A Method for Mapping Germ Line Sequences in *Tetrahymena thermophila* Using the Polymerase Chain Reaction

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ABSTRACT

A method for mapping DNA sequences to specific germinal chromosomes in the ciliated protozoan *Tetrahymena thermophila* has been developed. This mapping technique (PCR mapping) utilizes the polymerase chain reaction and template DNA derived from nullisomic strains to directly assign micronuclear DNA sequences to specific micronuclear chromosomes. Using this technique, a number of unique sequences and short repetitive sequences flanked by unique sequences have been mapped to four of the five germinal chromosomes.

THE ciliated protozoan *Tetrahymena thermophila* has two nuclei which differ in both structure and function. The diploid germinal micronucleus is transcriptionally inactive, and contains five pairs of chromosomes which undergo normal meiosis and mitosis. The polyploid macronucleus, which directs the phenotype of the cell, contains about 45 times the DNA content of the haploid genome, and is derived from the micronucleus during sexual reproduction (reviewed in GOROVSKY 1980). During this developmental process, approximately 150–200 macronuclear chromosomes are produced from the five pairs of micronuclear chromosomes, with a concomitant elimination of approximately 15% of the micronuclear DNA sequences (YAO and GOROVSKY 1974; ALTSCHULER and YAO 1985; CONOVER and BRUNK 1986).

Although the number of genes known in Tetrahymena has expanded considerably in the last several years (BRUNS and CASSIDY-HANLEY 1993), genetic analysis of this organism has been hindered by a number of problems. Traditional genetic techniques have failed to produce a linkage map of the micronuclear genome despite a number of efforts to demonstrate meiotic linkage among various markers (ALLEN 1964; DOERDER 1973; SONNEBORN 1975; MCCOY 1977). The use of cytological methods to assign genes to specific chromosomes has been hindered by the fact that all five meiotic chromosomes are metacentric and approximately the same size (BRUNS and BRUSSARD 1981). A novel mapping approach was introduced by BRUNS *et al.* (1983), who utilized the nuclear dichotomy of this system to produce a number of strains nullisomic for various micronuclear chromosomes. Since the macronucleus is responsible for the somatic functions of the cell, it is possible to manipulate the chromosomal content of the micronucleus without impairing cell viability, as long as the cells remain genetically competent. The nullisomic lines thus produced have greatly facilitated genetic mapping in Tetrahymena (ALTSCHULER and BRUNS 1984), and have been successfully used to map a number of germ line mutations to specific micronuclear chromosomes (BRUNS *et al.* 1982; COLE *et al.* 1987; BLEYMAN and SATIR 1990; GUTIERREZ and ORIAS 1992; BLEYMAN *et al.* 1992; J. FRANKEL, personal communication). However, mapping by crossing to nullisomic lines is frequently difficult and time consuming because of the low fertility of the nullisomic strains. The nullisomic lines have also been used to map cloned DNA sequences to micronuclear chromosomes by Southern hybridization (ALLITTO and KARRER 1986; ALLEN *et al.* 1984; YAO 1982; MARTINDALE *et al.* 1985). Although successful, this approach is also technically demanding and time consuming, and requires the isolation of highly purified micronuclei from each of the nullisomic strains. Recently, a new approach based on the random amplified polymorphic DNA (RAPD) system (WILLIAMS *et al.* 1990) has utilized nullisomic cell lines to map a number of strain polymorphisms (J. H. BRICKNER, T. J. LYNCH and E. ORIAS, in preparation; BRUNS and CASSIDY-HANLEY 1993).

We present here a quick, simple method for mapping both unique and short repetitive micronucleus specific DNA sequences to micronuclear chromosomes based on the use of the polymerase chain reaction (PCR mapping). In both cases, appropriate choice of primers can be used to limit amplification to sequences present only at specific sites in the micronucleus. This molecular approach utilizes DNA from vegetatively growing nullisomic cells and is therefore completely independent of the low fertility of the nullisomic lines. PCR mapping by this technique analyzes only the micronuclear genome, but can be performed on small amounts of total cell DNA, thus eliminating the need for isolating purified micronuclei.

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TABLE 1

Strains

Strain		Genotype (micronucleus)	Phenotype (macronucleus)	Reference
CU427	Diploid	*Chx/Chx*	cy-s, VI	
CU361	N*3*	*Chx/Chx, Mpr/Mpr*	cy-s, mp r, IV	Bruns *et al.* (1983)
CU362	N*3*	*Chx/Chx*	cy-s, VII	Bruns *et al.* (1983)
CU363	N*3*	*Chx/Chx, Mpr/Mpr*	cy-s, mp-s, III	Bruns *et al.* (1983)
CU357	N*4*	*Chx/Chx, Mpr/Mpr*	cy-s, mp-r, IV	Bruns *et al.* (1983)
CU367	N*4*	*Mpr/Mpr*	mp-s, VI	Bruns *et al.* (1983)
CU366	N*5*	*Mpr/Mpr*	mp-s, II	
CU368	N*5*	*Chx/Chx*	cy-s, II	Bruns *et al.* (1983)
CU389	N*1L2R5*	*Chx/Chx*	cy-s, VI	
CU423	N*1L2R345*	*Chx/Chx, gal/gal*	cy-s, gal-s, III	
CU370a	N*1L2R45*	*Chx/Chx, cdaA?*	cy-s, mp-r, tr III	Bruns *et al.* (1982)
CU371a	N*1L2R3(p)4*	*Chx/Chx*	cy-s, mp-r, IV	Bruns *et al.* (1982)
CU379	N*1R35*	*Mpr/Mpr*	mp-s, II	
CU376a	N*1R2L*	*Mpr/Mpr, cdaA/cdaA*	mp-r, tr, IV	Bruns *et al.* (1982)
CU411	N*12*		mp-s, cy-r, VI	
CU382	N*123R4L*		cy-s, mp-s, VII	
CU377	N*2L34L*	*Chx/Chx, Mpr/Mpr, cdaA/cdaA*	cy-s, mp-r, tr IV	Bruns *et al.* (1982)
CU416	N*23R*		cy-s, mp-r, ts, II	
CU380	N*3R45*	*Chx/Chx, Mpr/Mpr*	cy-s, mp-s, II	
CU404	N*235*	*gal/gal, ts11/ts11*	gal-r, ts, VII	
CU426	N*24Lp*	*gal/gal*	gal?, mp-r, ts, ?	
CU425	N*1L2R4Lp*	*Chx/Chx, gal/gal*	cy-s, gal?, ts, II	

We have utilized the PCR mapping technique to localize both unique and repetitive micronuclear DNA sequences. The unique sequences mapped in this study are derived from internal sequences eliminated during the differentiation of the macronucleus from its micronuclear precursor. Two of the internally eliminated sequences sites chosen, regions M and R, are well characterized, contiguous sequences (Austerberry *et al.* 1984; Yao *et al.* 1984; Austerberry and Yao 1987, 1988) which provide a good internal test for the accuracy of the PCR mapping technique. The other four unique micronucleus specific regions mapped were derived from the left and right ends of two unrelated internally eliminated micronuclear sequences.

