Developmental DNA Rearrangements and Micronucleus-specific Sequences in Five Species within the *Tetrahymena pyriformis* Species Complex

Piroska Huvos

Departments of Medical Biochemistry and Chemistry and Biochemistry, Southern Illinois University, Carbondale, Illinois 62901

Manuscript received April 15, 1995
Accepted for publication August 15, 1995

ABSTRACT

In *Tetrahymena thermophila,* the development of a transcriptionally active macronucleus from a transcriptionally inert micronucleus includes the elimination of many segments of DNA, the bulk of which belong to repetitive sequence families. Two approaches were used to study the interspecies variations in developmentally eliminated DNA segments. First, the occurrence of restriction fragments crosshybridizing to developmentally eliminated DNA segments isolated from *T. thermophila* was examined in other species of Tetrahymena. Most micronucleus-specific sequence families examined showed large differences in numbers and intensities of crosshybridizing bands in different species, indicating the possibility of gain or loss of repeats within each of the sequence families. Second, the presence of developmentally excisable DNA segments, *i.e.,* of rearrangement sites, was examined in the same set of species at a number of unique loci. This was carried out by comparing the hybridization patterns of seven unique macronucleus-retained sequences in the micro- and macronuclei of each of the species. Essentially all of the loci displayed variability with respect to the presence of rearrangement sites among the species examined. Results from the two approaches indicate that generation or loss of developmental rearrangements can occur among the species examined here.

CILIATES are unicellular organisms that have two different kinds of nuclei in their cytoplasm: a germ line diploid micronucleus, which is transcriptionally inactive, and a polyploid, transcriptionally active macronucleus. A new macronucleus (Mac) develops from the zygotic micronucleus (Mic) after each conjugation. Micronuclear DNA undergoes extensive rearrangement during development into a macronucleus, including the elimination of a large portion of the Mic genome (GOROVSKY 1980; BLACKBURN and KARRER 1986; BRUNK 1986; KLOBUTCHER and PRESCOTT 1987; YAO 1989).

In *Tetrahymena thermophila,* a representative of holotrichous ciliates, 10–20% of Mic DNA is eliminated during the formation of the Mac, and most of the eliminated DNA is repetitive (YAO and GOROVSKY 1974; YAO 1982; KARRER 1983). A large fraction of this Mic-specific DNA is eliminated internally, *i.e.,* through deletion-religation events. It has been estimated that there are at least 6000 internally eliminated sequences (IESs) in the entire genome that are deleted from the developing macronucleus within a time interval of about 2 hr (YAO *et al.* 1984; BRUNK 1986). The proposed signals for elimination have been found to be different at each of the few sites examined to date (AUSTERBERRY and YAO 1987, 1988; KATOH *et al.* 1993; HEINONEN and PEARLMAN 1994; WELLS *et al.* 1994), indicating that more than one elimination system is likely to exist. The only common feature is the presence of direct repeats at the rearrangement junctions.

No known function can be assigned to a specific sequence rearrangement, but it has been generally hypothesized (See ORIAS 1981; HOWARD and BLACKBURN 1985; AUSTERBERRY and YAO 1987) that they may be involved in transcriptional activation, or in the generation of phenotypic variation, or in the elimination of meiotic sequences that would be deleterious in the macronucleus. The micronuclear genome is transcriptionally silent during vegetative growth, but small portions of it are likely to be transiently expressed during development (SUGAI and HIWATASHI 1974). However, no Mic-specific sequences have been identified in cDNA libraries constructed using RNA from developing cells (MARTINDALE and BRUNS 1983), although there is one example of a Mic-specific sequence expressed during starvation (STEIN-GAVENS *et al.* 1987).

Recently, highly abundant Mic-specific sequences that resemble transposable elements have been described in the micronuclei of several species of hypotrichous ciliates (HERRICK *et al.* 1985; JAHN *et al.* 1988; BAIRD *et al.* 1989), and transposase genes have been identified within the repetitive elements (DOAK *et al.* 1994). Such transposons may survive in the genome by possessing the ability to be removed during macronuclear development (JAHN *et al.* 1989; TAUSTA and KLOBUTCHER 1989; WILLIAMS *et al.* 1993). Short IESs that occur frequently in hypotrichous ciliates have been hypothesized to be derivatives of transposons, because of similarities in the terminal structures and in excision

Corresponding author: Piroska Huvos, Department of Medical Biochemistry, Southern Illinois University, Carbondale, IL 62901.
E-mail: phardwicke@som.siu.edu

products (HERRICK *et al.* 1985; RIBAS-APARICIO *et al.* 1987). Thus, transposable elements may be intimately involved with developmental elimination in hypotrichous ciliates. Involvement of transposable elements in the developmental events in other organisms has also been described (GOLDEN *et al.* 1985; STRAGIER *et al.* 1989).

Gene organization and reorganization show some striking differences between the holotrich ciliates, such as *T. thermophila,* and the hypotrich ciliates (PRESCOTT 1994). In *T. thermophila,* IESs are often in the range of 1–3 kb, and are frequently composed of repetitive DNA, as opposed to the very short unique IESs found in hypotrichs (and also interestingly in a holotrichous ciliate, Paramecium, see STEELE *et al.* 1994; AMAR 1994; SCOTT *et al.* 1994). Mobile elements are likely to exist in holotrichous ciliates, since two transposon-like Mic-specific sequence families have been described in *T. thermophila* (CHERRY and BLACKBURN 1985; WYMAN and BLACKBURN 1991; WELLS *et al.* 1994); also, the chromosomal break sites produced during development seem to suggest a transposon-like deletion mechanism (SAVALIEV and COX 1995). However, apart from these, the holotrichous Mic-specific segments sequenced have no features characteristic of known transposable elements (such as inverted repeats, long direct repeats, transposase coding sequences) except for the short direct repeats at the rearrangement junctions (AUSTERBERRY and YAO 1987, 1988; KATOH *et al.* 1993; HEINONEN and PEARLMAN 1994). Moreover, one rearrangement site that has been studied in detail seems to contain signals for excision outside the eliminated DNA (GODISKA and YAO 1990), a fact that is not easily compatible with self-excision of a transposable element.

The present study addresses the question of variability in Mic-specific sequences among a group of closely related Tetrahymena species. The majority of the Mic-specific sequence families examined displayed large differences in numbers and intensities of crosshybridizing bands in different species, indicating that gain or loss of repeats may have occurred within these sequence families during evolution. Although alternative explanations are possible, these results may mean that these Mic-specific sequences have been derived from mobile elements in Tetrahymena species. The presence of developmental rearrangements was also examined at a number of single copy Mac-retained loci by comparing micro- and macronuclear DNA restriction patterns. All but one of the loci examined displayed some variability with respect to the presence of rearrangement sites among the species examined. These results indicate that some developmental rearrangements may have appeared or disappeared during evolution in this group of closely related species.

