/ml was the carrier DNA. Plasmid DNA to be used as a probe and pBR322 DNA to be used as a control were nick translated with [³H]dTTP (97 Ci/mmol) to specific activities of 6.5×10^6 and 5×10^6 dpm/µg, respectively. Hybridization solution (68 µl) was placed on a slide

and covered with a cover slip (22 by 50 mm), and the preparations were incubated in a moist chamber at 37°C for 16 h. Cover slips were removed by dipping the slides in 2× SSC. Slides were rinsed twice for 20 min in 2× SSC at 25°C, three times for 20 min in 3× SSC at 55°C, and once for 10 min in 2× SSC at 25°C and were dehydrated in ethanol. They were coated with NTB-2 emulsion, exposed for 10 to 30 days at 4°C, developed, and stained with Giemsa stain by the method of Pardue and Gall (24).

RESULTS

Fragments with homology to a cloned DNA fragment can be detected in restricted genomic DNA by sandwich hybridization. To obtain a source of DNA fragments which was representative of the sequences in the micronuclear genome, micronuclear DNA was digested with the restriction enzyme *Hind*III and cloned into the *Hind*III site of the plasmid vector pBR322. Recombinant plasmids were taken at random and screened for the presence of mic-specific DNA sequences.

Preliminary screening of the plasmid bank was done by a new method, which is described diagrammatically in Fig. 1. *Tetrahymena* nuclear DNA was digested with *Hind*III. The fragments were separated on a 0.7% agarose gel and transferred to a nitrocellulose filter by the method of Southern (28; Fig. 1a). The plasmid we used was pTtC6, which contained a 3.5-kb fragment of *Tetrahymena* DNA inserted at the *Hind*III site of pBR322. Digestion of the plasmid DNA showed that the insert did not contain any sites recognized by the restriction enzyme *Pst*I. The plasmid DNA was linearized by digestion with *Pst*I, which recognizes a single site in pBR322. The restricted plasmid DNA was denatured and hybridized to the homologous *Tetrahymena* DNA on the filter (Fig. 1b). Nick-translated pBR322 was subsequently hybridized to the vector portion of the plasmid (Fig. 1c) and detected by autoradiography.

Figure 2 shows that the presence of unlabeled plasmid DNA, with vector covalently linked to the cloned fragment, was required to obtain a signal by sandwich hybridization. Each of the three filters shown in Fig. 2 had bound to it DNA transferred from two lanes of an agarose gel, each containing 2 µg of *Tetrahymena* macronuclear DNA digested with *Hind*III. In every case, plasmid pBR322 restricted with *Hind*III was included in the DNA sample on the left as a positive control for hybridization. The filters differed in the presence or treatment of the plasmid DNA in the middle of the sandwich. They were all probed with radioactive pBR322 DNA.

The left lane of every pair had a band at 4.3 kb which resulted from hybridization of the nick-translated plasmid pBR322 to pBR322 DNA on

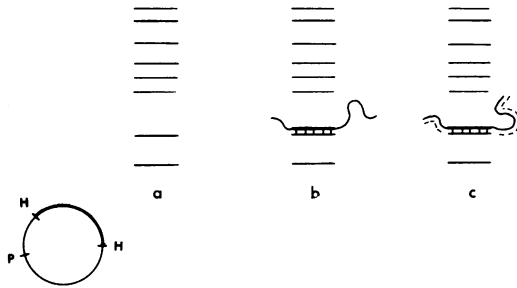


FIG. 1. Diagram of sandwich hybridization. The circle represents a fragment of *Tetrahymena* genomic DNA (heavy line) inserted at the *Hind*III site of plasmid pBR322 (light line). P indicates the presence of a *Pst*I site 779 base pairs from the *Hind*III site. (a) Restriction fragments of *Tetrahymena* genomic DNA immobilized on a filter; (b) insert portion of the plasmid hybridized to the homologous DNA on the filter; (c) nick-translated vector DNA hybridized to the vector portion of the plasmid.

the filter. Filter a in Fig. 2 was prehybridized in the absence of unlabeled plasmid DNA. The right lane of this filter shows that pBR322 has no detectable homology with *Tetrahymena* DNA under these conditions of hybridization.

