Identification, Mapping and Linkage Analysis of Randomly Amplified DNA Polymorphisms in *Tetrahymena thermophila*

Jason H. Brickner,[1] Timothy J. Lynch, Dawn Zeilinger and Eduardo Orias

Division of Molecular, Cellular, and Developmental Biology, Department of Biological Sciences, University of California, Santa Barbara, California 93106

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ABSTRACT

Using the random amplified polymorphic DNA (RAPD) technique and exploiting the unique genetics of *Tetrahymena thermophila,* we have identified and characterized 40 DNA polymorphisms occurring between two inbred strains (B and C3) of this ciliated protozoan. These RAPD markers permit the PCR amplification of a DNA species using template DNA from SB1969 (B strain) but fail to do so using DNA from C3-368-5 (C3 strain). Polymorphisms were mapped to chromosomes using a panel of monosomic strains constructed by crossing B strain-derived nullisomic strains to inbred strain C3. They map to all five chromosomes and appear to be evenly distributed throughout the genome. Chromosomal groups were then analyzed for linkage using meiotic segregants; four linkage groups were identified in chromosomes *1*R, 2L, *3* and *5*. The RAPD method appears useful for the construction of a genetic map of the Tetrahymena genome based on DNA polymorphisms.

THE ciliate *Tetrahymena thermophila* is a unicellular eukaryote that offers both the typical advantages of a microbial system (such as rapid growth rate and amenability to molecular manipulations) as well as the genetic, structural and physiological complexity of higher systems (*e.g.,* KRUGER *et al.* 1982; HOROWITZ and GOROVSKY 1985; YU *et al.* 1990). Like all sexual ciliates, Tetrahymena maintains both a micronucleus and a macronucleus, a property termed nuclear dimorphism (reviewed by RAIKOV 1976; BRUNS 1986). The micronucleus (MIC) is a transcriptionally silent, diploid, mitotically dividing germ line nucleus containing five pairs of chromosomes. The macronucleus (MAC) is a transcriptionally active, amitotically dividing nucleus that differentiates from a mitotic sister of the MIC after fusion of gamete pronuclei (for a review of ciliate genetics and molecular biology, see GALL 1986). MAC differentiation is characterized by DNA amplification and various site-specific DNA rearrangements resulting from chromosomal breakage, splicing, and deletion. The MAC contains many small chromosomes having telomeres but lacking centromeres, most of which are amplified to ~45-ploid (reviewed by YAO 1989).

Several unique and powerful genetic tools have been developed in *T. thermophila,* including the following: (1) genomic exclusion, by which whole-genome homozygotes can be attained in a single step (ALLEN 1967a,b), (2) the ability to maintain genotypically different MIC and MAC in the same cell, in the form of stable heterokaryons (BRUNS and BRUSSARD 1974; HAMILTON *et al.* 1988), and (3) a collection of nullisomic strains lacking both copies of certain germ line chromosomes or chromosome arms (BRUNS *et al.* 1982 and 1983), which can be utilized for genetic mapping (BRUNS *et al.* 1983; BLEYMAN *et al.* 1992; CASSIDY-HANLEY *et al.* 1994) and mutant analysis (ALTSCHULER and BRUNS 1984; GUTIERREZ and ORIAS 1992). The availability of a shuttle vector (YU *et al.* 1990) with which *T. thermophila* can be transformed by microinjection (TONDRAVI and YAO 1986) or electroporation (GAERTIG and GOROVSKY 1992) provides the means for molecular genetic manipulations.

Despite these tools, genetic work with Tetrahymena has been hampered by the paucity of detailed genetic maps (ALLEN 1964; MCCOY 1977; BRUNS and CASSIDY-HANLEY 1993). The development of nullisomic strains has made it possible to determine the chromosomal location of mutant genes (BRUNS and CASSIDY-HANLEY 1993), but the low density of these genes and the high frequency of recombination in Tetrahymena has precluded the detection of linkage between all but the most tightly linked markers (reviewed by BRUNS 1986). The identification and mapping of DNA polymorphisms is an attractive approach to increasing the density of genetic markers and establishing larger linkage groups. Screening for restriction fragment length polymorphisms (RFLPs), while fruitful for localized searches, is laborious, expensive, and unattractive for efficient large-scale mapping.

An alternative method of polymorphism detection, developed by WILLIAMS *et al.* (1990), is the random amplified polymorphic DNA (RAPD) technique, which utilizes 10-mer primers of arbitrary sequence in PCR to amplify genomic DNA from different inbred strains

Corresponding author: Eduardo Orias, Division of Molecular, Cellular, and Developmental Biology, Department of Biological Sciences, University of California, Santa Barbara, CA 93106.
E-mail: orias@lifesci.lscf.ucsb.edu.

[1] *Present address:* Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305.

TABLE 1

Strains used

Clone	Inbred strain	Micronuclear genotype	Mating type	Macronuclear phenotype	Reference
A*-III	A	Defective, not transmissible	III	Wild type	1
C3-368-5	C3	*mat-3/mat-3*	V	Wild type	1
SB1969	B	*mat-2/mat-2, ChxA2/ChxA2*	II	cycl-s	1
CU369	B	*mat-2/mat-2, ChxA2/ChxA2, Pmr-11/Pmr-11*	IV	cycl-s, pm-s	1
CU354	B	*mat-2/mat-2*, N-5, *ChxA2/ChxA2*	IV	cycl-s	2
CU357	B	*mat-2/mat-2*, N-*4*, *ChxA2/ChxA2*	IV	cycl-s	2
CU361sb	B	*mat-2/mat-2*, N-*3,4,*[a] *ChxA2/ChxA2*	IV	cycl-s	3
CU371	B	*mat-2/mat-2*, N-*1L, 2R, ChxA2/ChxA2*	IV	cycl-s	4
CU372	B	*mat-2/mat-2*, N-*1L, 3, ChxA2/ChxA2*	IV	cycl-s	4
CU374	B	*mat-null*, N-*2L,4L, ChxA2/ChxA2*	IV	cycl-s	1, 3
CU377	B	*mat-null*, N-*2L,3,4L, ChxA2/ChxA2*	IV	cycl-s	4
CU380sb	B	*mat-2/mat-2*, N-*3,4,5,*[b] *ChxA2/ChxA2*	II	cycl-s	5
CU389	B	*mat-2/mat-2*, N-*1L,2R,5, ChxA2/ChxA2*	VII	cycl-s	5
SB983	B/C3	*mat-2/mat-3, ChxA2/ChxA+, Pmr-11/Pmr+*	I	cycl-s, pm-s	1
SB990	B/C3	*mat-2/mat-2, ChxA2/ChxA+, Pmr-11/Pmr+*	VII	cycl-s, pm-s	1
SB1804	B/C3	*mat-2/mat-2, ChxA2/ChxA+, Pmr-11/Pmr+*	IV	cycl-s, pm-s	1