MATERIALS AND METHODS

Strains and cell culture: The strains used: strains CU427 and CU428 of *T. thermophila,* strains 23b and 44b of *T. malaccensis,* strain 4EA of *T. elliotti,* strain X4U2 of *T. borealis,* strain HG2 of *T. pigmentosa.* All strains used were maintained in 1% proteose peptone. Strains CU427 and 428 were derived by P. J. BRUNS, the rest of the strains are from a collection of ELLEN SIMON. For large scale harvesting, cells were generally grown in 2% proteose-peptone-0.1% yeast extract-0.003% sequestrine (PPYS). Strains of *T. elliotti* and *T. borealis* were grown in 2.5% PPYS.

Preparation of micro and macronuclei: The procedure used was that of GOROVSKY *et al.* (1975), based on differential centrifugation as modified by HOWARD and BLACKBURN (1985). Initially, difficulties were encountered in obtaining undegraded DNA from species other than *T. thermophila* and *T. malaccensis.* Several minor modifications were introduced to overcome this problem. Cells were poured on crushed ice before harvesting to cool them quickly (M. GOROVSKY, personal communication), and cultures with low cell density (1–2.0 × 10^5 cells/ml) were used for the preparations (S. ALLEN, personal communication). Most of the DNA prepared in this way migrated on agarose gels as a tight band with a molecular weight of ≥23 kb.

The micro- and macronuclear preparations were cross-contaminated with each other to varying extents. Micronuclear DNA preparations usually contained light bands of hybridization of macronuclear origin with most probes used, (see RESULTS). Occasionally, micronuclear preparations were heavily contaminated with macronuclei, as judged by hybridization results.

Plasmid DNA: All plasmid DNA was derived from *T. thermophila,* the reference species. The probes with which results are presented in this article are listed in Table 1. The hybridization patterns obtained in the present studies in *T. thermophila* were, in general, consistent with results presented in the original descriptions of the regions examined.

Preparation of plasmid DNA and isolation of the inserts from the plasmids: Plasmid DNA was either prepared by the polyethylene glycol (PEG) precipitation method as described by SAMBROOK *et al.* (1989), or by using the Qiagen maxi plasmid preparation kit, according to the manufacturers recommendations. For isolation of the inserts, the plasmids were digested with the appropriate restriction endonucleases, and electrophoresis was performed in 1% submarine agarose gels overnight at about 16 V, 30 mA. The inserts were isolated by binding DNA onto DEAE membrane. This was achieved after staining the gel with ethidium bromide, by placing a strip of DEAE NA 45 (Schleicher and Schuell) membrane in front of the band of interest in an incision, and continuing electrophoresis at about 30 V for an hour or until the stained band was visibly bound on the membrane. DNA was eluted by incubating the papers in 1 M NaCl, 1 mM EDTA, 0.05 M arginine-free base for 1 hr at 65°. The eluate was extracted three times with butanol, then once with phenol, and the DNA was precipitated by ethanol after addition of 10–20 μg nuclease-free glycogen from Boehringer-Mannheim as a coprecipitant. The DNA pellet was dissolved in 0.3 M Na acetate, reprecipitated with ethanol, and finally dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Restriction digestions and gel electrophoresis: Restriction endonucleases were purchased from GIBCO-BRL and digestions were performed under conditions recommended by the manufacturers. DNA was allowed to digest at least 4–6 hr in the presence of 5–10 units of enzyme for every microgram of DNA. Three to six micrograms DNA was layered on the gels, and submarine gel electrophoresis was performed overnight in 0.8–1% submarine agarose gels, at about 16 V, 30 mA in TEA buffer (40 mM Tris/20 mM Na acetate/2 mM EDTA, pH 8.0).

Southern transfer and hybridizations: These were per-

TABLE 1

Origins and characteristics of probes

Probe and locus Name	Presence of the sequence in Mac	Repetition in the *T. thermophila* genome	Chromosomal location	Origin of DNA from which the probe was derived	Probe length (kb)	Reference
Mic-specific probes						
IIA8 probe[a]	—	Repeated (~200 copies)	1, 2, 3, 4, 5	Mic	2	KARRER (1983)
C8b/4 probe[a]	—	Repeated (~8 copies)	ND	Mic	0.5	ROGERS and KARRER (1989)
Probes derived from Mac-retained sequences						
PSR-900 probe, Histone H1 locus	+	Single	ND	Mac	0.9	WU (1989); WU *et al.* (1986)
*Eco*RI-*Hin*dIII fragment probe, actin locus	+	Single	ND	Mac	0.3	CUPPLES and PEARLMAN (1986)
*Eco*RI fragment probe, PGK (phosphoglycerate kinase) locus	+	Single	ND	cDNA	0.9	VOHRA *et al.* (1992)
Calmodulin cDNA probe, calmodulin locus	+	Single	ND	cDNA	0.7	KATOH *et al.* (1994)
PCA102 probe, cTt455 locus	+	Single	4	Mac	1.9	GODISKA and YAO (1990) and refs CASSIDY-HENLEY *et al.* (1994)
IIC7.1a probe, IIC7 locus	+	Single[b]	3R	Mic[c]	0.9	WELLS *et al.* (1994)

[a] With the repeated sequences IIA8 and C8b/4 the locus (loci) cannot be identified.

[b] The IIC7.1a probe detected a single sequence in *T. thermophila* with high stringency washes, but additional hybridizing bands were present at low stringencies.

[c] IIC7.1a is a Mac-retained fragment subcloned from a micronuclear clone.

formed as described (HUVOS *et al.* 1988), but in some cases Immobilon N membrane (Millipore Corp.) was used according to the manufacturer's instructions instead of nitrocellulose (Schleicher and Schuell). The probe was labeled by random priming using the United States Biochemicals kit with [α-^{32}P]dATP or α-^{32}P[dCTP] according to the manufacturer's instructions, and hybridizations were performed at 40°, in 30%, instead of 50% formamide. Filters were first washed under moderate stringency conditions: 0.1× SSC, at 37°. In many cases this was followed by washes in 0.1× SSC at 50°, and at 60°.