The filters in Fig. 2b and c were prehybridized in the presence of unlabeled plasmid DNA consisting of the 3.5-kb fragment of *Tetrahymena* DNA cloned in pBR322. Plasmid DNA used to prehybridize filter b was linearized by digestion with the restriction enzyme *Pst*I. Prehybridization with unlabeled plasmid DNA permits sandwich hybridization of the pBR322 DNA to the *Tetrahymena* DNA on the filter and produces a band at 3.5 kb.

The critical control for this experiment is shown for filter c. This filter was prehybridized with the sample of plasmid DNA which had been digested with both *Pst*I and *Hind*III. Digestion with *Hind*III separates the vector portion of the plasmid from the insert. Thus, although the *Tetrahymena* DNA insert is presumably hybridized to the homologous DNA on the filter, the vector tails have been removed, preventing sandwich hybridization. The lack of a band at 3.5 kb in filter c strongly indicates that this band was a result of sandwich hybridization in filter b.

Preliminary characterization of a partial plasmid bank of *Tetrahymena* micronuclear DNA. The method of cloning we chose was expected to yield an assortment of plasmids which were a random sample of the sequences in the micronuclear (germ line) genome. To date we have analyzed 68 clones by the sandwich hybridization procedure. Since we have not yet been able to achieve reproducible single-copy sensitivity with this method, it was most useful for the detection of sequences which were repeated in the genome.

One of the 68 plasmids contained a sequence which was repeated in both micronuclear and macronuclear DNA. We have tentatively identified 16 clones (23% of the sample) as mic-specific. Each of the 16 was homologous to a different family of mic-specific DNA sequences which were repeated in the micronuclear genome as shown by the intensity of hybridization and by homology of the cloned DNA fragment to several fragments in restricted micronuclear DNA. An example of a cloned fragment which was shown by sandwich hybridization to belong to a family of mic-specific sequences is presented in Fig. 3. Two single-copy mic-specific sequences have been isolated by subcloning plasmids which contained repeated mic-specific elements.

Of the 51 cloned fragments which gave no signal in sandwich hybridization, 8 were rescreened by standard methods. Seven fragments hybridized to single bands of the same size in micro- and macronuclear DNA. One sequence hybridized to six *Hind*III fragments in micronuclear DNA which were rearranged to three different *Hind*III fragments in the macronuclear DNA. Rescreening did not reveal any additional single-copy mic-specific sequences.

The mic-specific sequences we have isolated are not homologous to mitochondrial DNA. To obtain

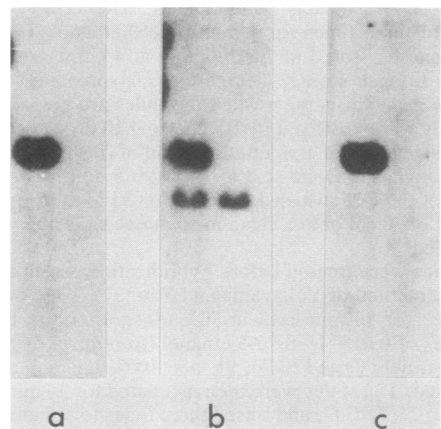


FIG. 2. Sandwich hybridization. Three identical filters with 2 μ g of *Hind*III-digested *Tetrahymena* DNA in each lane, transferred from an agarose gel by the method of Southern (28). The left lane on each filter also contains *Hind*III-digested plasmid pBR322 DNA as an internal control. Filter a was prehybridized in the absence of plasmid DNA. Filters b and c were prehybridized with DNA from the plasmid pTtIC6, a 3.5-kb fragment of *Tetrahymena* DNA inserted at the *Hind*III site of pBR322. In both cases, the plasmid DNA was digested with *Pst*I. The plasmid DNA used to prehybridize filter c was digested with *Hind*III in addition. Each filter was hybridized with 1.2×10^7 cpm of nick-translated pBR322 DNA.

micronuclear DNA for cloning, micro- and macronuclei were separated by differential centrifugation by the procedure of Gorovsky et al. (14). Since isolated mitochondria of *Tetrahymena pyriformis* have been reported to be nearly as large as the micronucleus (27), we were concerned that mitochondria might have copurified with the micronuclei and that some of the mic-specific DNA sequences we had identified might therefore actually be mitochondrial in origin.