We have also mapped a number of chromosome breakage sites associated with the formation of the ends of the macronuclear chromosomes. These micronuclear chromosomal breakage sites are uniquely characterized by the occurrence of a short repetitive sequence (Cbs). During the formation of the macronuclear chromosomes, this sequence plus a small number of surrounding nucleotides are eliminated and the upstream and downstream regions flanking the Cbs give rise to the ends of two different macronuclear chromosomes (Yao *et al.* 1987). Utilizing these flanking sequences as primer sources, we have mapped the associated Cbs regions to specific micronuclear chromosomes. In addition, the Cbs region at the left end of the ribosomal DNA gene provided an opportunity to map a sequence whose micronuclear chromosomal location had already been established by alternative procedures (Bruns *et al.* 1985; Spangler and Blackburn 1985; Bleyman *et al.* 1992).

MATERIALS AND METHODS

Strains: All of the strains used in this study are described in detail in Table 1. All strains were derived from *Tetrahymena thermophila* inbred strain B. Nullisomics were produced and characterized as described in Bruns *et al.* (1983). The designation Nulli *x*, where *x* represents the appropriate missing chromosome(s), is used to indicate the micronuclear chromosomal composition of each nullisomic line. *Chx* and *Mpr* are dominant mutations conferring resistance to cycloheximide and 6-methylpurine, respectively. *gal* is a recessive mutation conferring resistance to 2-deoxygalactose. Sensitive and resistant phenotypes are indicated by -s and -r, respectively. *ts11* and *cdaA* are recessive temperature sensitive mutations causing, respectively, abnormal growth and cell division arrest at 37°. The temperature sensitive phenotype is indicated by ts, while the ability to grow normally at the elevated temperature is indicated by tr. Roman numerals indicate mating type. Untested phenotypes are indicated by "?."

Media and growth: All cells were grown and maintained in Neff medium [0.25% proteose peptone, 0.25% yeast extract, 0.5% glucose, 33 μM ferric chloride, 250 μg/ml penicillin G, 250 μg/ml streptomycin sulfate, and 1.25 μg/ml amphotericin B (Fungizone, GIBCO)].

Karyotypes: Cells to be karyotyped were mated at 30° to a "star" line (containing a defective micronucleus) as previously described (Bruns and Brussard 1981). After mixing prestarved cells, samples were taken at intervals from 4 to 5.5 hr, placed in distilled water for 5–7 min, pelleted by gentle centrifugation for 1–1.5 min at 1150 rpm in an IEC tabletop centrifuge, and resuspended sequentially in 50% methanol, 70% methanol and 3:1 absolute methanol:acetic acid. The final pellet was resuspended in approximately 100 μl of fresh 3:1 absolute methanol:acetic acid. Cells were dropped onto clean glass slides from a height of 6–12 inches and air dried. Chomosomes were stained with 4,6-diamidino-2-phenylindole (DAPI) (Cole and Bruns 1992) visualized and photographed using a Zeiss photomicroscope III equipped with a UV light source.

TABLE 2

Origin of primer sequences

Origin of primer sequences	Primer set designation	Source
Micronucleus specific segment of internal deletion M	m and m′	AUSTERBERRY and YAO (1988)
Micronucleus specific segment of internal deletion R	r and r′	AUSTERBERRY and YAO (1987)
Sequence flanking Cbs site	Tt701 and rTt701	YAO *et al.* (1987)
Sequence flanking Cbs site	Tt814 and rTt814	YAO *et al.* (1987)
Sequence flanking Cbs site	Tt819 and rTt819	YAO *et al.* (1987)
Sequence flanking Cbs site	Tt826 and rTt826	YAO *et al.* (1987)
Sequence flanking Cbs site	Tt835 and rTr835	YAO *et al.* (1987)
Sequence flanking the micronuclear rDNA 3′ CBS site	rib1, rib3, and rib2′	KING and YAO (1982); M. C. YAO (unpublished data)
Sequences at the left and right boundaries of TLR1 rearrangement	TLR1LT,rTLR1LT TLR1RT,rTLR1RT	K. KARRER (unpublished communication)
Sequences at the left and right junctions of an IES sequence	MSE1LT,rMSE1LT MSE1RT,rMSE1RT	R. PEARLMAN (unpublished communication)

DNA isolation: Total DNA was isolated from 10-ml cultures during log phase growth by the method of AUSTERBERRY and YAO (1987). DNA was resuspended in approximately 100 μl of sterile distilled H_2O and a 1:10 dilution of this stock DNA was used directly for PCR at a concentration of approximately 5–20 ng per reaction.

Micronucleus specific sequences: Table 2 lists all of the micronuclear sequences used and their sources.

PCR primers: PCR primer sequences were selected on the basis of G-C content and predicted product size. Oligonucleotide primers were synthesized by the Cornell University Biotechnology Synthetic and Analytical Facility. Table 3 lists the sequence of each of the primers used, the size of the DNA fragment produced by each primer pair and the temperature at which the PCR was carried out.

PCR protocol using *Taq* polymerase: Each reaction mix consisted of 1 × PCR buffer (10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$), 200–250 μM each dATP, dTTP, dGTP, dCTP, 0.3–0.5 μg of each primer, 1.25–2.5 units *Taq* polymerase, 2–3 μl whole cell DNA (prepared as described above) and sterile H_2O to a final volume of 25 μl. Reactions were run for 35–40 cycles consisting of 1 min at 94° or 95°, 2 min at 45–55° (depending on the G-C content of the specific primers) and 2 min at 72°, plus a final cycle of 1 min at 94°, 2 min at 45° to 55° and 10 min at 72°.

PCR protocol using Vent polymerase: Vent DNA polymerase (New England Biolabs) was used to produce larger size product where indicated. For reactions using Vent polymerase, the following protocol was used: 1 × Vent PCR buffer [20 mM Tris-HCl(pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100], 100 μg/ml bovine serum albumin, 200 μM each dATP, dTTP, dGTP, dCTP, 0.3–0.5 μg each primer, 0.5 unit Vent DNA polymerase, 3 μl whole cell DNA, and sterile H_2O to a final volume of 25 μl. PCR was run for 40 cycles of 1 min at 94°, 1 min at 55° and 5 min at 72° plus a final cycle of 1 min at 94°, 2 min at 55° and 10 min at 72°.

Agarose gel electrophoresis: PCR products were visualized on horizontal agarose gels. Fragments smaller than 1 kb were run on 2% gels composed of a mix of 3 parts NuSieve (FMC) to 1 part Seakem agarose (FMC) in 0.5 × TBE buffer. Larger products were run on 1% Seakem agarose gels in 0.5 × TBE buffer. Gels were stained with ethidium bromide and visualized on a UV light box.

RESULTS

Oligonucleotide primers complementary to previously identified micronuclear DNA sequences were synthesized and used in a series of polymerase chain reactions utilizing whole cell DNA from various nullisomic and diploid strains as template. Each primer set produced a PCR fragment of predictable size with template DNA from diploid control strains. The presence or absence of this specific fragment was used to assign each primer set to one of the micronuclear chromosomes, since only nullisomics missing the chromosome or chromosome arm carrying the sequences homologous to the primer sequences consistently failed to produce a PCR fragment of the appropriate size. To confirm that the PCR product of predicted size was in fact produced from the site of interest, the fragments produced by eight of the primer sets, described in detail below (Tt826, Tt814, Tt819, Tt701, Tt835, rib, M and R), were run on an agarose gel, blotted and probed with oligonucleotides homologous to internal sequences. In all cases, the appropriate sized fragment hybridized successfully to the internal oligonucleotide (results not shown).

Mapping internally eliminated sequences: M and R are neighboring, micronucleus-specific sequences which are eliminated during the formation of the macronuclear chromosomes (AUSTERBERRY *et al.* 1984). Figure 1 illustrates this relationship and indicates the position of the associated primer sequences used in this study. Since the sequences are closely linked, independent PCR reactions for M and R primer sets should give the same positive or negative results using template DNA isolated from each of our nullisomic strains.