RESULTS

Distribution and conservation of repetitive Mic-specific sequences among five Tetrahymena species: The distribution of Mic-specific sequences among Tetrahymena species was studied by hybridizing micronuclear probes isolated from *T. thermophila* to restriction digests of micro- and macronuclear DNA prepared from the five species of Tetrahymena. The species studied were, in increasing phylogenetic distance from the reference species, *T. thermophila: T. malaccensis, T. elliotti, T. borealis* and *T. pigmentosa*. Their phylogenetic relationship

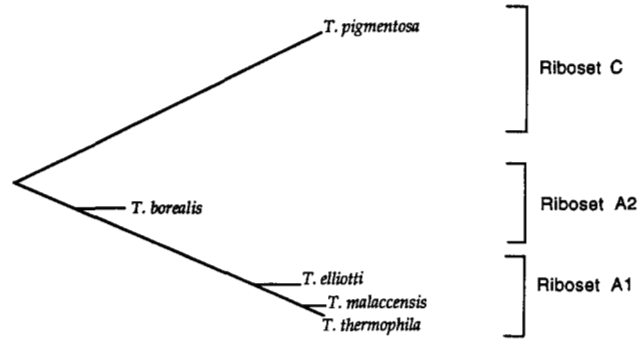


FIGURE 1.—Phylogenetic relationship among the five species studied. The branching order of the five species studied is redrawn from SOGIN *et al.* (1986), PREPARATA *et al.* (1989), NANNEY *et al.* (1989), BRUNK *et al.* (1990), and SADLER and BRUNK (1992). The drawing is according to the topology of Figure 3 in NANNEY *et al.* (1989), but the branch lengths do not represent exact distances. Assignment of the species in "riboset" groups according to NANNEY *et al.* (1989) also illustrates their phylogenetic relationships. For a discussion on comparison of the trees based on different molecules, see SADLER and BRUNK, (1992).

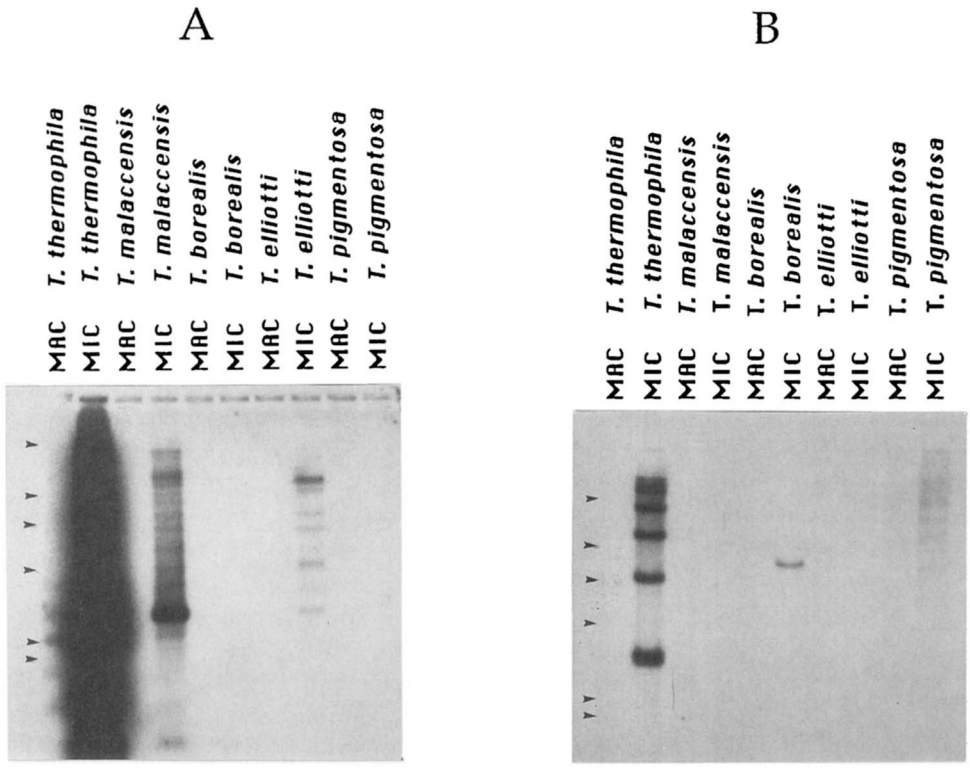


FIGURE 2.—Hybridization of micronuclear sequences to DNA from five Tetrahymena species. Preparation of micro- and macronuclei, DNA extraction, Southern blotting, hybridization and washing conditions were as described in MATERIALS AND METHODS. The following probes were used: IIA8 (A) and C8b/4 (B). The enzymes used for digestion were *Eco*RI (A) and *Hha*I (B). The source of DNA, *i.e.,* Mic and Mac DNA, and the five species are indicated on the top. It should be noted that the order of samples for the DNA of the five species on the blots was not strictly in increasing phylogenetic distance from *T. thermophila.* Filters were washed in 0.1× SSC, 0.1% SDS at 37°. *Hind*III-digested λ DNA was used as standards. Arrowheads show the positions of bands with MWs of 23.3, 9.5, 6.5, 4.3, 2.3, and 2.0 kb. In some experiments, the 0.5-kb band is indicated.

is represented in Figure 1. All species are from within the *T. pyriformis* species complex. The Mic-specific probes with which results are shown here, are described in Table 1, together with some Mac-retained probes that were used to obtain results presented in the next section.

All of the Mic-derived sequences tested hybridized to more than one band in restriction digests of Mic DNA from *T. thermophila* (see examples in Figure 2). This is to be expected, since a large fraction of Mic-specific sequences are repetitive. Moreover, most of the Mic-specific sequences used in this study originated from a collection of Mic-specific clones, prepared by K. KARRER (1983), using a method that selected for repetitive sequences from micronuclear DNA.

There was a great deal of variation in the hybridization of the various Mic-specific probes. Most probes tested (seven out of 13), hybridized with closely related species but only sparingly or not at all with more distant species. This type of distribution is illustrated in Figure 2A with probe IIA8. With this probe, hybridization was observed to Mic DNA from *T. thermophila, T. malaccensis* and *T. elliotti,* respectively, with the hybridization intensity decreasing as the phylogenetic distance increased. No hybridization was observed with the more distantly related *T. borealis* and *T. pigmentosa.* Two of the probes hybridized with comparable intensities to DNA from all species except *T. pigmentosa,* the most distant relative (data not shown).

Four of the probes tested showed hybridization to DNA of some of the more distant relatives of *T. thermophila,* while hybridization was undetectable to the more closely related species. A striking example of this type of distribution is shown in Figure 2B with probe C8b/4. This probe hybridized to 6–8 bands in *T. thermophila,* to one band in *T. borealis* and to a smear in the DNA from *T. pigmentosa,* (see Figure 2B). No hybridizing bands were detectable in the more closely related *T. malaccensis* or *T. elliotti.*

In summary, the distribution of the repetitive Mic-specific sequences among the five species examined was variable, and different for each sequence family examined. The decrease in hybridization intensity in related species compared with the reference species is likely to be due to variation in numbers of crosshybridizing sequences among the five species and also to decreased hybridization efficiency due to divergence.