To eliminate this possibility, we prepared mitochondria by the procedure of Schwab-Stey et al. (27) and isolated the DNA. The DNA was identified as mitochondrial by comparing its restriction map with the published maps for the restriction enzymes *EcoRI* and *HindIII* (23). Clones of putative mic-specific DNA were digested with *HindIII*. The DNA was electrophoresed on an agarose gel and transferred to a nitrocellulose filter with restricted mitochondrial

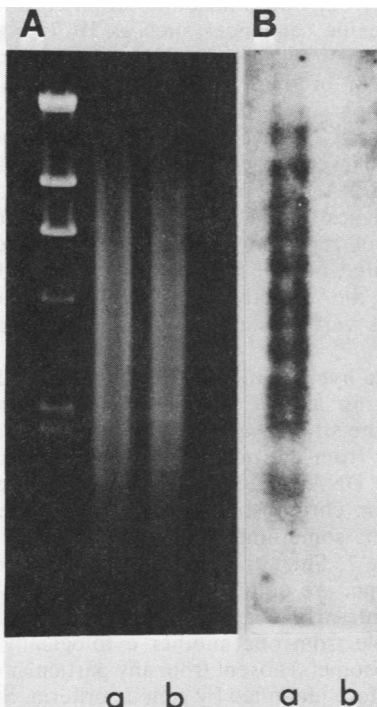


FIG. 3. *HindIII* digests of equal amounts of *Tetrahymena* micro- and macronuclear DNA. The fragments were separated by electrophoresis on an agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized by the sandwich procedure to nick-translated plasmid pBR322 in the presence of the pBR322 plasmid carrying the *Tetrahymena* DNA insert IC1. (A) Ethidium bromide-stained gel of *HindIII*-digested λ phage DNA, micronuclear DNA (a), and macronuclear DNA (b). (B) Autoradiogram of the filter after hybridization. There is no hybridization of the mic-specific sequence IC1 to macronuclear DNA.

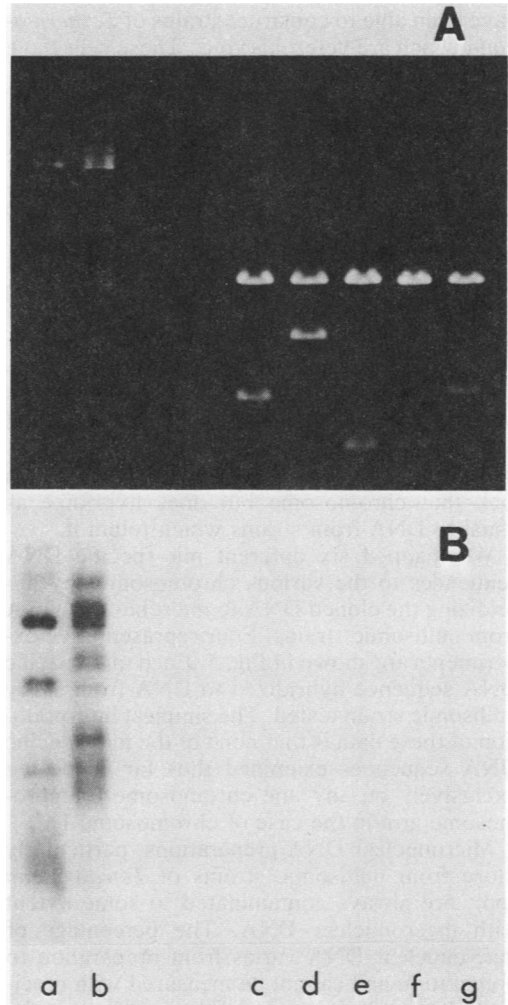


FIG. 4. Mitochondrial DNA (lane a) and several plasmids containing mic-specific *Tetrahymena* DNA sequences (lanes c through g) digested with *HindIII* and run on an agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized with nick-translated mitochondrial DNA. (A) Ethidium bromide-stained gel. The upper band in lanes c through g is pBR322, and the lower band is the *Tetrahymena* DNA insert (c, IIA8; d, IIB4; e, IC1; f, ID10; g, IIC7). (B) Autoradiogram of the filter after hybridization with mitochondrial DNA. There is no homology with putative mic-specific DNA sequences, indicating they are not mitochondrial in origin. Lane b contains mitochondrial DNA partially digested with *EcoRI*.

DNA as a positive control. Nick-translated mitochondrial DNA did not hybridize to any of the mic-specific DNA sequences tested (Fig. 4).