As a further test of reproducibility, a larger fragment bridging the two regions should also be produced using the outside primers from each set (primers m and r′, see Figure 1). The large PCR fragment produced by these two primers should map to the same chromosome as the fragments produced by the m-m′ and r-r′ primer sets individually.

As shown in Table 4 and Figure 2, A and B, primer sets from both region M and region R consistently produced PCR fragments of the predicted size with all template

TABLE 3

Primer sequences

Primer name	Primer sequence 5′ → 3′	Predicted PCR product size (bp)	PCR temperature (°C)
m	GAGGTTGCTATCCTGTACTT	476	50
m′	TGCTGACTGGATACATAATG		
r	ATGAACGAAGTATTTCCGTT	918	50
r′	TTGAGTAACTATGTGTATTA		
Tt835	CTAACAGTTTAATTAATAAG	249	45
rTt835	TTTATCTATTATTTAAAAAC		
Tt701	CTTACCACTTTGTTTGATTA	238	55
rTt701	CTAAACATATTTTGTTCATA		
rTt819	GATCAAATTCATTTTAATTA	503	50
rTt819	GATCAAACTGAGTACTCACT		
rib1	TGATAATGCATAAGTAGCAT	~430	55
rib2′	ATAGCAAATTGTTATATAGA		
rib3	GGGTTTTAACTTATTTTTAA	~510	55
rib2′	ATAGCAAATTGTTATATAGA		
Tt814	CTACATTAAAAATGATAAAA	258	50
rTt814	CATGCATTTTTAATTTTGAG		
Tt826	CTTGAATTAATTCTTATTCT	345	50
rTt826	TTTGTTCTTCATAATCTGAA		
MSE1RT	CTTTCCCTAAGAGAAAATGA	691	55
rMSE1RT	CTTGTTTGGTTTTTTACTTG		
MSE1LT	TCTTTTCACTTAAATTTCAA	~721	55
rMSE1LT	GTACTTTTATGTCGATAACA		
TLR1RT	CGTGTATCAGAAAGAAATGA	786	55
rTLR1RT	AATGTGAATTTCGATTCGAT		
TLR1LT	ACTCTTTTGACTAAAGTTAC	1730	55
rTLR1LT	CTGTTTAGAAGATTTGATGA		

Whenever possible, primer sequences were selected to maximize GC content. Predicted PCR product size is based on known sequence, or, in the case of the rib primers and the MES primers, estimated from the size of micronuclear clones from which partial sequence has been determined.

DNAs except those from lines nullisomic for chromosome *4*. Also, as shown in Figure 2C, the two outside primers (m and r′) produce the expected 4.5-kb fragment when template DNA from diploid controls is used, but fail to produce the expected fragment when template DNA from a line nullisomic for chromosome *4* is used in the reaction. Thus both region M and region R can be assigned to chromosome *4*. M and R were further localized to the left arm of chromosome *4* based on PCR with DNA from nullisomic strains CU377 and CU382. Each of these lines has been shown by current genetic tests to be missing the left arm of chromosome *4*, but to have retained the right arm of that micronuclear chromosome (results not shown). In both instances, M and R primers failed in repeated tests to produce the predicted PCR fragment, indicating that the homologous region must lie on the missing left arm.

Mapping the ribosomal DNA locus: The micronuclear ribosomal DNA (rDNA) has been previously mapped to the left arm of chromosome *2* (BRUNS *et al.* 1983, 1985; SPANGLER and BLACKBURN 1985) and thus provides an opportunity to test PCR mapping using a gene whose location has already been established. Figure 3 shows the organization of the micronuclear DNA immediately adjacent to the rDNA genes. The primer sets rib1 and rib2′ define a portion of the micronuclear genome originally thought to be eliminated from developing macronuclei (YAO *et al.* 1987). Initial estimates based on the size of the clone containing the micronuclear rDNA gene predicted a PCR fragment about 400 bp long. Our single PCR product appears to be about 430 bases in length. Surprisingly, however, PCR using template DNA from all of our nullisomic strains gave a positive band of the appropriate size (Figure 4A) indicating the presence of the homologous sequence in all of the strains. Since the template DNA included both micronuclear and macronuclear DNA, and since all nullisomic lines must have completely euploid macronuclei to survive, we conclude that the DNA acting as template for the production of this PCR product must also be present in the macronucleus.

The observation that the micronuclear sequence just flanking the rDNA is actually found in the macronucleus was confirmed by Southern hybridization. The PCR fragment was synthesized using wild-type DNA from CU427 as template and rib1 and rib2′ as primers, and then used as a probe to hybridize with macronuclear DNA prepared from purified macronuclei. The results indicate

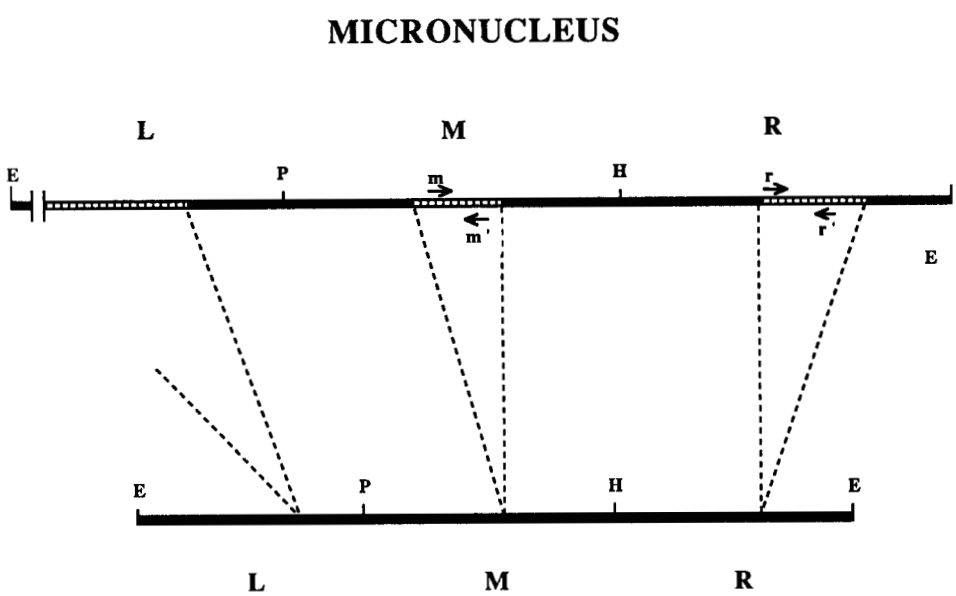

FIGURE 1.—Diagrammatic representation of a defined DNA rearrangement involving elimination of adjacent internal micronucleus specific sequences. Relevant features of an *Eco*RI fragment of the micronuclear DNA and the corresponding region of the macronuclear DNA (YAO *et al.* 1984; AUSTERBERRY and YAO 1987) are shown. Both sequences are bounded by *Eco*RI sites (E) and can be conveniently divided into three regions (L, M, and R) by the restriction enzymes *Hpa*II (P) and *Hha*I (H). Within each region, a segment of micronucleus specific sequence, indicated by the vertically hatched line, is deleted in the macronucleus. The dashed lines indicate the junctions of the deleted segments. The left junction of the deletion in region L has not yet been characterized. The location and 5′ to 3′ orientation of the primers used for PCR (m, m′, r, and r′) are indicated by arrows.

that the sequence is indeed present as a single copy sequence in the macronuclear genome (data not shown). Its flanking restriction sites are also in good agreement with what is known for this region (YAO 1981). Thus the 3-kb DNA segment previously shown to be eliminated from this region during macronuclear development (YAO 1981) does not include this sequence, and cannot be a simple terminal deletion associated with chromosome breakage.