Detection of developmental rearrangements in the flanking regions of seven Mac-retained loci by comparison of micro- and macronuclear DNA restriction patterns in related Tetrahymena species: In these studies, seven single-copy loci were examined in four species, in addition to *T. thermophila,* the reference species. In order to make a survey of such a large number of loci feasible, a number of single restriction digests from the micro- and macronuclear DNAs of the respective species were hybridized with probes derived from the single-copy Mac-retained genes studied. Since Mac-retained DNA segments are present in both the micro- and macronucleus, they can be used to characterize the micronuclear and macronuclear versions of a locus by hybridization. The presence of a Mic-specific segment in the micronuclear version of DNA but not in the macronuclear version, *i.e.,* of a developmental re-

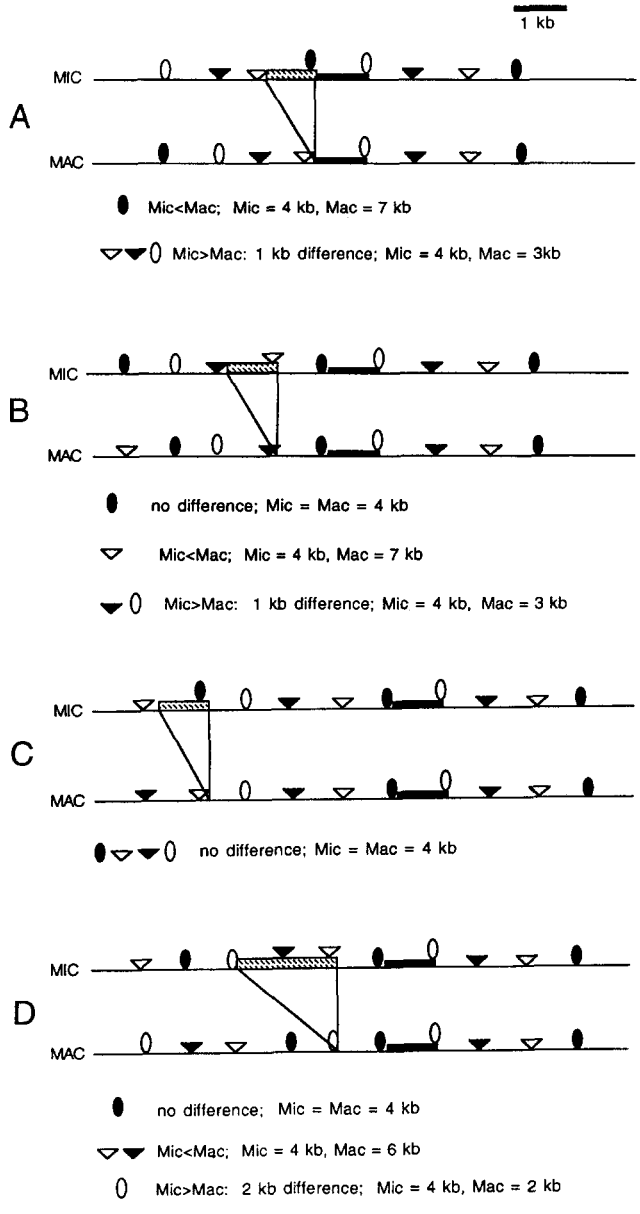


FIGURE 3.—Detectability of Mic-specific sequences, *i.e.*, rearrangements between Mic and Mac, by hybridization to a Mac-retained sequence. The stippled box represents the Mic-specific fragment, and the thick line represents the sequence used as the probe. The different symbols represent restriction sites for different endonucleases. Four situations are shown here. The distance of the Mic-specific segment from the sequence used as a probe varies in A, B, and C. The Mic-specific segment is longer in D (2 kb) than in A, B, and C (1 kb). The location of restriction sites in relation to the sequence used as a probe is kept constant. The four types of restriction site are distributed evenly in the region, at 1-kb intervals, and the restriction fragments produced by a particular enzyme are all the same length in the Mic (4 kb). In A, where the Mic-specific segment is directly flanking the sequence used as a probe, a difference between Mic and Mac hybridization pattern is detectable with all the restriction enzymes used. The numerical values of the differences converge around a single value for three of the enzymes used, indicating the existence of a single Mic-specific segment, and defining its length. In C, where the distance of the Mic-specific fragment from the probe sequence is the same as the restriction fragment length, *i.e.*, 4 kb, none of the restriction enzymes used detect a difference. In general, the closer the Mic-specific sequence is located to the sequence used as a probe, the greater the probability that it will be detected in the form of a restriction fragment length difference between the Mic and the Mac. The proportions of enzymes that detect a rearrangement decrease from 4/4 in case A, to 3/4 in case B, to 0/4 in case C, because the distance of the rearrangement from the probe increases. Rearrangements in B and D are detected by three out of four enzymes tested because they are at the same distance from the probe. Once the length of the Mic-specific segment exceeds a minimal length necessary for detection, its real length is more likely to be determined by restriction analysis if it is short enough not to contain restriction sites for the enzymes used (*cf.* cases B and D).

arrangement, was inferred from differences observed between the sizes of hybridizing bands in micro- and macronuclear DNA, as described by YAO *et al.* (1984). (This is analogous to the detection of an insertion of a sequence in one strain or isolate compared to another, through detection of differences in hybridizing bands on Southern blots.) The most frequent developmental event leading to differences between the micro- and macronuclear DNAs is deletion-religation (YAO *et al.* 1984). Differences in restriction patterns are therefore interpreted in terms of this type of change. Other types of changes that can cause differences between the restriction patterns of micro- and macronuclear DNA are summarized here. Deletion-chromosome breakage events, which lead to the formation of subchromosomal Mac fragments, are about 20 times less frequent than deletion-religation events in Tetrahymena, so they are likely to make only a minor contribution to changes observed. Differences between micro- and macronuclear patterns can also arise because of methylation. In *T. thermophila*, 0.6–0.8% of adenines are methylated in the NAT sequence in the Mac, but not in the Mic (BROMBERG *et al.* 1982). Therefore, the source of difference between Mic and Mac DNA cannot be unambiguously determined if the Mic band is shorter than the Mac band in an enzyme digest that is sensitive to methylation at A in an AT recognition sequence (*Eco*RI, *Eco*RV and *Xba*I in the present study), and such results were disregarded. Another possible source of difference between the Mic and Mac could be due to the presence of two alleles in the diploid Mic, but of only one allele in the Mac, because of phenotypic assortment of subchromosomal fragments occurring during amitotic division (ALLEN and GIBSON 1973). This situation might lead to more bands being present in the Mic than in the Mac.