Mic-specific DNA sequences are present on more than one chromosome. Certain features of the genetics of *T. thermophila* allow one to map any particular DNA sequence to one or more of the five micronuclear chromosomes by Southern blot analysis. Peter Bruns and his colleagues

have been able to construct strains of *T. thermophila* which are heterokaryons. These cells have the normal complement of DNA in the macronucleus but are lacking one or more pairs of chromosomes in the micronucleus (8). Since the macronucleus is the site of most, if not all, of the transcriptional activity in a vegetative cell (13, 19), the cells are viable as long as they are maintained in the vegetative state.

The availability of these cell lines provides an opportunity for mapping DNA sequences to the five *Tetrahymena* chromosomes. In this method, a particular DNA sequence is hybridized to micronuclear DNA from various strains which are lacking different combinations of chromosomes. Any DNA sequence which is derived from a particular chromosome does not hybridize to micronuclear DNA from strains which lack that chromosome but does hybridize as usual to DNA from strains which retain it.

We mapped six different mic-specific DNA sequences to the various chromosomes by hybridizing the cloned DNA to micronuclear DNA from nullisomic strains. Four representative experiments are shown in Fig. 5. Each mic-specific DNA sequence hybridized to DNA from every nullisomic strain tested. The simplest interpretation of these data is that none of the mic-specific DNA sequences examined thus far is located exclusively on any one chromosome (or chromosome arm in the case of chromosome 1).

Micronuclear DNA preparations, particularly those from nullisomic strains of *Tetrahymena* spp., are always contaminated to some extent with macronuclear DNA. The percentage of macronuclear DNA varies from preparation to preparation and cannot be measured with precision. Thus, it is not useful to compare the intensities of hybridization from lane to lane. It is, however, valid to compare the relative intensities of several bands within a lane.

Changes in the relative intensity of various bands from one nullisomic strain to another are indicative of unequal distribution of the members of a family of repeated sequences on the five micronuclear chromosomes. The mic-specific sequence IIA8, for example, hybridized primarily to two *Hind*III fragments in genomic DNA of 2 and 4 kb. Both of these fragments are repeated in the micronuclear genome as judged by the intensity of hybridization. In the micronuclear DNA from wild-type cells and from most nullisomic strains, the probe hybridized with slightly greater intensity to the 2-kb band. However, hybridization to the 4-kb band was stronger in micronuclear DNA from strain CU373, which is nullisomic for 1R, indicating that the 2-kb fragment is present in a disproportionately large amount on the right arm of chromosome 1 (Fig. 5A, lane b).

A similar observation was made in the case of the mic-specific sequence IB6 (Fig. 5B). This clone hybridized strongly to a band at 1.3 kb in micronuclear DNA from wild-type and from most nullisomic strains. In the micronuclear DNA from these strains, there was also hybridization with much lower intensity to several other bands (arrows), including one at 1.6 kb. In the micronuclear DNA from strain CU373, however, the band at 1.3 kb hybridized at lower intensity relative to the larger fragments, particularly the fragment at 1.6 kb. The simplest interpretation of these data is that the copy number of the 1.3-kb IB6 fragment was reduced in the strain lacking the right arm of chromosome 1. The same result was obtained in two independent experiments and was therefore unlikely to be due to incomplete digestion of the DNA.

For those mic-specific sequences which are members of large families of repeated sequences, the patterns of hybridization were very similar from one nullisomic strain to another. Mic-specific sequences such as IIC7 (Fig. 5C) hybridized to a large number of fragments so that most of them were not resolved on the autoradiogram. In the case of the mic-specific sequence IC1, however, (Fig. 3 and 5D), individual bands were absent from or underrepresented in the DNA from particular nullisomic strains. This suggests that the various members of the family of repeated sequences were unequally distributed on the five chromosomes and supported the hypothesis that mic-specific sequences were present on more than one chromosome.

In situ hybridization demonstrates that IIA8 is present on all five *Tetrahymena* chromosomes. Given the strains we have examined thus far, it is clear from the nullisomic mapping that mic-specific DNA sequences are not located exclusively on chromosome 2, 3, 4, or 5. There is, however, some ambiguity with regard to chromosome 1. Since the chromosomes of *Tetrahymena* spp. are quite small and since all five are metacentric (Fig. 6a), they are not readily distinguishable from one another cytologically. The chromosome(s) absent from any particular strain is therefore identified by genetic criteria. Strains which are identified as lacking one chromosome arm, such as CU371 and CU373, may well retain any part of that arm for which there are no genetic markers, such as centromeric heterochromatin. Thus, one possible, albeit unlikely, interpretation of the data in Fig. 5 is that mic-specific DNA sequences are located exclusively in centromeric heterochromatin on chromosome 1.