Only those genotypes and phenotypes relevant to this study are described above; see BLEYMAN *et al.* 1992 for more complete information. *ChxA2* and *Pmr-11* are alleles that confer dominant resistance to cyclohexamide (cycl) and paromomycin (pm), respectively. Wild-type alleles (+) of both genes confer sensitivity. References: 1, BLEYMAN *et al.* 1992; 2, BRUNS *et al.* 1983; 3, GUTIERREZ and ORIAS 1992; 4, BRUNS *et al.* 1982; 5, CASSIDY-HANLEY *et al.* 1994.

[a] CU361 has been described as missing only chromosome *3* (see reference 1 and 5); our stock of this strain (CU361sb) is missing both *3* and *4* (ref. 3).

[b] CU380 is listed as N*3R,4,5* in reference 5; the N*3,4,5* assignment for the strain frozen in our lab under the same name (now designated CU380sb) was as originally communicated to us by the BRUNS lab and avoids a discrepancy in the mapping of the *EstB* gene (S. L. ALLEN, D. ZEILINGER and E. ORIAS, unpublished results).

(reviewed in TINGEY and DEL TUFO 1993). Reactions generally amplify several discrete DNA fragments. Polymorphisms are identified by the amplification of a fragment using template DNA from one strain but not another (due to primer annealing site differences, insertions, deletions, etc.). The pattern of bands produced is primer-specific and sensitive to single-base pair changes in primer DNA (WILLIAMS *et al.* 1990). Polymorphisms identified by this method segregate as Mendelian alleles and the utility of the technique does not seem to be affected by genome size (WILLIAMS *et al.* 1990). DNA fragments linked to the identified polymorphisms can be readily cloned (MARCHUK *et al.* 1990) for use in chromosome walks.

Comparison of the inbred strains B and C3 offer a generous source of polymorphisms (ALLEN *et al.* 1984; LARSON *et al.* 1986; LUEHRSEN 1986; LUEHRSEN *et al.* 1987, 1988). RAPD reactions using DNA from these two inbred strains of Tetrahymena readily detect DNA polymorphisms (LYNCH *et al.* 1995). In a genome-wide screen, we have used the RAPD technique and a novel mapping strategy to identify and map 40 DNA polymorphisms, both augmenting the collection of genomic markers in Tetrahymena and demonstrating the utility of these techniques for a larger mapping effort.

MATERIALS AND METHODS

Strains and culture conditions: All of the strains used in this study are listed in Table 1. Inbred strains B and C3 have been described previously (ALLEN and GIBSON 1973). Nullisomic strains, lacking both copies of particular chromosomes or chromosome arms, were generously provided by Drs. VIRGINIA MERRIAM and PETER BRUNS (Cornell University). The nullisomic assignments in Table 1 were based on published work (BRUNS *et al.* 1982, 1983; BLEYMAN *et al.* 1992; GUTIERREZ and ORIAS 1992; CASSIDY-HANLEY *et al.* 1994). The nullisomic strains used in this study are heterokaryons at the *Chx* locus: their MACs express cyclohexamide sensitivity, but their MICs are homozygous for *ChxA2*, a dominant resistance allele (see Table 1). Monosomic strains were constructed by crossing B strain-derived nullisomic strains to C3-368-5 in mass culture and selecting for resistance to cyclohexamide (the monosomic strains are denoted by replacing the CU of the nullisomic strain name with an M; *e.g.*, M380 is the monosomic derivative of CU380). Likewise, the B/C3 heterozygote used as a positive control in monosomic mapping reactions was constructed by crossing SB1969 and C3-368-5 and selecting for cyclohexamide-resistant progeny in mass culture. In both cases, DNA preparations were made as early as practicable after cyclohexamide selection to prevent phenotypic assortment.

The 32 round II meiotic segregants used in this work are a subset of panel 2 described in LYNCH *et al.* (1995). They were obtained from B/C3 heterozygous F_1 clones SB983 and SB990 by genomic exclusion. The resulting round II clones are whole-cell homozygotes representing single independent meiotic products and are expected to segregate the B- and C3-derived alleles at each locus in a 1:1 ratio. Of this panel of 32 clones, 28 were derived from SB990: SB23 (69P, 70C, 71P, 73A, 74C, 77C, 78A, 79P, 80P, 86P, 88P, 91C, 93P, and 95P), SB24 (00C, 01C, 02C, 03C, 06A, 07C, 09A, 11C, 12C, 17C, 29C, 32A, 35C, and 37C). The remaining four panel members were derived from SB983: SB23 (52A, 55A, 61A, and 64C). A detailed explanation of the isolation of these

Primer	Sequence	Primer	Sequence	Primer	Sequence
A01	CAGGCCCTTC	A13	CAGCACCCAC	B06	TGCTCTGCCC
A02	TGCCGAGCTG	A14	TCTGTGCTGG	B07	GGTGACGCAG
A03	AGTCAGCCAC	A15	TTCCGAACCC	B11	GTAGACCCGT
A04	AATCGGGCTG	A16	AGCCAGCGAA	B12	CCTTGACGCA
A05	AGGGGTCTTG	A17	GACCGCTTGT	B15	GGAGGGTGTT
A06	GGTCCCTGAC	A18	AGGTGACCGT	B17	AGGGAACGAG
A07	GAAACGGGTG	A20	GTTGCGATCC	B20	GGACCCTTAC
A08	GTGACGTAGG	B01	GTTTCGCTCC	C03	GGGGGTCTTT
A09	GGGTAACGCC	B02	TGATCCCTGG	C05	GATGACCGCC
A10	GTGATCGCAG	B03	CATCCCCCTG	C06	GAACGGACTC
A11	CAATCGCCGT	B04	GGACTGGAGT	C15	GACGGATCAG
A12	TCGGCGATAG	B05	TGCGCCCTTC	C17	TTCCCCCCAG

FIGURE 1.—Sequences (5′ to 3′) of 10-mer primers used to identify the RAPDs in this study. The primers were supplied by Operon Technologies, Inc., and the numbers are those designated by the manufacturer, except that the "OP" prefix has been omitted.

clones and their nomenclature is given in LYNCH *et al.* (1995). Each of the four genotypic combinations of cyclohexamide and paromomycin resistance or sensitivity was equally represented to ensure that the panel was unbiased in regions linked to these loci. Panel members were grown in 50 ml of 2% proteose peptone to a cell density of 2×10^5 cells/ml for DNA preparation.