The proportion of restriction digests that detect a rearrangement depends on the distance of the rearrangement from the probe and on the size of the restriction fragments. In general, the closer the Mic-specific sequence, *i.e.*, the rearrangement, to the probe used, the higher the proportion of restriction enzymes that is likely to detect the presence of this sequence

(see Figure 3, which shows models of regions containing Mic-specific segments at different distances from the probe used). An alteration in the proportion of restriction digests that show differences between micro- and macronuclear DNAs in two species can therefore be taken as an indication that the locus examined had Mic-specific segments (*i.e.,* rearrangement sites) at different distances from the probe in the two species examined (see Figure 3).

The length of the Mic-specific, *i.e.,* eliminated, DNA segment at a given locus can be determined from the difference between the sizes of the hybridizing micronuclear and macronuclear bands, if the Mic band is longer than the corresponding Mac band. The difference between Mic and Mac bands at a particular locus can only be accepted as the real length of the fragment eliminated from the Mac, if several different restriction enzymes give the same result. If differences between Mic and Mac band lengths are variable with different restriction endonucleases, there may be recognition sites present for some of the restriction endonucleases used within the eliminated DNA, or multiple rearrangements may occur in the flanking region of the locus examined. If the Mic bands have a smaller size than the Mac bands, it necessarily means that restriction sites for the enzymes used are present within the eliminated region, precluding any conclusion about the size of the eliminated DNA (see model cases presented in Figure 3).

Of the seven loci examined, data from only the PSR900 region are presented here in detail. Results from the other loci are described briefly. All results are summarized in Table 2.

The PSR900 region near the histone H1 locus: The PSR900 probe is a 0.9-kb fragment almost immediately downstream from a micronucleus-limited sequence, and 3 kb upstream from the H1 histone gene in *T. thermophila* (M. Wu, personal communication). Hybridization of this probe to DNA of the five species is shown in Figure 4. The sequence was detectable and was present as a single band, *i.e.,* in a single copy in all five species examined.

In *T. thermophila,* the results showed that the difference between the corresponding Mic and Mac bands was about 1.5 kb in six different restriction endonuclease digests. Since all digests showed a difference between Mic and Mac bands, it was concluded that the Mic-specific fragment, *i.e.,* the rearrangement site, is likely to be in close proximity to the probe used. The convergence of the differences between micro- and macronuclear band sizes around a single value indicates that all digests show the real length of the Mic-specific DNA. These results are consistent with the 1.2-kb deletion in the immediate flanking region of the PSR900 fragment, (M. Wu, personal communication).

In *T. malaccensis,* as in the reference species, all digests showed a difference between the corresponding Mic and Mac fragments, indicating that the position of the Mic-specific fragment is likely to be in close proximity to the PSR900 probe. The difference between the Mic and Mac fragment lengths was about 1.2 kb, with the exception of the *Hin*dIII digest, in which the Mac band was longer than the Mic fragment. Thus, both the length and the position of the eliminated sequence is similar in *T. thermophila* and *T. malaccensis,* and therefore it is likely that the rearrangements detected in these two species are homologous to each other, *i.e.,* the Mic-specific DNA segments in *T. thermophila* and *T. malaccensis* are derived from a common progenitor that was present before the divergence of the two species.

In *T. elliotti,* of six restriction digests, none detected a difference between the Mic and Mac bands. Therefore, in this species no rearrangement site is likely to be present in this region.

In *T. borealis,* one out of five restriction digests showed a difference between Mic and Mac patterns. In this case the micronuclear band was longer than the macronuclear one by 1.2 kb, which is consistent with a 1.2-kb deletion.

In *T. pigmentosa,* no difference between Mic and Mac band length was observed in five restriction digests. (In the *Eco*RV digest the Mic band is shorter than the Mac band; therefore it cannot be determined from this result whether the difference is due to methylation or to the presence of a Mic-specific fragment.)

In summary, based on the proportion of restriction enzymes that detect a difference between micro- and macronuclear DNA, the position of the eliminated sequence is similar in *T. thermophila* and *T. malaccensis.* Therefore, it is likely that the rearrangements detected in these two species are homologous to each other. On the other hand, in *T. borealis* the rearrangement is likely to be displaced further from the sequence crosshybridizing to the PSR900 probe than in *T. thermophila* and *T. malaccensis.* Homologous rearrangements, *i.e.,* Mic-specific segments derived from a common progenitor, can be assumed to be at the same distance from a particular probe in different species if the macronuclear genome organization is essentially unchanged. Macronuclear organization may change during evolution since insertions and deletions can occur in the vicinity of rearrangements, especially in nontranscribed regions. Nevertheless, a comparison of the distribution of micro- and macronuclear sequences among the five species (*cf.* Figures 2 and 4) shows that the frequency of insertions-deletions associated with micronuclear sequences is likely to be much higher than of those occurring in macronuclear sequences. Therefore, the rearrangements in *T. borealis* that are at a larger distance from the probe used are not likely to be homologous with those in *T. thermophila* and *T. malaccensis. T. elliotti* and *T. pigmentosa* may not have any rearrangements at the PSR900 region. For a summary of results, see Table 2, where the number of digests in which differences

TABLE 2

Restriction fragment length differences between Mic and Mac at selected loci in five Tetrahymena species

	T. thermophila		*T. malaccensis*		*T. elliotti*		*T. borealis*		*T. pigmentosa*	
	D	Mic-Mac (kb)	D	Mic-Mac (kb)	D	Mic-Mac (kb)	D	Mic-Mac (kb)	D	Mic-Mac (kb)
PSR locus	7/7		6/6		0/6		1/5		0/6	
		1.6 (3)		1.5 (2)				1.2		
		1.5 (3)		1.25						
		1.4		1.2 (2)						
Actin locus	0/7		3/7		0/7		1/7		5/7	
				4.5						1.0
				1.2						
				1.0						
PGK locus	5/6		2/6		0/4		0/6		3/6	
		3.5		4.0						2.0
		2.0								1.1
		1.5 (2)								1.0
		1.3								
Calmodulin locus	4/6		2/6		3/6		0.7		5/6	
		3.5		2.0 (2)		1.0				
		1.4								
		1.3								
cTt455 locus	6/6		3/6		6/6		2/5		1/6	
		6.		3.0		6.0				
		3.2		2.3		3.2				
		1.0 (2)		1.2		2.3				
		0.9				1.6				
						1.5				
IIC7 locus	3/3		2/4		1/1		0/1		ND	
		0.8								

D is the differences found for each locus in each species, expressed as number of restriction digests in which a difference between Mic and Mac was observed/total number of restriction digests tested. The numerical values for differences between Mic and Mac bands are listed in kilobases (kb) in cases where the Mic band was longer than the Mac band, and therefore the difference may represent the length of the eliminated Mic-specific DNA. Numbers in parentheses show the number of cases in which the same difference in MWs was found. Numerical values for the differences in MWs are not listed in cases in which more than one band was observed in both micro- and macronuclear digests, or if the Mic band was shorter than the Mac band, and thus no conclusion about the length of the eliminated DNA could be drawn.