To ensure the production of a PCR product from micronucleus specific template within this region, we chose a PCR primer set that required DNA synthesis across the Cbs (chromosome breakage sequence) associated with the 3′ end of the micronuclear ribosomal DNA. Since all the Cbs sequences are micronucleus specific (YAO *et al.* 1987), PCR reactions primed by sequences flanking a Cbs region must yield unique products synthesized solely from micronuclear DNA template, even if both primer sequences are found in both micronuclear and macronuclear DNA. As Figure 3 indicates, the first 3 bases of the 5′ end of primer rib3 overlap the last 3 bases of the 3′ end of the rDNA gene itself and all 20 bases of the primer sequence are on the 5′ side of the Cbs associated with this region. Primers rib3 and rib2′ were then used with the same set of nullisomic template DNAs. Since this primer is 79 bases upstream of primer rib1, we predicted a PCR fragment of approximately 510 bp in length. As shown in Table 3 and Figure 4B, these primers produced the appropriate fragment, which hybridized to an internal sequence from within this region, with all template DNAs except those derived from lines that were nullisomic for all of chromosome *2* or missing the left arm of chromosome *2*. Thus, when primers flanking the Cbs region are used, the 3′ end of the micronuclear rDNA gene maps by PCR to the left arm of chromosome *2*. This result demonstrates the utility of choosing oligonucleotides that prime DNA synthesis across a region known to be eliminated in the macronucleus.

It should be noted that when chromosome 2L is missing, a second band of slightly smaller size is produced by the primer set. This band is not present in detectable amounts at the DNA concentrations run on our gels when chromosome 2L, bearing the rDNA genes, is present in the template DNA, but has been observed in reactions using template DNA from 12 strains missing chromosome *2* or 2L, regardless of the nullisomic condition of the remaining chromosomes (results not shown). The source of this second band is unknown. However, since we know the size of the fragment produced by these primers from the correct template, the production of an alternate band of different size in the absence of that template does not interfere with the use of this primer set as an indicator of the presence or absence of chromosome 2L.

Mapping repetitive sequences which mark the breakage sites giving rise to the ends of macronuclear chomosomes (Cbs sequences): YAO *et al.* (1987) isolated a number of micronuclear clones containing chromosome breakage sequences. One of these clones, Tt701, contained the micronuclear version of a chromosome breakage site which was flanked by two macronuclear chromosome ends which had also been cloned and sequenced. This fortuitous event allowed the exact determination of the boundaries of the micronucleus specific DNA eliminated during the formation of the two ma-

TABLE 4

PCR results with various nullisomics

Strain	Chromosome constitution	Primer set: MSE1Rt *1*L	MSE1Lt *1*L	Tt826 *1*R	rDNA 2L	Tt814 2R	Tt701 *3*L	Tt819 *3*R	TLR1Rt *3*R	TLR1Lt *3*R	M *4*L	R *4*L	Tt835 *4*L
CU427	Diploid	+	+	+	+	+	+	+	+	+	+	+	+
CU361	N*3*	+	+	+	+	+	–	–	–	–	+	+	+
CU362	N*3*	+	+	+	+	+	–	–	–	–	+	+	+
CU363	N*3*	+	+	+	+	+	–	–	–	–	+	+	+
CU357	N*4*	+	+	+	+	+	+	+	+	+	–	–	–
CU367	N*4*	+	+	+	+	+	+	+	+	+	–	–	–
CU366	N*5*	+	+	+	+	+	+	+	+	+	+	+	+
CU368	N*5*	+	+	+	+	+	+	+	+	+	+	+	+
CU389	N*1L2R5*	–	–	+	+	–	+	+	+	+	+	+	+
CU423	N*1L2R345*	–	–	+	+	–	–	–	–	–	–	–	–
CU370a	N*1L2R45*	–	–	+	+	–	+	+	+	+	–	–	–
CU371a	N*1L2R3(p)5*	–	–	+	+	–	–	–	+	+	+	+	+
CU379	N*1R35*	+	+	–	+	+	–	–	–	–	+	+	+
CU376a	N*1R2L*	+	+	–	–	+	+	+	+	+	+	+	+
CU411	N*12*	–	–	–	–	–	+	+	+	+	+	+	+
CU382	N*123R4L*	–	–	–	–	–	+	–	–	–	–	–	–
CU377	N*2L34L*	+	+	+	–	+	–	–	–	–	–	–	–
CU416	N*23R*	+	+	+	–	–	+	–	–	–	+	+	+
CU380	N*3R45*	+	+	+	+	+	+	–	–	–	–	–	–
CU404	N*235*	+	+	+	–	–	–	–	–	–	+	+	+
CU426	N*24L(p)*	+	+	+	–	–	+	+	+	+	–	+	–
CU425	N*1L2R4L(p)*	–	–	+	+	–	+	+	+	+	–	–	+

Production of the predicted PCR fragment with each of the primer sets is shown. "+" or "–" indicates the presence or absence of the predicted fragment in PCR utilizing template DNA from the nullisomic line shown in the "Strain" column and the primers listed in the "Primer set" row. Negative results were confirmed by several independently repeated reactions. The "a" designation following some of the strain names indicates that the chromosomal composition of that strain has changed since previous publication. The additional chromosome loss is underscored. The loss of chromosome *5* is based on genetic analysis.

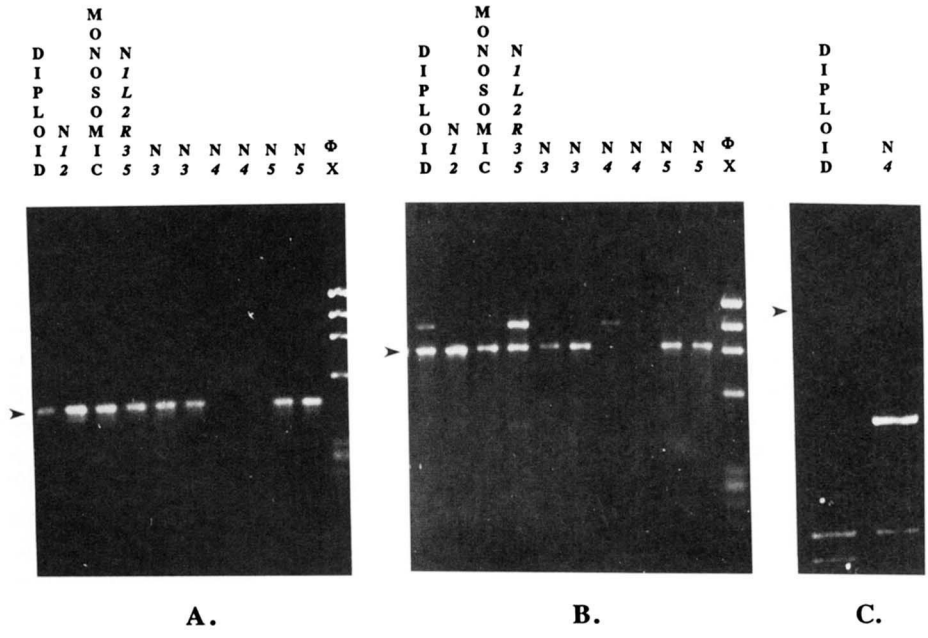

FIGURE 2.—Analysis of the PCR fragments produced using micronucleus specific primers from (A) region M and (B) region R. Whole cell DNA from the strains indicated was used as template in a series of PCR reactions with primers from each region, as described in MATERIALS AND METHODS. *Hae*III cut ϕX174 was run as a size standard. Arrows indicate the predicted PCR products based on the location of the primers within each region. Negative results were confirmed by several separate reactions. (C) The linkage between M and R was demonstrated with template DNA from a normal diploid strain by the production of the predicted large fragment using the external primers from M and R in a single reaction. As shown in the right lane, template DNA from a strain nullisomic for chromosome *4* failed to produce the M-R fragment.