To eliminate the ambiguity in the nullisomic mapping, we mapped the repeated sequence

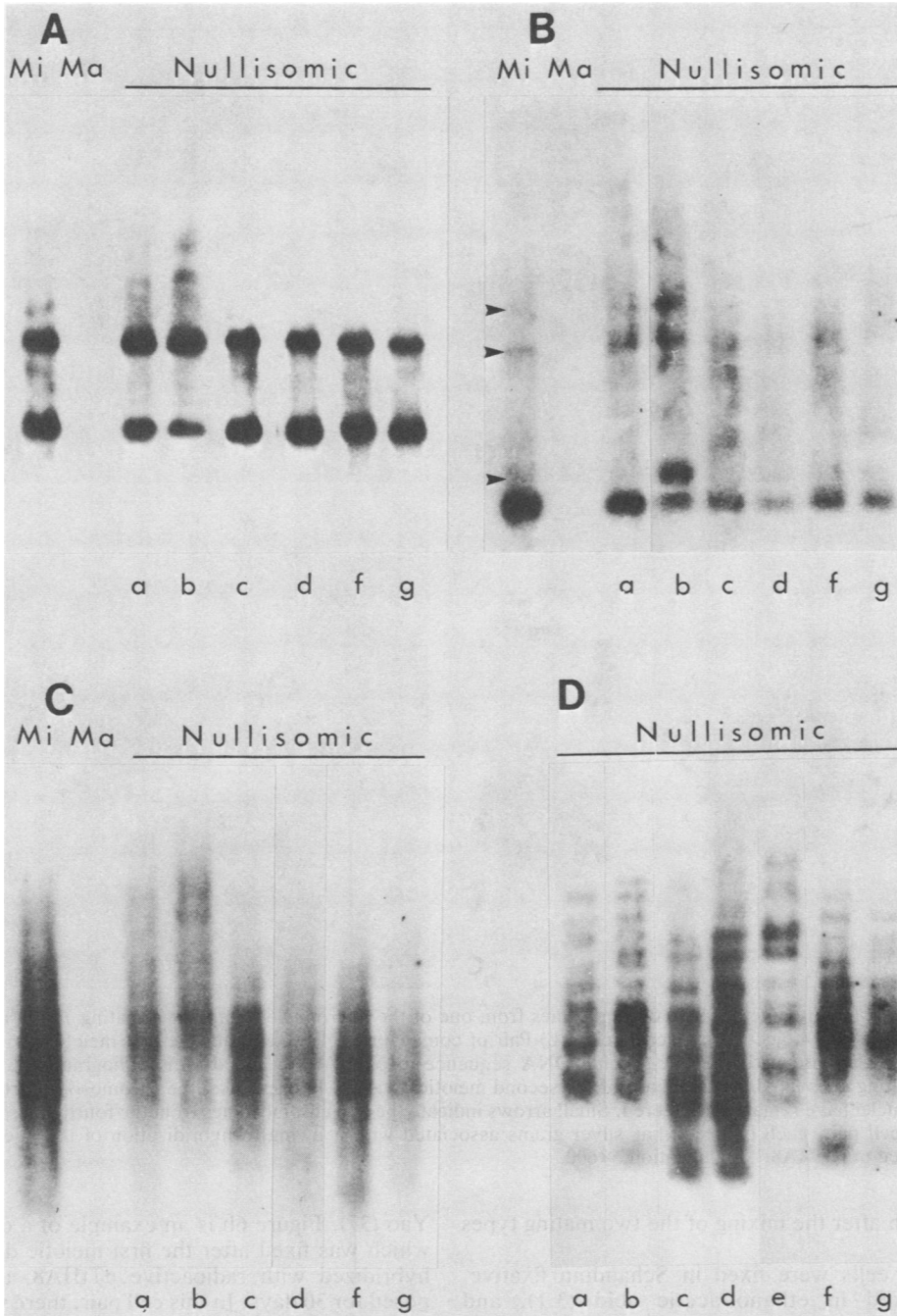


FIG. 5. Hybridization of mic-specific DNA sequences to *Hind*III-digested micronuclear and macronuclear DNA and to micronuclear DNA from strains nullisomic for chromosome(s) as follows: lane a, 1L, 2R; lane b, 1R; lane c, 2, 3, 5; lane d, 3, 4, 5; lane e, 3; lane f, 4; lane g, 5. The nick-translated plasmids used to probe the filters were pTtIIA8 (A), pTtIB6 (B), pTtIIC7 (C), and pTtIC1 (D).