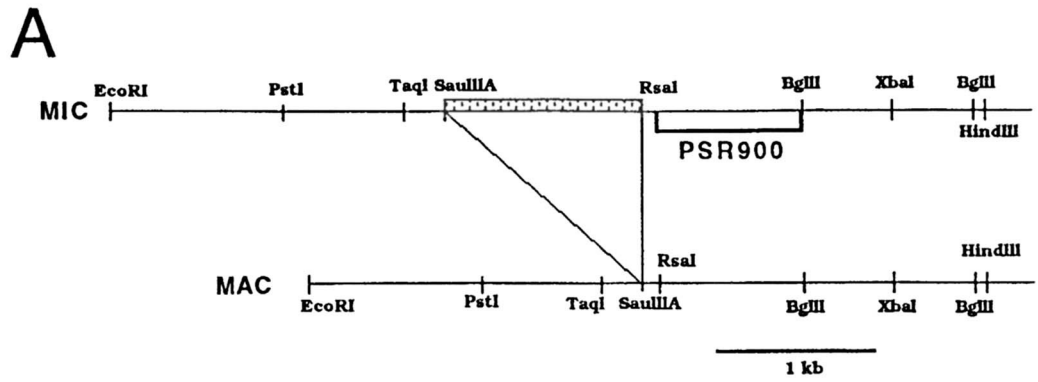
were observed between macro- and micronuclear fragments/total numbers of digests tested are listed for each species. The values for the molecular weight differences between Mic and Mac bands are listed only for digests where micronuclear bands are longer than the macronuclear ones.

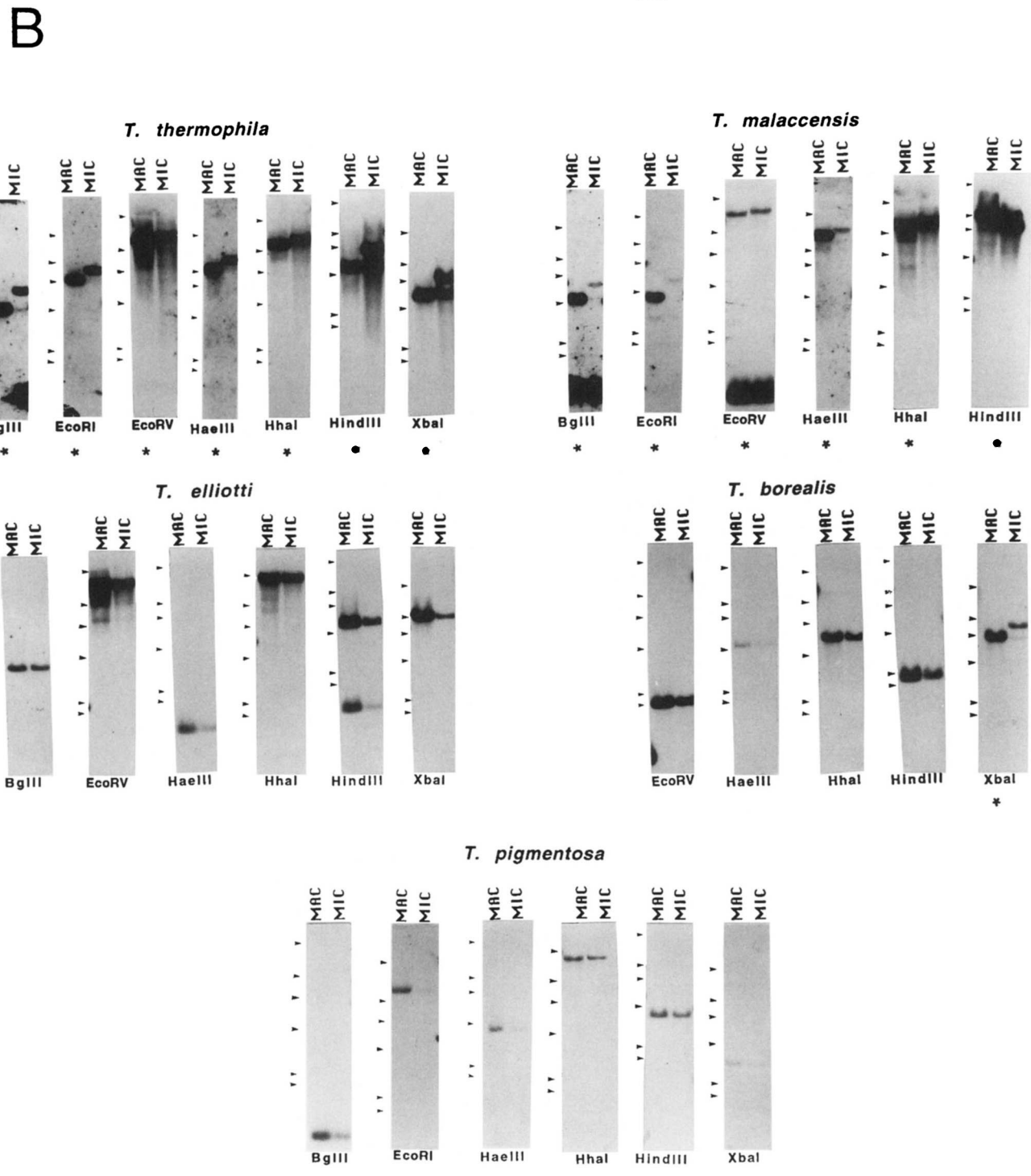
The actin locus: Essentially no difference between micro- and macronuclear hybridization patterns was found in the reference species, indicating that there are probably no rearrangements in the vicinity of the actin gene in *T. thermophila,* as observed by R. PEARLMAN (personal communication). In *T. malaccensis,* differences between Mic and Mac were observed with three enzyme digests out of a total of seven tested, indicating the existence of a Mic-specific fragment(s), *i.e.,* of a rearrangement site(s), in the flanking regions of this gene. None of the enzymes used detected a difference in *T. elliotti,* whereas one and five digests out of seven total tested detected differences between Mic and Mac in the more distantly related *T. borealis* and *T. pigmentosa,* respectively (Table 2).