cronuclear chromosome ends. In this case, a 54-bp segment is eliminated during the formation of the ends of the two macronuclear chromosomes. It was therefore possible to select primers within the macronuclear sequences flanking both sides of the eliminated micronucleus specific region to determine if such primers, sharing homology with both macronuclear and micronuclear DNA sequences, would give rise to a unique micronuclear PCR fragment when whole cell DNA was used as template. Figure 5 shows the arrangement of this region and the location of the primers used.

The results of PCR using these primers with various template DNAs are shown in Table 4 and Figure 6. A fragment of the correct size was produced in all reac-

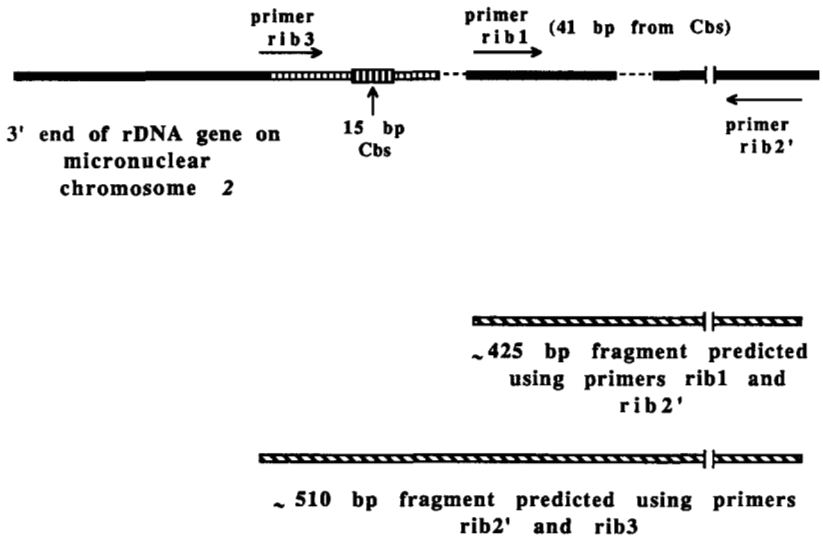

FIGURE 3.—Schematic representation of the 3′ end of the micronuclear ribosomal DNA region. The location of each of the primers used for PCR analysis of this region are shown, as well as their relationship to the Cbs region associated with the 3′ end of the ribosomal DNA. The sequence of the 3′ end of the micronuclear ribosomal gene and the location of the adjacent Cbs region have been determined (KING and YAO 1982; YAO *et al.* 1987). In addition, the micronuclear region 3′ to the Cbs has been cloned and partially sequenced (C.-H. YAO, unpublished communication), providing a downstream primer site and an estimate of the approximate size of the PCR fragments expected from each set of primers. The micronucleus specific sequence that is removed during the formation of the macronuclear chromosome is indicated by the vertically hatched line. Since the exact length of the micronuclear eliminated sequence is unknown, the connection to sequence present in the macronuclear DNA is shown as a dashed line.

tions except those involving template DNA from lines nullisomic for chromosome *3*. This micronuclear breakage site must therefore be on chromosome *3*. Tt701 has been further localized to the left arm of chromosome *3* based on PCR results using template DNA from three lines, CU380, CU382 and CU416. These lines have been shown by genetic analysis to be missing the right arm of chromosome *3*, but have not yielded analyzable progeny in matings with strains carrying genetic markers on *3*L. Since Tt701 primers produce the expected signal with template from these three lines, but fail to give the predicted signal with lines solely missing all of chromosome *3*, the Tt701 sequence must lie on a region of left arm of chromosome *3* present in all three of the above lines.

We conclude that micronuclear mapping can be accomplished using unique primer sequences which are present both in the micronucleus and in the macronucleus when such primers span an eliminated micronuclear chromosomal region. Such primer sets will produce a PCR fragment of the correct, predictable size only in the presence of micronuclear DNA template which contains both primer sites and the appropriate spanning region.

Mapping other chromosome breakage sites: In a similar fashion, four other chromosome breakage sequences, Tt835, Tt819, Tt814 and Tt826 (YAO *et al.* 1987), were mapped to specific micronuclear chromosomes using primers derived from flanking sequences upstream and downstream of the actual 15-bp Cbs. Although corresponding macronuclear clones are not available for these sites to define the extent of macronuclear eliminated sequences around the Cbs, the results from the previous two sections indicate that it does not matter if the primers are homologous to both micronuclear and macronuclear sequences as long as the two primer sets lie on opposite sides of the Cbs.

Primers derived from Tt835 produce the expected 249-bp fragment with all template DNAs except those derived from strains nullisomic for micronuclear chromosome *4*. Therefore we have assigned assign Tt835 to that chromosome. We have further localized Tt835 to the left arm of chromosome *4* based on the PCR results with two nullisomic strains, CU377 and CU382, shown by current genetic tests to have lost the left arm of chromosome *4*, but to have retained the right arm of that micronuclear chromosome. Tt835 primers consistently failed to produce the predicted PCR fragment with all of these strains, indicating that the homologous region lies on the missing left arm.

Interestingly, when these results are compared to those obtained for M and R (also on chromosome *4*L) there are two discrepancies, CU425 and CU426. DNA from strain CU425 does not produce the expected fragment with primers from either M or R, but does yield a fragment of the correct size with Tt835 primers. We interpret this to mean that CU425 is missing part of chromosome *4*L, and that M and R are located on the missing segment, while Tt835 is located on the part of chromosome *4*L remaining in this strain. Cytological analysis of line CU425 (Figure 7D) indicates that this line does in fact contain two chromosomes missing the end of one arm. Cytological analysis of CU426 indicates that this line has undergone chromosome fragmentation (Figure 7C). The PCR results indicate that the region containing Tt835 is lost in that line, as is region M, although R is retained. Loss of complete micronuclear chromosomes or parts of chromosomes in the course of routine strain transfer and vegetative culture has been previously reported (ALLEN *et al.* 1984).