The panel used to analyze "anomalous polymorphisms" on chromosome *3* (described later) was also derived from the panels described in LYNCH *et al.* (1995) and included meiotic segregants derived from B × C3 F_1 strain SB1804 (see footnote for Table 5). These clones were chosen without regard to their drug-resistance alleles, since neither *ChxA* nor *Pmr* are located on chromosome *3* (BRUNS and CASSIDY-HANLEY 1993).

In all cases, whole cell DNA was prepared for use in PCR as previously described (LARSON *et al.* 1986). Procedures for long term stock maintenance under liquid nitrogen (FLACKS 1979), cell culture and crosses in Petri dishes (ORIAS and BRUNS 1975) or 96-well plates (BLEYMAN *et al.* 1992) and mating type testing (ORIAS and BAUM 1984) have also been described.

RAPD PCR and gel electrophoresis: Reagents and concentrations used in RAPD PCR amplification were exactly as described (WILLIAMS *et al.* 1990), except that two primers were used in each reaction rather than one (LYNCH *et al.* 1995). The temperature cycle used was also that described by WILLIAMS *et al.* (1990) (5 min at 94°, followed by 45 cycles, each with 1 min at 94°, 1 min at 36°, and 2 min at 72°, ending with an additional 12 min at 72°.) The sequence of DNA primers from the 'A', 'B' and 'C' kits (available from Operon Technologies, Inc.) used to identify the polymorphisms described appear in Figure 1. PCR products were size-fractionated in 1.5% agarose gels submerged in Tris-acetate EDTA electrophoresis buffer and run until the bromophenol blue dye front was ~7.5 cm from the wells. This distance was increased to ~8.5 cm for "subtle" polymorphisms. Gels were then stained in 0.5 μg/ml ethidium bromide for 45 min and destained in water for 1.5 hr before photographing.

Clones used in the screen for RAPD polymorphisms: Initially only one B clone (SB1968) and one C3 clone (C3-368-5) were used in the RAPD screening. However, since some of the *1R* polymorphisms (to be described in the RESULTS section), which were positive in all of the monosomic strains, could have been due to an alternative deletion event (YAO 1989) unique to the macronuclear differentiation of that B-derived strain rather than an interstrain polymorphism. To circumvent this problem, pools of newly obtained clones of inbred strains B and C3, with independently differentiated new MACs, were later used. All of the polymorphisms described herein were either identified or retested and confirmed using DNA from SB1969- and C3-368-5-derived clone pools.

The two clone pools were obtained by crossing SB1969 and C3-368-5 to A* for two rounds of genomic exclusion (ALLEN 1967a). The SB1969-derived clone pool was produced from a round II mass culture by selection for cyclohexamide resistance (see Table 1 for MIC genotypes). Since C3-368 is *ChxA*$^+$ (*i.e.*, cycl-s) in both MAC and MIC, progeny from this cross were identified differently. After crossing to A* for one round, conjugating pairs were isolated, several pairs per drop, and allowed to separate and grow in 2% bacterized peptone. After 2 days, they mated again [round II of genomic exclusion (ALLEN 1967a)]. Thus, each line likely represents a mixture of independent meiotic products. Single cells were then isolated from each microtiter well and tested for mating competence. Sexually immature lines were transferred by serial replicating to maturity, subcloned, and then tested for mating type as described (ORIAS and BAUM 1984). Clones exhibiting a different mating type from parental strains (*i.e.*, neither III nor V) were saved. This produced a set of nine clones called the C3-491 series. Whole cell DNA was prepared from each of the nine, pooled in equal concentrations, and used as C3 DNA in PCR reactions.

RESULTS

Identification of polymorphisms using RAPD in Tetrahymena: PCR amplification of whole cell DNA from *T. thermophila* using DNA 10-mers of arbitrary sequence as primers generally produced several DNA species seen as discrete bands in an ethidium bromide-stained gel. When such primers were used to amplify DNA from inbred strains B and C3, reactions typically produced several bands, most of which were common to both strains. In some reactions, a band was amplified in one strain but not the other, representing a DNA polymorphism (Figure 2; compare the B and C3 lanes for second primer A11). We chose to pursue polymorphisms that produced a band using template DNA from B strain but not using DNA from C3 strain for mapping-related reasons (discussed later). Two primers were used because they generally produced a pattern totally unlike that seen with either primer alone (LYNCH *et al.* 1995; compare the first two lanes in Figure 2, in which only A10 was used, to the reactions in which a second primer was added), enabling us to screen for more potential polymorphisms using fewer primers.

Using this strategy, we identified 40 RAPD polymorphisms between B and C3, which are described in Table 2. All polymorphic PCR products were confirmed by repeating the reaction. A preliminary estimate of the frequency of reproducible B+, C3− polymorphisms was ~0.34 per primer combination (37 polymorphisms

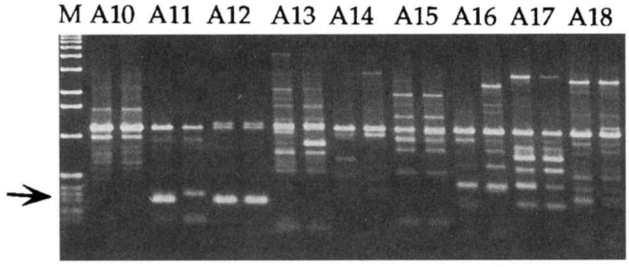

FIGURE 2.—A representative gel from the RAPD polymorphism screen. The products of PCR reactions using template DNA from either B strain (first lane of each pair) or C3 DNA (second lane of each pair) and two primers were separated on an agarose gel as described in MATERIALS AND METHODS. The A10 primer was common to all reactions and the second primer is indicated above each pair of lanes. M, 1-kb ladder, Bethesda Research Laboratories, Gaithersberg, MD. The arrow indicates the 0.3-kb B+/C3- PCR product that defines the 1JB15 polymorphism (compare the fourth and fifth lanes from the left) that is produced in reactions using the A10-A11 primer combination.

identified in 109 primer combinations). Of the first 50 polymorphic bands identified, 13 (26%) were irreproducible. Polymorphisms described as subtle are difficult to resolve from the common bands and were run further before staining (see MATERIALS AND METHODS).