Thus, the appearance of developmental rearrangements among the five species is "patchy," *i.e.,* incongruent with the phylogenetic tree. These results are consistent with multiple changes occurring in this region during the evolution of the five species that led to repeated generation and/or disappearance of developmental rearrangements. These considerations also apply to the PSR region discussed above.

The phosphoglycerate kinase (PGK) locus: Multiple changes also occur in developmental rearrangements at the PGK locus (see Table 2). At the PGK locus, differences between micro- and macronuclear hybridization patterns, *i.e.,* DNA rearrangements, were detectable in all species apart from *T. elliotti* and *T. borealis.* A change in the organization of this locus between *T. thermophila* and *T. malaccensis* was also indicated by the higher proportion of enzymes detecting a difference between Mic and Mac bands in *T. thermophila* than in *T. malaccensis.*

The calmodulin locus: A patchy appearance of rearrangements was also observed among the species examined at the calmodulin locus (see Table 2). In *T.*


A
MIC
EcoRI
PstI
TaqI
SauIIIA
RsaI
BglII
XbaI
BglII
HindIII
PSR900
MAC
RsaI
HindIII
EcoRI
PstI
TaqI
SauIIIA
BglII
XbaI
BglII
1 kb


B
T. thermophila
T. malaccensis
T. elliotti
T. borealis
T. pigmentosa
MAC
MIC
BglII
EcoRI
EcoRV
HaeIII
HhaI
HindIII
XbaI

thermophila, the data are consistent with an approximately 1.4-kb deletion in the vicinity of the calmodulin gene, as shown by KATOH *et al.* (1993). Rearrangements in the vicinity of the calmodulin locus were indicated in *T. malaccensis, T. elliotti* and *T. pigmentosa* but not in *T. borealis,* a species that is more closely related to *T. thermophila* than *T. pigmentosa.*

The cTt455 region: The cTt455 region contains three segments of Mic-specific DNA in *T. thermophila* (YAO *et al.* 1984; AUSTERBERRY and YAO 1987, 1988; AUSTERBERRY *et al.* 1989; GODISKA and YAO 1991). The existence of Mic-specific DNA segments was indicated in all species examined. The proportion of enzyme digests in which a difference between micro- and macronuclear hybridization is detectable was lower in most of the other species than in *T. thermophila,* indicating that some of the Mic-specific segments present in *T. thermophila* may be absent in the other species examined.

The IIC7 locus: The IIC7 locus contains a long (>13 kb) Mic-specific fragment (STEIN-GAVENS *et al* 1987; WELLS *et al.* 1994). Rearrangements were also detected in *T. malaccensis* and *T. elliotti,* but not in *T. borealis* (see Table 2). The IIC7.2 sequence, which is a 200-bp fragment of the Mic-specific segment, is apparently a single sequence in *T. malaccensis,* but is represented by many crosshybridizing sequences in *T. thermophila,* and in *T. elliotti* as well (WELLS *et al.* 1994 and data not shown). Thus, the evolution of the IIC7 region seems to have been complex, and no inference can be made as to whether the rearrangements in *T. elliotti* and *T. malaccensis* are homologous to the one in *T. thermophila.*

The isoleucyl-tRNA synthetase locus (CSANK and MARTINDALE 1992): Except for one digest in *T. malaccensis,* no differences were found in the isoleucyl-tRNA synthetase gene region between micro- and macronuclear hybridization patterns in any of the species examined.

In summary, with the possible exception of the IleutRNA synthase gene, none of the loci examined had a Mic-specific DNA segment present in all five species, *i.e.,* at six loci the presence of developmental rearrangements was variable among the five species. Thus, changes in the micronuclear genome that lead to the appearance/disappearance/inactivation of developmental rearrangements seem to have occurred frequently during the evolution of the five species.

DISCUSSION

In the present study, the distribution of several developmentally eliminated, *i.e.,* Mic-specific sequences, was examined in five species within the *T. pyriformis* complex of species. In addition, the locations of developmentally eliminated sequences relative to a number of single-copy Mac-retained genes were examined in the same species.

In Tetrahymena, many of the Mic-specific sequences belong to repetitive sequence families (YAO 1982; BRUNK *et al.* 1982; KARRER 1983; HOWARD and BLACKBURN 1985; TSAO *et al.* 1992). Middle, or moderately, repetitive sequences are found in the genomes of most organisms. One of the main characteristics of middle repetitive sequences in a large variety of organisms is that they can rapidly increase and decrease in numbers among closely related species or strains, unlike conventional single-copy genes.

As shown in Figure 2 and in the accompanying text, the Mic-specific sequences show large differences in numbers and intensities of crosshybridizing bands among the species examined and are often undetectable in some species. This is in striking contrast to Mac-retained sequences, most of which are present as single crosshybridizing bands, *i.e.,* as single copies in *T. thermophila* and its relatives, and are detectable in all species examined. Conservation in copy numbers is not only true for Mac-retained sequences that are known to be expressed, but also for sequences that are probably not expressed, such as the PSR sequence (see Figure 4), and for a repeated Mac-retained sequence representing a member of a cysteine protease family (data not shown, see also KARRER and STEIN-GAVENS 1990). It should be noted that not all subchromosomal fragments are necessarily present in the same numbers in the polyploid Mac, which results in variations in the relative abundance of single copy genes in the Mac. This has been observed for the histone H4 genes through detecting variations of hybridizing band intensities (BRUNK and NAVAS 1992). Such variations were not examined in the present work.

The individual sequence families have different and characteristic evolutionary histories. Most of the sequences are detectable in three or four of the species examined, with the hybridization intensities decreasing with increasing phylogenetic distance (see Figure 2A). These results can be explained by loss or gain of members within these sequence families and/or by rapid divergence such that some members are undetectable by hybridization. Similar patterns of distribution were found for repetitive sequences in other organisms (MOORE *et al.* 1981; DOWSETT 1983; ABAD *et al.* 1991). A transposon-like sequence in *T. thermophila* was undetectable in *T. pigmentosa* (CHERRY and BLACKBURN

FIGURE 4.—Comparison of micro- and macronuclear versions of the PSR900 region in five Tetrahymena species. (A) Restriction map of the PSR900 region in *T. thermophila.* The map is redrawn from M. WU (personal communication). The positions of the PSR900 probe, and the Mic-specific segment (a stippled box) are indicated. (B) Hybridization of the pSR900 probe to micro- and macronuclear DNA from five Tetrahymena species digested with the indicated restriction endonucleases. Preparation of samples, hybridization and washing conditions were as described in the legend to Figure 1. Asterisks under each pair of digests indicate that a difference is found between the micro-and macronuclear hybridization patterns.