Cbs sequence Tt819 has been mapped to chromosome *3* (see Table 3). Primers from this region produce the predicted 503 bp fragment with all template DNAs

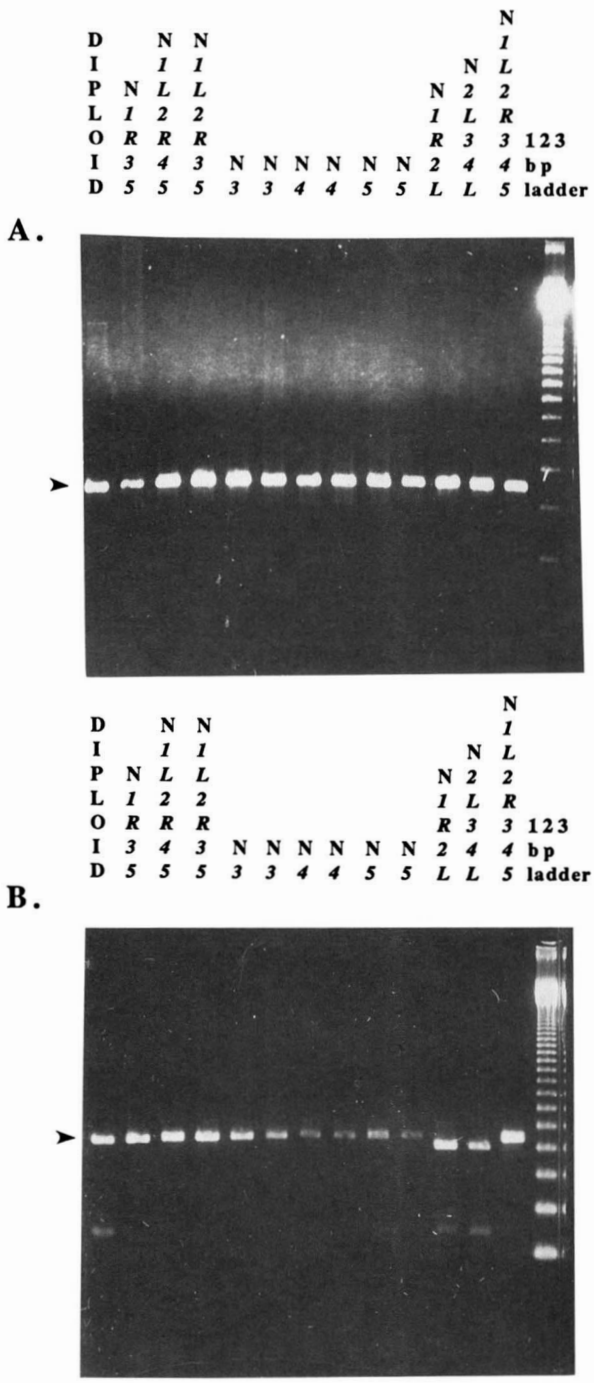

FIGURE 4.—Analysis of the PCR fragments produced using primers from the 3′ region adjacent to the micronuclear ribosomal DNA. Whole cell DNA from strains nullisomic for the chromosomes indicated was used as template in a series of PCR reactions as described in MATERIALS AND METHODS. A 123-bp ladder was run as a size standard. (A) Results using primers located 3′ to the Cbs region, (B) results using primers located on opposite sides of the Cbs region. As shown, a slightly smaller secondary band is often seen in reactions which do not produce the primary product.

except those derived from strains nullisomic for micronuclear chromosome *3*. Tt819 has been mapped to the right arm of chromosome *3*, based on the failure of primers from this region to yield product with template DNA from the three strains nullisomic for the right arm of chromosome *3* (CU382, CU380 and CU416) which give a positive signal with Tt701.

Mapping regions which lie on chromosomes *1* and *2* is more complex. We do not yet have lines singly nullisomic for either of these chromosomes. All of the lines missing chromosome *1* or *2* are multiply nullisomic, and cytological and genetic analysis indicate that many of these lines are missing individual chromosome arms rather than entire chromosomes. It is therefore necessary to compare results among a number of different lines to map a micronucleus specific region to either of these chromosomes.

PCR using primers from the Tt814 region produces the expected fragment with template DNA from lines nullisomic for chromosomes *3*, *4* or *5* (Table 4). Therefore Tt814 is not on any of these micronuclear chromosomes. The predicted Tt814 fragment is not produced in reactions using DNA from lines previously shown by genetic analysis to be missing either all of chromosome *2*, or only the right arm of that chromosome. Reciprocally, the expected fragment is produced with template DNA from lines which by previous analysis are missing the right or left arms of chromosome *1*. Since the PCR fragment expected using Tt814 primers fails to be made only when chromosome *2* is absent, we assign Tt814 to chromosome *2*. Furthermore, since the predicted fragment is made when only the left arm of chromosome *2* is missing, but fails to be produced when only the right arm is absent, we further assign Tt814 to the right arm of chromosome *2*.

Primers from the Tt826 region produce the predicted 345 bp fragment with all template DNAs derived from lines nullisomic for chromosomes *2*, *3*, *4* or *5* and the left arm of chromosome *1* (Table 3). However, DNA from lines missing chromosome *1* or *1*R do not support the production of the expected fragment. These results place Tt826 on the right arm of micronuclear chromosome *1*.

Mapping the left and right ends of internally eliminated sequences: We have mapped the left and right ends of two internally eliminated sequences, MSE1 (*m*icronuclear *s*equence *e*lement 1, provided by R. PEARLMAN) and TLR1 (formerly region IIC7, provided by K. KARRER). Both primer sets derived from the MSE1 sequences (Figure 8) were used in a series of PCR reactions using as template DNA from a variety of nullisomic lines. Based on the results of these reactions, both primer sets map to the left arm of chromosome *1* (Table 4). This result also confirms that the two sides of the macronuclear junction which are brought into juxtaposition by this developmental process originate from the same micronuclear chromosome arm.

The TLR1 sequences (Figure 9), although known to be adjacent to the macronuclear sequences flanking a junction site, have not yet been linked to each other through sequencing or restriction analysis, raising the

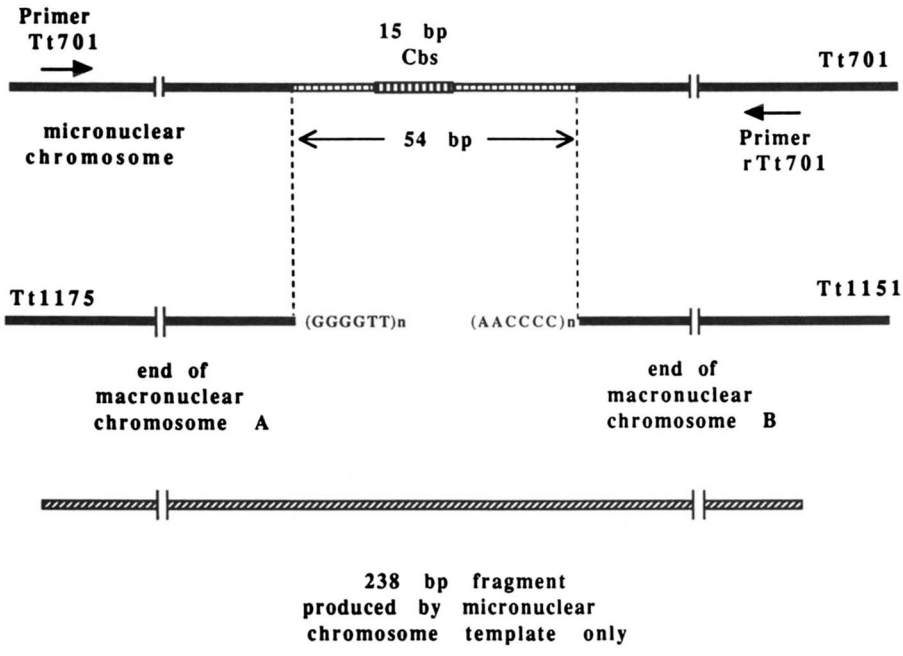

FIGURE 5.—Diagrammatic representation of the micronuclear and macronuclear DNA associated with a Cbs breakage site (clone Tt701). Both the micronuclear and macronuclear versions of the DNA associated with this breakage site have been cloned (YAO *et al.* 1987). As indicated by the vertically hatched line, the micronuclear version contains a 54-nucleotide sequence, including the Cbs, which is not retained in the macronucleus. The breakage at this junction gives rise to the ends of two macronuclear chromosomes as indicated by the vertical dotted lines. The relative positions of the primers used for PCR are indicated, along with the size of the expected micronucleus specific PCR product.