Monosomic mapping: To map polymorphisms to their chromosomal locations, we used a panel of monosomic strains obtained by crossing nullisomic B strains to inbred strain C3 (see MATERIALS AND METHODS). Nullisomic strains are heterokaryons lacking certain chromosomes or chromosome arms in their MICs but having normal MACs (permitting viability) (BRUNS *et al.* 1982). The progeny from such a cross are heterozygous B/C3 for all chromosomes present in the MIC of the parental nullisomic strain but are hemizygous, possessing only C3 DNA, for chromosomes absent from the MIC of the nullisomic parent (Figure 3A). Such a gross chromosomal imbalance would be lethal in most diploid organisms but is tolerated in Tetrahymena, perhaps due to compensatory amplification of C3 DNA in the MAC to produce the normal dosage of hemizygous chromosomes (see discussions of ploidy-related MAC regulation in NANNEY and PREPARATA 1979, and of replicative regulation of MAC genetic balance in PREER and PREER 1979).

The monosomic strains are effectively stable deletion mutants for B DNA (Figure 3A). All of the selected polymorphisms produce PCR products in the B strain but not in C3 and act as completely dominant markers. Polymorphisms on heterozygous chromosomes will produce the diagnostic band. The monosomic strains lacking regions of the B genome allow the recessive C3 allele phenotype (*i.e.*, the lack of a band) to be uncovered. PCR amplification of DNA from monosomics that are hemizygous for the chromosomal location of such a polymorphism will not produce the diagnostic PCR product (Figure 3).

A representative monosomic mapping result is shown in Figure 3B. Shown is 1JB21, a polymorphism that produces a 500-bp band in B but not in C3. A B/C3 heterozygote template (positive control) and C3 template (negative control) were included for all primer combinations. Reactions using the primers A4 and A9 did not amplify the polymorphic band in reactions using DNA from M361, M372, M377 and M380. Since the B-derived chromosome *3* is the only one missing in every member of this subset, 1JB21 must lie on chromosome *3*. Consistent with this assignment, all of the monosomic strains that produced the 500-bp fragment (B/C3, M354, M357, M371, M374 and M389) possess a B strain-derived copy of chromosome *3*. Furthermore, the other chromosomes missing in M361, M372, M377, and M380 are all excluded as possible locations by the production of the band by strains having chromosome *3* but lacking these chromosomes. Using this redundant panel of monosomic strains, we were able to map 39 polymorphisms to chromosomes or chromosome arms (Table 2). An earlier version of these chromosome assignments was communicated to BRUNS and CASSIDY-HANLEY (1993).

The panel of nine monosomic strains we used included overlapping deletions covering every part of the genome except for the right arm of chromosome *1* (see Table 1). Since none of the nullisomic strains used in this study was missing *1*R, RAPD markers that produced a band using DNA from all of the monosomics were assigned to *1*R by default. This assignment is validated by the observation that some of these markers showed linkage to one another and to *ChxA*, which is known to be on the right arm of chromosome *1* (see below; BRUNS and CASSIDY-HANLEY 1993). However, the *1*R assignment of polymorphisms not in the *ChxA* linkage group must be considered preliminary.

The monosomic pattern for 1JB12 was ambiguous; it was not amplified in reactions using DNA from some, but not all, of the monosomic strains lacking chromosomes *3* or *4* (Table 2). Since RAPD polymorphisms are dominant markers, a positive score with any monosomic excludes the chromosomes hemizygous in that strain as possible locations for the RAPD. A negative score, however, is ambiguous. Chromosome *1*R was the only assignment consistent with the monosomic mapping data for 1JB12; the polymorphic PCR product was produced by monosomics lacking every chromosome except *1*R (Table 2). Consistent with this assignment, 1JB12 exhibits linkage to other markers that map to *1*R (S. L. ALLEN, D. ZEILINGER and E. ORIAS, unpublished results). This also indicates that CU361 and CU380 must lack the region of *1*R containing 1JB12. Neither CU361 nor CU380 are entirely missing *1*R, however, since other RAPDs on *1*R are present in these strains (Table 2). A resolution of this inconsistency awaits a more refined genetic map.

Meiotic segregant mapping: Once organized into chromosomal groups, some of the polymorphisms in Table 2 were analyzed for linkage using whole cell DNA