1985). In other groups of organisms, a large fraction of middle repetitive sequences are assumed—and are often found—to be derivatives of mobile elements. By analogy, the high interspecies variability of Mic-specific DNA in Tetrahymena make it likely that these sequences may also be derivatives of mobile elements. However, it should be noted that repeat numbers in sequence families may change through transposition-independent mechanisms, such as unequal crossing over, inversion, translocation, *etc.* Also, of the five sequenced IESs in *T. thermophila,* only one has been found to contain long inverted repeats (WELLS *et al.* 1994). The other four 1-kb Mic-specific segments have no features characteristic of transposons, apart from direct repeats at the ends (AUSTERBERRY and YAO 1987, 1988; KATOH *et al.* 1993; HEINONEN and PEARLMAN 1994). Nevertheless, defective elements with very little sequence similarity to functional copies may exist, as has been hypothesized for the relationship between IESs and transposable elements in hypotrichous ciliates (HERRICK *et al.* 1985; RIBAS-APARICIO *et al.* 1987).

A somewhat stronger indication that a sequence may be derived from a mobile element was obtained from our studies in the case of the C8b/4 sequence. Sequences crosshybridizing to the C8b/4 probe are found in distantly related, but not in closely related species (Figure 2B). This scenario is often observed with transposable elements, (see *e.g.,* MARUYAMA and HARTL 1991). There are two major ways this pattern of evolution could have arisen: either by stochastic loss of the element from *T. malaccensis* and *T. elliotti,* or by interspecific transfer of the sequence from *T. borealis* or *T. pigmentosa* (or another distantly related taxon) to *T. thermophila.* Other complex situations can also be envisaged (see CAPY *et al.* 1994).

Whatever their origins, middle repetitive sequences are, in general, dispersed in the genome, as has also been shown for some of the repetitive Mic-specific sequences of *T. thermophila* (KARRER 1983). Therefore, interspecies changes in numbers of sequences must result in insertion and deletion of DNA segments during the evolution of closely related species. If the repetitive sequences are developmentally eliminated as in the case of Tetrahymena, their insertion and deletion can result in changes in the locations of developmental rearrangements among closely related species, unless insertion of Mic-specific repeats is restricted to preexisting Mic-specific sequences.

As shown in RESULTS (see Figure 4 and Table 2), essentially all of the loci displayed some variability with respect to the presence of Mic-specific DNA in the species examined. In fact, at a number of loci, the distribution of developmental rearrangements was incongruent with the phylogeny of the five species. For example, rearrangements were detectable at the actin locus in *T. malaccensis, T. borealis* and *T pigmentosa,* but not in *T. thermophila* or *T. elliotti* (Table 2). A parsimonious explanation for this situation would be that the ancestor of the five species had a rearrangement that is still detectable in *T. malaccensis, T. borealis* and *T. pigmentosa,* but that the rearrangement has disappeared in two independent lineages, (those leading to *T. thermophila* and *T. elliotti*). This interpretation would mean that the Mic-specific sequences that are deleted during these rearrangements are derived from a common ancestor in *T. malaccensis, T. borealis* and *T. pigmentosa.* Another possible explanation is that the ancestor of the five species did not contain a rearrangement and that independent genomic changes occurred in lineages leading to *T. malaccensis, T. borealis* and *T. pigmentosa.* This latter explanation is more likely, because the proportions of enzymes that detect differences between Mic and Mac are 3/7, 1/7 and 5/7 in the three species, indicating that rearrangements are at different distances from the actin gene. This makes it likely that unrelated rearrangements are present in these species, although changes in macronuclear DNA cannot be excluded as a source of variation in the distance between the rearrangement and the actin gene. Only detailed mapping and sequencing can determine whether the rearrangements are related to each other in the different species.

Some of the developmental rearrangements may be invariant in some of the species, as is likely to be the case for the PSR900 region in *T. thermophila* and *T. malaccensis* (Figure 4). It is also possible that the locations of some developmental rearrangements are conserved between species, even though insertion of other Mic-specific sequences into these segments occurs, generating composite elements. The occurrence of such events is indicated by the existence of clustered and scrambled sequences (YAO 1982; WHITE *et al.* 1985).

In summary, results from these studies provide circumstantial evidence that generation or loss of developmental rearrangements can occur within the genome of the five Tetrahymena species studied here. The present studies examine a relatively large portion of the genomes of the five species, and should be useful as a framework and basis for further detailed studies. Detailed comparisons of individual loci through mapping and sequencing will be necessary in different species to determine the molecular basis of changes shaping the micronuclear genome and its developmental reorganization. Further studies will concentrate on the sequencing of homologous loci in the five species examined. This will determine the types of change that occur within IESs and at the rearrangement junctions, and is expected to reveal whether—and how—IESs may be generated or lost.

Dr. ELLEN SIMON, University of Illinois, Urbana, is gratefully acknowledged for the donation of strains of different Tetrahymena species and continued advice during these studies. Most of the Mic-specific clones including the IIA8 and C8b and the IIC7 probes are from Dr. KATHLEEN KARRER, Marquette University; the actin and PGK

probes from Dr. R. PEARLMAN, York University, Toronto; the PSR900 clone from the histone H1 region from Drs. MIN WU and MARTIN GOROVSKY, University of Rochester; the PCA102 probe from the cTT455 region from Dr. MENG-CHAO YAO, Fred Hutchinson Cancer Research Center, Seattle; the calmodulin probe from Drs. T. TAKEMASA and Y. WATANABE, University of Tsukuba; the isoleucyl-tRNA synthetase gene from Drs. CSILLA CSANK and DUANE MARTINDALE, McGill University. I thank the above groups for discussion and reading the manuscript, in particular Dr. K. KARRER for continued support, help and interest in this work. Suggestions on the manuscript by Drs. C.F. BRUNK, L.A. KLOBUTCHER, G. HERRICK and the anonymous reviewers are also gratefully acknowledged. This work was supported by National Science Foundation ROW BSR 8909603 and by the Office of Research and Development at SIU. The following undergraduates are thanked for participation at different stages of this work: CHRIS SKINNER, CINDY JARVIS, JIM PERSINGER, BECKY BESHAM, RICH ZWICKLINSKY, BRAD MCCRARY, GERALD JONES, AMY HOERR, JERRY GRANA and CHRIS RYAN.

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Communicating editor: S. L. ALLEN