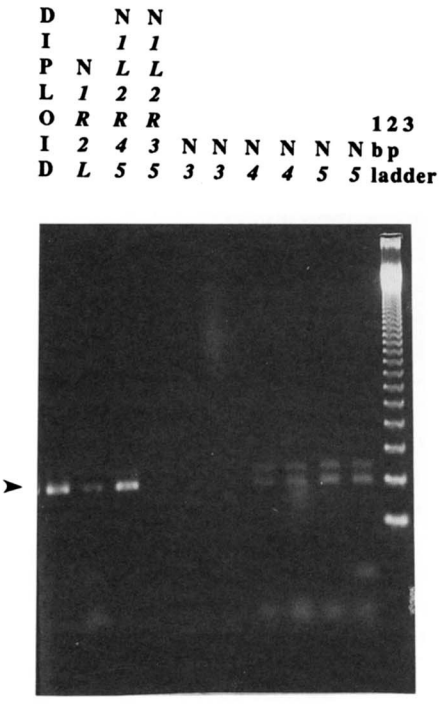

FIGURE 6.—Analysis of the PCR fragments produced with primers flanking the Cbs site isolated in clone Tt701. Whole cell DNA from the nullisomic strains indicated was used as template in a series of PCR reactions as described in MATERIALS AND METHODS. A 123-bp ladder was used as a size standard. Negative results were confirmed by several separate reactions. Extraneous bands of varying sizes were considered secondary PCR products and were not considered in the analysis.

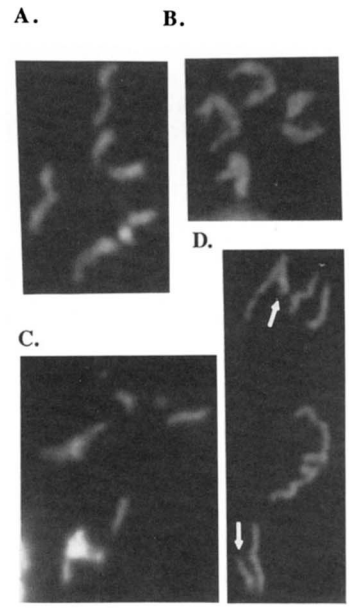

FIGURE 7.—Karyotypes of (A) normal diploid cell, (B) a standard nullisomic line missing chromosome *3*, (C) strain CU426 and (D) strain CU425. A and B show the typical thickened appearance of condensed chromosomes at metaphase I in *T. thermophila* under the conditions used in this study. C illustrates the frequent breakage occurring in CU426 which results in fragmentation of the chromosomes. D typifies the chromosomal constitution of CU425. The thin chromosome morphology of this line is suggestive of monosomy, and several of the chromosomes are abnormal (arrows indicate broken arms which are typically observed in this line).

question whether the macronuclear chromosome might be produced by the joining of sequences arising from different micronuclear chromosomes. As seen in Table 4, primer sets derived from both of these regions gave identical results with all nullisomic template DNAs tested, mapping to chromosome *3*R. Thus it is clear that both ends of the macronuclear junction originate from the same micronuclear chromosome arm.

DISCUSSION

We have developed a quick and relatively easy method of mapping micronuclear DNA sequences to specific mi-

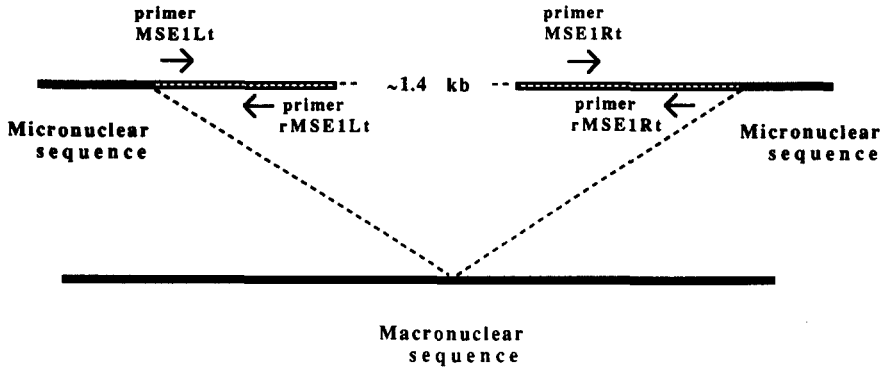

FIGURE 8.—Schematic representation of the region containing the internally eliminated sequence from which the MSE1 primers were derived (R. PEARLMAN, unpublished communication). Sequences for all primers were chosen from within the micronuclear specific region, indicated by the vertically hatched line. PCR reactions were conducted with primer sets derived from both the left and the right ends of this eliminated sequence as shown.

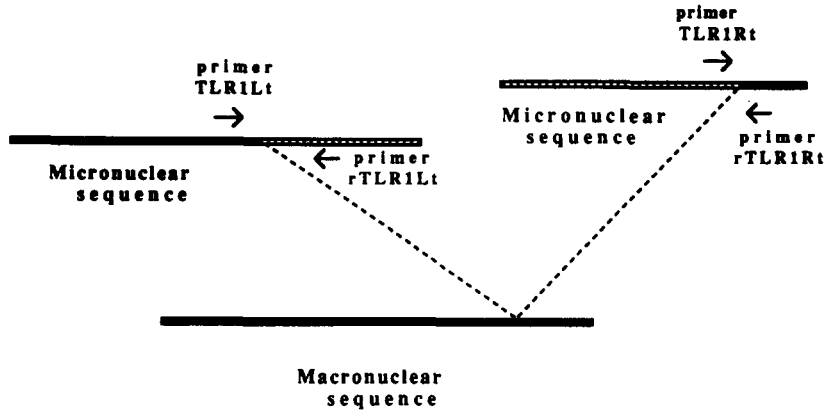

FIGURE 9.—Schematic representation of the TLR1 rearrangement from which the TLR primers were derived (K. KARRER, unpublished communication). Primer sets were selected from the right and left ends of this rearrangement. As shown, one primer from each set was derived from micronuclear specific sequence, indicated by the vertically hatched line, and one from the adjacent macronuclear region. Micronuclear sequences are not drawn colinearly because, prior to PCR mapping, the left and right sequences had not been linked by other analytical methods.

cronuclear chromosomes using the polymerase chain reaction. In this technique, pairs of primers homologous to sequences including but not necessarily restricted to micronucleus specific sequences, are used in a series of individual polymerase chain reactions using as template whole cell DNA from a panel of nullisomic strains. The size of the PCR fragment synthesized from the correct site on the micronuclear DNA is predictable since the original sequence is known. The production of such a PCR fragment is completely dependent on the presence in the micronucleus of a template sequence containing regions homologous to both primers and separated by the appropriate distance. The DNA sequence containing each primer set is assigned to a specific chromosome or chromosome arm based on the presence or absence of this unique PCR product. DNA from all strains except those missing the specific chromosome or chromosome arm bearing the sequence in question should function as template for the PCR production of the predicted fragment. Absence of an expected product using DNA from specific nullisomic strains as template can be used to map chromosomal location. The production of secondary PCR products is generally not a problem since the exact size of the fragment of interest is known and other size fragments can be ignored. Agarose gel electrophoresis of PCR products provides a simple visual assay for the presence or absence in each template DNA of the primer associated micronuclear sequence. It is important to note that for each analysis, several lines nullisomic for a given chromosome were used and each line was assayed at least twice. Accurate mapping is dependent on consistent results with multiple lines nullisomic for the same chromosome.