TABLE 2

Chromosomal mapping

Name[a]	Primers[b]	Size (kb)	Monosomic strains[c]									Chromosome
			5	*4*	*3,4*	*1*L, *2*R	*1*L, *3*	*2*L, *4*L	*2*L, *3,4*L	*3, 4 5*	*1*L, *2*R, *5*	
1JB05	A2/A3	0.37	+	+	+	+	+	+	+	+	+	*1*R
1JB08*	A2/A13	1.3	+	+	+	+	+	+	+	+	+	*1*R
1JB12	A9/B4	0.35	+	+	−	+	+	+	+	(−)	+	*1*R
1JB14	A3/A10	0.32	+	+	+	+	+	+	+	+	+	*1*R
1JB22	A4/A13	1.15	+	+	+	+	+	+	+	+	+	*1*R
1JB28*	A16/B1	0.45	+	+	+	+	+	+	+	+	+	*1*R
1JB30	A7/A15	0.75	+	+	+	+	+	+	+	+	+	*1*R
1AS2	A12/C5	1.0	+	+	+	+	+	−	−	+	+	2L
1EO1	A5/A6	1.3	+	+	+	+	+	−	−	+	+	2L
1JB03*	A1/A9	1.0	+	+	+	+	+	−	−	+	+	2L
1JB11*	A2/A20	0.45	+	+	+	+	+	−	−	+	+	2L
1KF2	A2/C6	0.6	+	+	+	+	+	−	−	+	+	2L
1PM8	B17/B20	0.5	(+)	+	+	+	+	−	−	+	+	2L
1JB10*	A2/A9	1.2	(+)	+	+	+	+	−	+	+	−	2L/R[d]
1JB15*	A10/A11	0.3	+	+	−	+	−	+	−	−	+	*3*
1JB16*	A11/A14	0.9	+	+	−	+	−	+	−	−	+	*3*
1JB21	A4/A9	0.8	+	+	−	+	−	+	−	−	+	*3*
1JB26	B1/B5	0.35	+	+	−	+	−	+	−	−	+	*3*
1JB35*	A8/A17	0.25	+	+	−	+	−	+	−	−	+	*3*
1JB36	B1/B15	0.5	+	+	−	+	−	+	−	−	+	*3*
1JB40	A9/A15	0.25	+	+	−	+	−	+	−	−	+	*3*
1AS1*	A7/C15	1.5	+	−	−	+	+	−	−	−	+	4L
1JB06	A4/A7	0.8	+	−	−	+	+	−	−	−	+	4L
1JB07	A7/A15	0.9	+	−	−	+	+	−	−	−	(+)	4L
1JB18*	A6/A7	0.9	+	−	−	+	+	−	−	−	+	4L
1JB31	A7/A16	1.3	ND	−	−	+	ND	−	−	−	+	4L
ND1JB37	B2/B7	1.25	+	−	ND	+	+	−	−	−	+	4L
1KF3	A2/C5	0.55	+	−	−	+	+	−	−	−	+	4L
1KF4	A8/C17	0.95	+	−	−	+	+	−	−	−	+	4L
1JB19*	A6/A9	0.6	+	−	−	+	+	+	+	−	+	*4*R
1JB01	A1/A7	1.1	−	+	+	+	+	+	+	−	−	*5*
1JB02	A7/A11	0.6	−	+	+	+	+	+	+	−	−	*5*
1JB04*	A1/A4	1.1	−	+	+	+	(+)	+	+	−	−	*5*
1JB17*	A6/A15	0.45	−	+	+	+	+	+	+	−	−	*5*
1JB23*	A7/A20	0.75	−	+	+	+	+	+	+	−	−	*5*
1JB24	A7/A15	2.9	−	+	+	+	+	+	+	−	−	*5*
1JB29	A9/B1	0.9	−	+	+	+	+	+	+	−	−	*5*
1JB32*	A12/B3	0.45	−	+	+	+	+	+	+	−	−	*5*
1JB33	A8/A9	1.3	−	+	+	+	+	+	+	−	−	*5*

"Subtle" polymorphisms are indicated by an asterisk (see MATERIALS AND METHODS). ND, not determined.

[a] Polymorphism names indicate the lab in which they were identified (prefix number 1 = ORIAS lab) and the person who identified them: AS, ANITA SUCHARCZUK; CH, CHRISTIAN HEID; EO, EDUARDO ORIAS; JB, JASON BRICKNER; KF, KENNETH FERGUSON; PM, PUNAM MATHUR. Non-JB polymorphisms were identified in a search for markers linked to the *mat* locus (LYNCH *et al.* 1995).

[b] Sequences shown in Figure 1.

[c] Nullisomic strains used to create these monosomics are listed in Table 1.

[d] Exhibits linkage to markers in 2L; see Table 4.

from a panel of 32 meiotic segregants (see MATERIALS AND METHODS). Polymorphisms segregated in a 1:1 ratio in this panel, with the exception of a group in chromosome *3* described below. Data from this panel were used to analyze linkage between the polymorphisms, without regard to their chromosomal assignments, and to order the linkage groups, using MAPMAKER (LANDER *et al.* 1987). Segregation data for *mat, ChxA, Pmr-11* were also included in the analysis. Statistically significant linkage was detected among markers mapped to chromosomes *1*R, 2L, *3*, and *5* (Tables 3–6). Accepted linkage groups had a log likelihood (LOD) ≥ 3. These linkage groups were ordered to create the maps seen in Figure 4. The order of the markers within these groups is that that gave the highest log-likelihood for the recombination data from the meiotic segregants but is not statistically significant (the maximum LOD score is less than three log units greater than that of the next best order; LYNCH