IIA8 to all five *Tetrahymena* chromosomes by in situ hybridization. Optimal morphology chromosome was obtained by fixing conjugating cells during meiosis. Strains CU399 (mating type VI) and CU401 (mating type VII) were starved over-

night in 10 mM Tris, pH 7.4, and mixed to induce conjugation (7). We obtained the best chromosomes from cells at metaphase of the second meiotic division. To maximize the number of cells at this stage, mating pairs were fixed

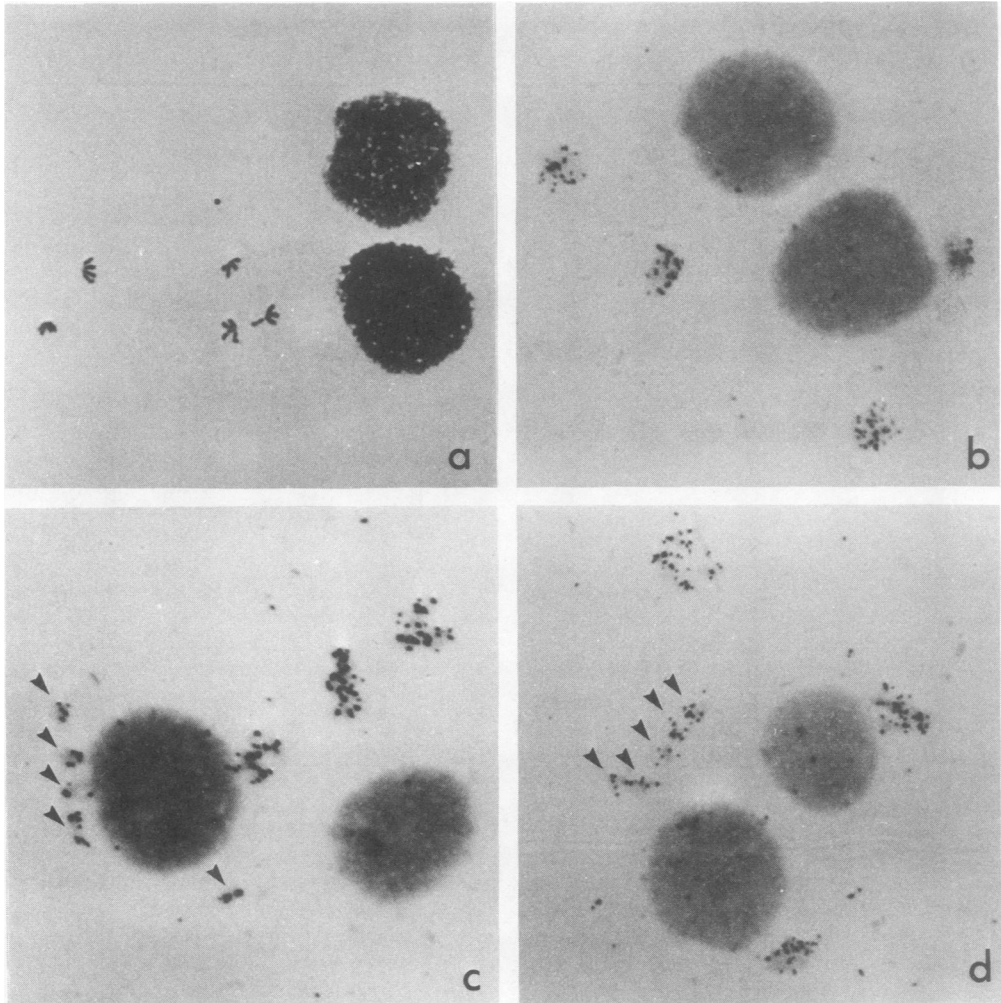


FIG. 6. (a) Five metacentric chromosomes from one of the micronuclei in a pair of mating *T. thermophila*. The large round objects are macronuclei. (b) Pair of conjugating cells fixed after the first meiotic division and hybridized in situ with the mic-specific DNA sequence in pTtIIA8. (c and d) Autoradiographs of pairs of conjugating cells fixed at metaphase of the second meiotic division. In each case, the chromosomes from three micronuclei have remained clustered. Small arrows indicate the five chromosomes from the fourth micronucleus in the cell pair, each of which has silver grains associated with it owing to hybridization of the mic-specific sequence in pTtIIA8. Magnification, $\times 600$.

at 4.5 h after the mixing of the two mating types (18).