Mapping accuracy is also dependent on maintaining stable nullisomic lines. There has been loss of whole chromosomes or, much more commonly, chromosome arms in some of our strains following their initial genetic characterization. It is important to note that, except when kept in continuous vegetative culture over a long period of time, singly nullisomic lines are completely stable. None of the singly nullisomic strains used in this study have changed from their original characterization. However, some of the multiply nullisomic lines have changed over time. In the majority of instances where change has occurred, strains in which a single chromosome arm was initially characterized as missing have lost a second chromosome arm. Additional individual chromosomes were also lost from some of the multiple nullisomics. To corroborate the PCR analysis, we have retested some of the strains which show a changed chromosomal composition for the loss of genetic markers carried on the newly missing chromosome arms. For example, CU376a and CU379, originally characterized as N*2L* and N*3,5*, respectively (BRUNS *et al.* 1982; P. J. BRUNS, personal communicaton) have by PCR analysis now lost the right arm of chromosome *1*. Both of these strains originally carried the gene for cycloheximide resistance on *1*R and the gene for 6-methylpurine resistance on *2*R. Progeny from outcrosses of each of these two lines exhibit resistance to 6-methylpurine, but are sensitive to cycloheximide, thus substantiating the loss of chromosome *1*R as indicated by the PCR

analysis. CU411, originally designated N*1R* (P. BRUNS, unpublished communication), and now characterized as N*1,2*, was similarly examined for the loss of the 6-methylpurine resistance gene carried on *2*R (G. HERRICK and J. WARD, unpublished communication). All of the progeny from an outcross of this line to a heterokaryon carrying resistance to paromomycin in its micronucleus were resistant to paromomycin, but sensitive to 6-methylpurine, again substantiating the PCR analysis.

Since it is known that cells in continuous culture do lose micronuclear chromosomes and parts of chromosomes (ALLEN *et al.* 1984), we assume that the chromosomal losses observed occurred in the period between the development of the nullisomic lines and the time when the lines used in this analysis were frozen. We have since developed a technique for long term storage in liquid nitrogen which yields a dependably high recovery of viable cells (manuscript in preparation) and recommend that all strains used as a source of template DNA for PCR, especially the multiple nullisomics, be maintained frozen in liquid nitrogen, and not exposed to long term continuous culture. We have designated all the previously published stocks whose chromosomal composition has changed with the original stock number accompanied by an "a." The changes in nullisomy are underlined in Table 4.

We have mapped twelve DNA regions to four of the five micronuclear chromosomes. Six of these regions are associated with internal micronuclear sequences that are eliminated during macronuclear differentiation. Six others are micronuclear sequences associated with the formation of the ends of the macronuclear chromosomes during development, including the region associated with the formation of the 3′ end of the macronuclear rDNA chromosome. Two of the micronuclear internally eliminated sequences, M and R, provide an excellent internal control for the PCR mapping procedure. The consistent results obtained with these two contiguous regions in PCR reactions involving a variety of nullisomic lines, as well as the accurate assignment of the 3′ end of the micronuclear rDNA to its known micronuclear chromosomal location, indicate that mapping with PCR is both reliable and consistent with the results obtained from other mapping procedures.

The localization of the TLR1 and MSE1 sequences exemplify the utility of the PCR mapping approach in resolving mapping questions not easily amenable to other techniques. Using this procedure, it is possible to examine the micronuclear origins of DNA sequences at macronuclear junctions even when internal segments of the micronuclear genome are lost during the formation of a macronuclear chromosome. The mapping of these regions also further substantiates the effectiveness of using either two micronuclear specific primers or one micronuclear primer and one primer which occurs on both the micronuclear and the macronuclear chromosomes.

Results obtained with all 12 primer sets tested thus far indicate that the production of a specific PCR product from a micronuclear template is not affected by the high concentration of macronuclear DNA present in total genomic DNA, nor by variations in the number of nontarget chromosomes in multiply nullisomic micronuclei. In all cases, the consistent production of the PCR fragment of appropriate size was uniquely associated with the presence or absence of a specific micronuclear chromosome or chromosome arm. It is important to note that the presence of macronuclear DNA completely homologous to both primers did not interfere with the desired reaction when those primer sequences were located on the ends of separate macronuclear chromosomes and only linked in the micronuclear DNA. This suggests that, at least in the instance where the homologous macronuclear regions were physically separated in a manner which prevented the geometric accumulation of product, competition for primers between micronuclear and macronuclear DNA does not seem to be a problem. Therefore it should be possible to map even very short internal micronucleus specific sequences by using one primer from the micronucleus specific region and one homologous to both micronuclear and macronuclear sequences.

Although the PCR conditions used in these experiments were fairly stringent, secondary bands were often produced, presumably as a result of partial sequence homology between the primers and other parts of the genome. The occurrence of multiple bands does not affect the interpretation of PCR results since the distance between the two primers, and therefore the exact size of the desired product, is known. PCR products of any other size can be ignored since they do not reflect the sequence of interest, and the region can be mapped to the appropriate micronuclear chromosome based solely on the production of the fragment of the correct size.

The chance production of a secondary fragment exactly the same size as the expected product is remote, but it is necessary to consider the possible effects of such an occurrence on future uses of our mapping strategy. If, due to partial homology, a sequence in the macronucleus acted as a successful template for the production of a PCR fragment the exact same size as that made from a known micronuclear sequence, DNA from all of the nullisomic lines would show a PCR product of the predicted size. Initially, such a result would suggest a macronuclear location for the sequence in question. However, selection of a second set of primers within the micronuclear region and subsequent retesting of the template efficiency of the nullisomic panel would resolve this problem, since it is highly unlikely that a micronucleus specific sequence would give rise to two unrelated primer sets both of which would spuriously produce secondary products of exactly the predicted

size from macronuclear DNA sequences. This same strategy would also resolve the problem introduced when a set of primers obtained from one micronucleus specific sequence show partial homology to sequences on another micronuclear chromosome. Initially, DNA from all singly nullisomic lines would produce the expected fragment, and only multiply nullisomic lines missing all the relevant chromosomes would not. Retesting with a second primer set should resolve the actual location of the DNA region in question. Instances of exact duplication could be resolved with strains nullisomic for multiple chromosomes.

The micronucleus specific regions we have examined so far seem to be randomly spread throughout the micronuclear genome. The fact that we have not yet detected a sequence associated with chromosome *5* is probably not significant, given the small number of regions we have mapped, but more work is needed to establish unequivocally that chromosome *5* does contain Cbs and other micronucleus specific regions. This method of mapping DNA sequences to specific micronuclear chromosomes is simple and quick, and should allow the routine mapping of any known micronucleus specific sequence to the appropriate chromosome or chromosome arm. In addition, known primers can be used to determine the chromosome content of any nullisomic strain, both to develop new nullisomic lines and to insure the stability of existing lines. We are in the process of identifying and localizing more micronuclear sequences to develop a panel of molecular markers for all of the micronuclear chromosomes.

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