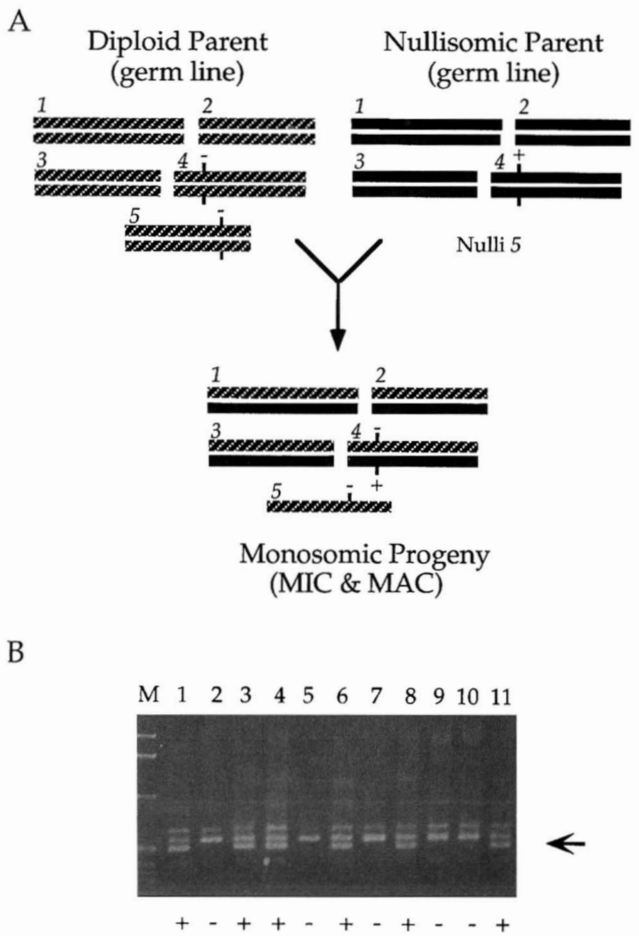

FIGURE 3.—Monosomic mapping. (A) Schematic representation of monosomic mapping. The nullisomic parent strain lacks both copies of chromosome *5* in its MIC (germ line nucleus). When crossed to diploid C3, the resulting monosomic progeny are hemizygous, possessing only C3 (hatched) DNA at chromosome *5*. RAPD markers on heterozygous chromosome *4* will be amplified. However, since C3 does not produce any of the dominant RAPD markers, the diagnostic band for a polymorphism on chromosome *5* will not be amplified using DNA from this strain. (B) Results of a typical mapping test. RAPD PCR reactions, using template DNA from the monosomic strains listed below and primers A4 and A9, were separated on an agarose gel and stained with ethidium bromide. The 500-bp polymorphic PCR product (indicated by the arrow) defines 1JB21. Lanes: M, 1-kb ladder; 1, B/C3 heterozygote; 2, C3; 3, M354, mono *5;* 4, M357, mono *4;* 5, M361a, mono *3, 4;* 6, M371, mono *1*L, 2R; 7, M372, mono *1*L, *3;* 8, M374, mono 2L, *4*L; 9, M377, mono 2L, *3, 4*L; 10, M380, mono *3, 4, 5;* 11, M389, mono *1*L, 2R, *5*. The presence (+) or absence (−) of the polymorphic band is indicated below. Since every monosomic strain containing B-derived chromosome *3* generates the band, while every strain lacking this chromosome does not, these data map 1JB21 to chromosome *3*.

et al. 1995). We have subjected these markers to more extensive linkage analysis to create maps for 2L (LYNCH *et al.* 1995) and *1*R (S. L. ALLEN, D. ZEILINGER and E. ORIAS, unpublished results). The orientation of groups with respect to the centromere is unknown. These linkage groups were entirely consistent with chromosomal assignments and provide independent confirmation of the monosomic mapping for these markers.

The meiotic segregation pattern of four of the seven markers mapping to chromosome *3* was aberrant. Instead of the expected 1:1 pattern, 1JB15, 1JB26, 1JB35, and 1JB36 segregated as follows: the 28 clones of the panel derived from SB990 (see MATERIALS AND METHODS) exhibited the C3 parental genotype, while the four derived from SB983 segregated 1:1, as expected (data not shown). We postulated that SB990 might lack B strain-derived DNA in this part of chromosome *3*. We tested these anomalous primer combinations in reactions with DNA from 35 additional meiotic segregants (data not shown) derived from three F_1 clones: SB983, SB990, and SB1804 (LYNCH *et al.* 1995). These polymorphic PCR products were not produced by any of the five additional SB990-derived clones, were amplified by all 15 SB1804-derived clones, and segregated randomly among the 15 additional clones derived from SB983. Thus, part of the B strain-derived chromosome *3* DNA has been lost in SB990 and an overlapping part of the C3-derived chromosome *3* DNA has been lost from SB1804. Micronuclear mitotic recombination or deletion of this region in one of the homologous chromosomes (ALLEN *et al.* 1984) before the isolation of these F_1s may have lead to this frequent loss of micronuclear heterozygosity. Understanding the basis of this segregation pattern will require more investigation.

As a result of this aberrant segregation pattern, linkage analysis for markers on chromosome *3* had to be assessed separately within each of the two groups: those that segregated among SB990-derived strains and those that segregated among SB983-derived strains. Only the latter group is included in Table 5. The two markers that segregated 1:1 among the original 32 panel members (1JB16 and 1JB21) did not exhibit linkage to one another or to any markers mapped to other chromosomes (data not shown). As seen in Table 5 and Figure 4, tight linkage (no recombination) was detected between 1JB15 and 1JB36, indicating that they, and presumably the other chromosome *3* markers, were correctly mapped. 1JB26 remains to be mapped with the additional panel of 20.

DISCUSSION

RAPD markers are useful in Tetrahymena: The data presented here will aid future genetic studies in Tetrahymena. The 40 polymorphisms mapped herein will substantially augment the current collection of 100 genetic markers assigned to chromosomes (BRUNS and CASSIDY-HANLEY 1993). The genetic and molecular techniques used here provide a practical framework for future large-scale efforts toward the identification and mapping of such greatly needed markers. The RAPD method has proven a fast, dependable, and safe method for identifying polymorphisms in many organisms.

RAPD polymorphisms are dominant genetic markers. With the exception of the chromosome *3* polymor-

TABLE 3

Recombination frequencies between markers on chromosome *1*

	Marker 1							
Marker 2	JB08	JB12	JB14	JB20	JB22	JB30	*ChxA*	KN2[a]
JB05	>0.50[b]	>0.50	>0.50	>0.50	>0.50	>0.50	>0.50	>0.50
	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00	<0.00
JB08		0.31	0.22	0.48	0.03	0.19	0.19	0.06
		1.00	2.33	0.01	7.41	2.93	2.93	3.48
JB12			0.16	0.35	0.29	0.19	0.19	0.47
			3.61	0.58	1.22	2.93	2.93	0.01
JB14				0.45	0.19	0.03	0.03	0.24
				0.06	2.72	7.70	7.70	1.09
JB20					0.47	0.48	0.48	>0.50
					0.03	0.01	0.01	<0.00
JB22						0.16	0.16	0.06
						3.38	3.38	3.47
JB30							0.06	0.18
							6.38	1.68
ChxA								0.24
								1.09

In Tables 3–6, the top number in each intersecting pair is the recombination frequency between marker 1 and marker 2, and the bottom number is the LOD score. Recombination frequencies and LOD values were obtained from the panel of 32 meiotic segregants described in MATERIALS AND METHODS (see exceptions in Table 5). Recombination frequencies and LOD scores indicating statistically significant linkage are underlined.

[a] KN2 was identified by KATHY NAKANO and produces a 0.3-kb band using primers A2/A17. It was mapped to *1*R by meiotic linkage to *ChxA* (T. J. LYNCH and E. ORIAS, unpublished results) but has not been mapped using monosomics.

[b] In cases in which the recombinant type was >50%, a LOD score of <0.00 was arbitrarily assigned.

phisms mentioned above, the identified markers segregated with the expected 1:1 ratio among segregants. We have yet to encounter any RAPD marker that behaved abnormally due to rare, special genetic phenomena of the macronucleus (*e.g.*, alternative deletions during MAC differentiation; see detailed discussion in LYNCH *et al.* 1995), nor have we found any polymorphic bands produced by the ribosomal or mitochondrial DNA, despite their high copy number.