The cells were fixed in Schaudinn fixative, postfixed in ethanol-acetic acid (3:1), and dropped onto a clean slide. Under these conditions, the bivalents from each of the micronuclei in a cell pair tended to remain clustered. This was important for the identification of the five chromosomes which compose one complete set among all of the chromosomes from the several micronuclei in a given cell pair.

The plasmid pTtIIA8 was nick translated with [^3H]dTTP and hybridized to the cytological preparations by the method of Yokoyama and

Yao (37). Figure 6b is an example of a cell pair which was fixed after the first meiotic division, hybridized with radioactive pTtIIA8, and exposed for 30 days. In this cell pair, there were 15 to 34 silver grains exposed in the photographic emulsion over each of the four micronuclei, whereas the macronuclei had background levels of 2 and 4 silver grains. This result is representative of the cell pairs observed on several different slides. The mean number of silver grains counted over micronuclei was 27 ± 8 , and over macronuclei in the same cell pairs only 5 ± 3 (Table 1), even though the macronuclei contain 20 times as much DNA at this stage. Preferential

labeling of micronuclei by in situ hybridization was consistent with the molecular data, which indicated that IIA8 is a mic-specific sequence.

Figures 6c and d are examples of cell pairs fixed at metaphase II and hybridized with radiolabeled pTtIIA8. In each cell pair, the chromosomes from three of the micronuclei remained closely associated, and chromosomes within a micronucleus were not distinguishable under the silver grains in the overlying emulsion. The five bivalents from the fourth micronucleus (Fig. 6c and d, arrows) were more widely separated, and 4 to 10 silver grains were associated with each of them (Table 1, cells pairs 6), indicating that the mic-specific sequence pTtIIA8 is present on each of the five micronuclear chromosomes in *T. thermophila*. Since the chromosomes are very small, the resolution of this technique does not allow us to draw any conclusions regarding the localization of this sequence along the length of the chromosomes.

As a control, slides were prepared and hybridized in parallel with radiolabeled pBR322. These preparations did not show significant numbers of silver grains over the micronuclei or the micronuclear chromosomes. This strongly suggested that the hybridization observed with the plasmid pTtIIA8 can be ascribed to the inserted fragment of *Tetrahymena* DNA and cannot be accounted for by nonspecific association of the vector DNA sequences with anything in the cytological preparation.

DISCUSSION

We have been studying the structure and chromosomal location of germ line-specific DNA in the micronucleus of the ciliated protozoan *Tetrahymena* spp. To this end, we have constructed a partial plasmid bank of *T. thermophila* micronuclear DNA digested with *Hind*III and cloned into the *Hind*III site of plasmid pBR322. This method is expected to produce clones which are a random representation of the sequences in the *Tetrahymena* genome. Preliminary screening of the plasmid bank was done by sandwich hybridization. Of the 68 clones tested by this method, 16 (23%) showed mic-specific hybridization. One cloned fragment hybridized to DNA which was repeated in both the micro- and macronucleus. These results are consistent with the data of Yao and Gorovsky (34), who concluded from renaturation kinetics that moderately repeated sequences in the micronucleus were absent or underreplicated in the macronucleus.

The major goal of this work was to map the chromosomal location of mic-specific DNA sequences in *Tetrahymena* spp. Cytological analysis indicates that the germ line-limited DNA sequences in most organisms are organized as

TABLE 1. In situ hybridization of mic-specific sequence pTtIIA8 to conjugating *T. thermophila*

Slide	Cell pair	No. of silver grains					
		Micronuclei				Macronuclei	
		1	2	3	4	1	2
1	1	15	34	27	26	4	2
	2	29	28	40	33	3	4
	3	30	37	23	31	2	3
	4	22	21	25	25	5	3
	5	23	20	36	10	4	3
	6	33	38	26	6+10+4+6+5	1	12
2	1	23	30	43	21	4	9
	2	12	20	16	13	4	6
	3	29	24	17	27	7	8
	4	40	33	31	27	8	3
	5	25	21	31	29	3	4
	6	37	27	34	5+6+5+8+8	3	7

large blocks of contiguous sequences. At one extreme are several members of the insect family *Cecidomyiidae*, which eliminate entire chromosomes from the soma (1, 22). In the nematode *Ascaris* spp. (5), in which most of the germ line-specific DNA belongs to families of short, tandemly repeated sequences (26), and in the crustacean *Cyclops* spp. (2), the bulk of the eliminated DNA sequences are present in the germ line as blocks of heterochromatin at the termini or near the centromeres of the chromosomes. We wanted to determine whether the germ line-specific DNA in *Tetrahymena* spp. is distributed over the chromosomes, as in the nematodes and crustaceans, or concentrated on a germ line-specific chromosome, as in the insects.