The RAPD markers identified in this work are distributed evenly among all five chromosomes (see Table 2), suggesting a random distribution of polymorphisms throughout the genome, which will aid in mapping efforts in the future. The only obvious bias in their distri-

TABLE 4

Recombination frequencies between markers on chromosome *2*

	Marker 1							
Marker 2	JB03	JB10	JB11	KF2	PM8	*mat*	*Pmr*	AS2
EO1	0.22	0.06	0.07	0.00	0.03	0.03	0.09	0.03
	2.33	6.38	5.84	9.63	7.41	7.70	5.31	7.70
JB03		0.16	0.20	0.22	0.26	0.19	0.12	0.19
		3.61	2.51	2.33	1.64	2.93	4.40	2.93
JB10			0.07	0.06	0.10	0.03	0.03	0.03
			5.84	6.38	5.05	7.70	7.70	7.70
JB11				0.07	0.03	0.03	0.10	0.03
				5.84	7.13	7.13	4.80	7.13
KF2					0.03	0.03	0.09	0.03
					7.41	7.70	5.31	7.70
PM8						0.06	0.13	0.07
						6.11	4.51	6.11
mat							0.06	0.00
							6.38	9.63
Pmr								0.06
								6.38

See Table 3 for explanation.

TABLE 5

Recombination frequencies between markers on chromosomes *3* and *4*

	Marker 1	
Marker 2	JB35	JB36
	Chromosome *3*[a]	
JB15	0.21	0.00
	1.28	5.12
JB35		0.21
		1.28
	Marker 1	
Marker 2	KF3	KF4
	Chromosome *4*	
AS1	0.19	0.22
	1.46	1.28
KF3		0.33
		0.52

See Table 3 for explanation.

[a] Recombination data for the markers on chromosome *3* were obtained from the following meiotic segregant clones, all derived from SB983: SB18 (41, 42, 43, 50, 52, and 53), SB23 (02P, 04C, 07P, 08P, 15C, 17A, 18P, 24C, 27C, 52A, 55A, 61A, and 64C) [see LYNCH *et al.* (1995) for derivation nomenclature].

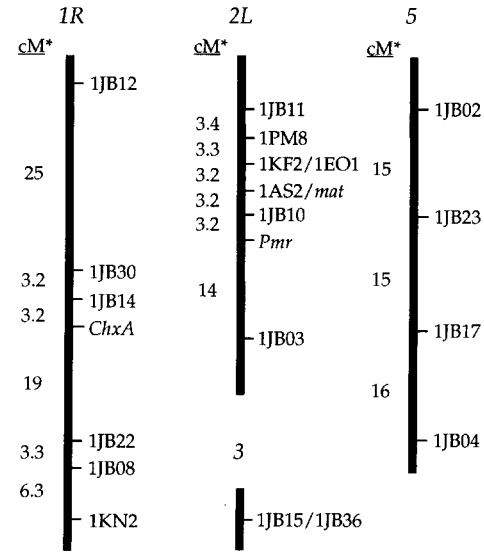

FIGURE 4.—Linkage groups. Each map represents the region of the *T. thermophila* genome indicated. *, the recombination frequencies reported in Tables 3–6 differ from cM values generated by MAPMAKER [due to the Haldane correction for multiple crossovers (LANDER *et al.* 1987)]. Classical genetic loci appearing in the maps are as follows: *ChxA*, cyclohexamide resistance; *mat*, mating type locus; *Pmr*, paromomycin resistance (rDNA) (BRUNS and CASSIDY-HALEY 1993). A map of chromosome 2L based on more extensive linkage data is presented in LYNCH *et al.* (1995).

bution is among chromosome arms, for which there are several possible explanations. First, although all five chromosomes appear to be metacentric, there may be differences in the lengths (target sizes) of the arms of these chromosomes not detectable by cytological analysis. Second, there may be clustering of RAPD polymorphisms within chromosomes. Third, the nullisomic assignments may be oversimplified. Although the data in Table 2 are consistent with the loss of arms, the borders of the deletions might not coincide exactly with the centromere. Additional work is required to distinguish between these possible explanations.

Mapping RAPD polymorphisms in Tetrahymena: The use of monosomic strains to assign polymorphisms to chromosomes is a powerful and simple tool for mapping in Tetrahymena. As a form of deletion mapping, it has served to confirm the validity of RAPD linkage groups. It has also provided a way to target a search for

TABLE 6

Recombination frequencies between markers on chromosome *5*

	Marker 1						
Marker 2	JB02	JB04	JB17	JB23	JB24	JB29	JB33
JB01	>0.50	0.34	0.39	0.47	0.18	0.42	0.40
	<0.00	0.62	0.35	0.03	2.72	0.15	0.03
JB02		0.23	0.28	0.13	>0.50	>0.50	0.37
		1.95	1.38	4.15	<0.00	<0.00	0.47
JB04			0.13	0.21	0.31	>0.50	0.25
			3.91	2.31	0.86	<0.00	1.59
JB17				0.13	0.36	>0.50	0.33
				4.15	0.50	<0.00	0.74
JB23					0.44	>0.50	0.34
					0.07	<0.00	0.62
JB24						0.48	0.32
						0.01	0.79
JB29							>0.50
							<0.00

See Table 3 for explanation.

DNA polymorphisms to a particular chromosome arm (LYNCH *et al.* 1995). This deletion mapping approach has also provided a chromosome assignment to "isolated" RAPD markers, *i.e.,* those not yet connected to a linkage group. This will aid in searching for linkage to conventional genetic markers: the *ad hoc* meiotic segregant panel need only be tested with RAPD markers assigned to the same chromosome or chromosome arm.

In deciding whether to use template DNA from the purified MICs of nullisomic strains or whole cell DNA from B/C3 monosomic strains, we opted for the latter because whole cell DNA could be prepared more quickly, easily, and in larger quantities than highly purified MIC DNA. Also, the sensitivity of PCR would have made chromosomal assignments using nullisomic MIC DNA more difficult, since these assignments can only be made with confidence when based upon positive scores. The production of a polymorphic band in such nullisomic reactions as a result of contaminating MAC DNA would have confounded mapping efforts. A third possible advantage of monosomic DNA- over nullisomic DNA-templated reactions is the comparable DNA complexity, provided by whole cell monosomic DNA, necessary for the parallel reactions required for mapping. This is in contrast to the unique pattern of bands that might result from each different nullisomic MIC DNA missing, in some cases (*e.g.,* CU377 and CU389), up to 40% of the genome.