Hybridization of mic-specific DNA sequences to micronuclear DNA from nullisomic strains of *Tetrahymena* spp. suggests that each of the six sequences we have tested by this method is present on more than one chromosome. One mic-specific DNA sequence has been shown by in situ hybridization to be present on all five chromosomes. These data place certain constraints on the possible models for the arrangement of germ line-specific sequences in the micronucleus and for the mechanisms by which they are eliminated from the macronucleus.

The evidence clearly excludes a model whereby *Tetrahymena* spp. eliminates one of the five germ line chromosomes and supports a model of chromosome fragmentation similar to the one originally proposed by Yao and Gall (33). Thus, the characteristics which distinguish mic-specific DNA from sequences which are retained in the developing macronuclear anlagen must operate subchromosomally, perhaps at the level of DNA sequence or modification. Yao (30)

reached this conclusion by the analysis of linkage relationships between members of a family of mic-specific DNA sequences associated with C_4A_2 repeats.

Yokoyama and Yao (37) have presented evidence that mic-specific DNA is replicated a few times early in the formation of the macronuclear Anlagen and is then eliminated, presumably by specific degradation, before any nuclear division occurs. Two laboratories (21, 25) have estimated the size of the macronuclear DNA to be about 600 kb. The simplest model which is consistent with these data and with the presence of mic-specific DNA sequences on all five chromosomes is one in which in the micronucleus, relatively long stretches of mic-specific DNA alternate with DNA sequences which are retained in the macronucleus. Mic-specific DNA would be excised and degraded, and the remaining DNA would be replicated in the macronucleus, perhaps, although not necessarily, without extensive rearrangement.

We can calculate the average length of a contiguous stretch of mic-specific DNA which we would expect to find in the micronuclear genome according to the above model. The DNA content of the haploid micronucleus as determined microspectrophotometrically by Woodard et al. (J. Woodard, M. Gorovsky, and E. Kaneshiro, *J. Cell Biol.* **39**:182a, 1968) is 0.23 pg or about 2.1×10^5 kb. Assuming that about 20% of the micronuclear genome is absent from the macronucleus, as suggested by our data, then the complexity of macronuclear DNA (which does not contain many repeated DNA sequences) is about 1.7×10^5 kb. If the macronuclear DNA is present in 600-kb subchromosomal fragments, this would suggest that each of the five *Tetrahymena* chromosomes is fragmented into about 55 pieces in the macronucleus (25). If we postulate that the fragmentation of macronuclear DNA is largely the result of elimination of alternating mic-specific DNA sequences which are scattered over all the chromosomes, it would follow that the 4×10^4 kb of mic-specific DNA is in 250 to 300 tracts of approximately 140 kb each. The existence of relatively long stretches of mic-specific sequences in the micronuclear genome would be consistent with the observation of Yao (30) that six of seven randomly selected phages containing mic-specific DNA had only mic-specific DNA.

There is at least one family of repeated DNA sequences in the *Tetrahymena* genome, the repeated hexanucleotide C_4A_2 , which undergoes extensive rearrangement between the micro- and macronuclear DNA (31). In the original model of Yao and Gall (33), it was proposed that fragmentation of the micronuclear chromosomes occurred at or near C_4A_2 repeats. Since subse-

quent experiments show that most of the micronuclear C_4A_2 repeats seem to be flanked on both sides by mic-specific DNA (6, 30), it is likely that the macronuclear C_4A_2 repeats, which are largely present at the termini of macronuclear DNA (6, 36), may be added to the ends of the macronuclear DNA as the analogous C_4A_4 repeats are in the hypotrichous ciliate *Oxytricha* spp. (4). This could occur either enzymatically or by rearrangement of micronuclear C_4A_2 repeats. Whether the micronuclear C_4A_2 repeats are eliminated or rearranged to the termini of macronuclear DNA does not affect the model offered here regarding the interspersions of mic-specific and macronucleus-retained DNA sequences in the micronuclear genome.

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