One potential disadvantage to monosomic mapping is that alleles on the B/C3 heterozygous chromosomes in monosomic strains are subject to phenotypic assortment, leading to allele purity at a locus after extensive vegetative growth. When this assortment is coupled to a loss of diversity due to subcloning or differential growth of subclones in mass culture, the loss of a band in a monosomic caused by phenotypic assortment could be mistaken for hemizygosity. This problem was minimized by using monosomic progeny derived from multiple pairs in mass crosses and by preparing DNA from the resulting culture as early as possible.

A disadvantage of either deletion mapping approach (using nulli- or monosomic strains) is that the nullisomic strains are available only in inbred strain B genetic background. Thus the dominance of the RAPD markers limited our monosomic mapping effort to markers that produced a band using template DNA from strain B and not in reactions using DNA from strain C3 (B+, C3-). The wealth of polymorphisms between the two inbred strains makes this a minor consideration, especially in light of the important advantages of deletion mapping. Furthermore, the reciprocal RAPD markers (C3+, B-) can be assigned to linkage groups by typing the panel of 32 meiotic segregants and analyzing them for linkage to all isolated RAPDs and to suitable representatives from known linkage groups.

The main obstacle encountered in using the nullisomic strains has been changes in the assignments of their missing chromosomes, presumably due to micronuclear chromosome loss (ALLEN *et al.* 1984) since their isolation in the early 1980s. The original assignment for CU361(N*3*) has recently been confirmed (CASSIDY-HANLEY *et al.* 1994). Our stock (CU361sb) was shown to lack chromosome *4* as well (GUTIERREZ and ORIAS 1992), a conclusion that is supported by the data in Table 2. CU380sb appears to lack all of chromosome *3* (see Table 2; S. L. ALLEN, D. ZEILINGER and E. ORIAS, unpublished results). Data from the other strains used in this study were consistent with either the original assignments, recently confirmed by PCR (CASSIDY-HANLEY *et al.* 1994), or the revised assignments (BLEYMAN *et al.* 1992), probably due to their maintenance under liquid nitrogen since shortly after they were isolated by MERRIAM and BRUNS. It should be noted that, as indicated by the "sb" suffix, the assignments in Table 1 that differ from other published assignments (*e.g.,* ALLEN *et al.* 1984; CASSIDY-HANLEY *et al.* 1994) may only apply to our frozen stocks of these strains.

Linkage groups in four chromosomes were detected. These linkage groups have proven useful to workers mapping genes in these regions (S. L. ALLEN, D. ZEILINGER and E. ORIAS, unpublished results) and will be similarly useful in future work. The relatively small panel of 32 meiotic segregants that we used had two consequences. At LOD $\geq$ 3 level of statistical significance, it limited detection of linkage between markers to those $< \sim$20 cM apart (although, given the very high frequency of meiotic recombination in Tetrahymena, this may be of the order of only one or very few megabases; LYNCH *et al.* 1995) and the reliable ordering of linked markers. The map order for each linkage group in Figure 4 thus represents the best one (*i.e.,* the maximum likelihood) among a number of acceptable orders. The genetic distance between *mat* and *Pmr* in Figure 4 is less than that reported previously based on an analysis of >200 segregants (BLEYMAN *et al.* 1992; LYNCH *et al.* 1995). We believe that this discrepancy is the result of the lower than average recombination frequency observed in panel 2 (16 cM for *mat-Pmr*; LYNCH *et al.* 1995, Table 2), from which the panel of 32 is derived, coupled with statistical sampling variation (two recombinants observed *vs.* five expected).

Our decision to use a relatively small panel of 32 was guided by the consideration that the total work required to map a RAPD polymorphism is nearly proportional to the number of meiotic segregant clones that are typed. We believe that, at this juncture and for the same total amount of work, it is more generally useful to have a greater number DNA polymorphisms in linkage groups than to know the order of markers within fewer and smaller linkage groups more precisely. This initial effort will enable workers to map genes of interest through linkage analysis to polymorphisms of known location, and to use the techniques and data presented here in directed screens for new markers (*e.g.,* LYNCH

et al. 1995). Determination of a statistically reliable order of polymorphisms within a linkage group using a larger panel of meiotic segregants can be more easily justified when some interesting gene, mutant or cloned DNA insert must be mapped, as in the case of the *mat* locus (LYNCH *et al.* 1995).

The Tetrahymena Genome Project: This work can be considered the beginning of the Tetrahymena Genome Project, a global mapping effort using RAPD markers. *T. thermophila* possesses characteristics that will prove very useful in the construction of a global RAPD map. In addition to the requisite features (the ability to cross polymorphic strains to generate meiotic segregants, as well as the ability to harvest sufficient quantities of DNA from culture), Tetrahymena possesses many genetic features unique to ciliates and to this organism. Creating whole-genome homozygotes through genomic exclusion (ALLEN 1967a,b) offers a superior alternative to selfing for production of homozygous meiotic products of independent origin. This is very useful in light of the complete dominance of RAPD markers. Also, the availability of stable large-scale deletion strains (nullisomics) will expedite both global mapping efforts and local directed searches for polymorphisms. Use of an ordered panel of mitotic recombinants would allow both the detection of mitotic linkage between MIC markers over intermediate distances and the determination of their order with respect to the centromere. Eventually, we hope to use these and other techniques to create a global genetic and physical map of the Tetrahymena genome. With 2000 randomly distributed markers, the probability of a new gene being within 150 kb of any marker would be 95%. A high density map could be used to study phenotypic coassortment of linked markers, the frequency of macronuclear recombination, the size of the Tetrahymena genome in cM, and the colinearity of MIC and MAC DNA. Such a map will be of great use in genetic and molecular studies of Tetrahymena. Coupled with the genetic and molecular tools unique to this organism, this map would clearly complement Tetrahymena's demonstrated utility as an experimental system in cellular and molecular biology.

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