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Genetic Map of Randomly Amplified DNA Polymorphisms Closely Linked to the Mating Type Locus of *Tetrahymena thermophila*

Timothy J. Lynch, Jason Brickner, Kathy J. Nakano and Eduardo Orias

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

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ABSTRACT

We have used the PCR-based randomly amplified polymorphic DNA (RAPD) method to efficiently identify and map DNA polymorphisms in the ciliated protozoan *Tetrahymena thermophila*. The polymorphisms segregate as Mendelian genetic markers. A targeted screen, using DNA from pooled meiotic segregants, yielded the polymorphisms most closely linked to the *mat* locus. A total of 10 polymorphisms linked to the *mat-Pmr* segment of the left arm of micronuclear chromosome *2* have been identified. This constitutes the largest linkage group described in *T. thermophila*. We also provide here the first crude estimate of the frequency of meiotic recombination in the *mat* region, 20 kb/cM. This frequency is much higher than that observed in most other eukaryotes. Special features of Tetrahymena genetics enhanced the power of the RAPD method: the ability to obtain in a single step meiotic segregants that are whole-genome homozygotes and the availability of nullisomic strains permitting quick deletion mapping of polymorphisms to micronuclear chromosomes or chromosome segments. The RAPD method appears to provide a practical and relatively inexpensive approach to the construction of a high-resolution map of the Tetrahymena genome.

SEVEN mating types, designated I–VII, are known in *Tetrahymena thermophila*, a microbial (unicellular) eukaryote belonging to the ciliated protozoa. A cell of this species carries in its germline (micronucleus) the potential for several (up to all seven) mating types but normally expresses only one. Which mating type the cell expresses is determined by a heritable differentiation of the somatic nucleus (macronucleus). Mating type determination is developmentally programed: it is a differentiation of the new macronucleus, which occurs at a postzygotic stage of conjugation. The spectrum of mating type potentialities is determined by a micronuclear locus called *mat* (NANNEY *et al.* 1955; NANNEY 1959). Homozygotes for the *mat-1* or *mat-3* alleles express any mating type except IV or VII, while *mat-2* homozygotes express any mating type except I. The *mat* locus is on chromosome *2L*, linked by ~30 cM to *Pmr* (BLEYMAN *et al.* 1992). *Pmr* is a mutation in the segment of the ribosomal RNA gene coding for the small subunit RNA; it confers resistance to paromomycin (BRUNS *et al.* 1985; SPANGLER and BLACKBURN 1985). The life cycle, conjugation events, basic genetics and mating type determination of Tetrahymena have been reviewed (ORIAS 1981, 1986; BRUNS 1986).

We are interested in characterizing the *mat* locus and its role in mating type determination. The aim of the present work was to find DNA polymorphisms near the *mat* locus that could aid its molecular cloning. Two recent advances facilitated the search for DNA polymorphisms in Tetrahymena: the development of efficient methods for identifying and cloning DNA polymorphisms and the finding that certain inbred strains of *T. thermophila* are a rich source of DNA polymorphisms.

RAPD (randomly amplified polymorphic DNA) is a recently developed, efficient method for detecting DNA polymorphisms (WILLIAMS *et al.* 1990; TINGEY and DEL TUFO 1993). As described, 10-mers of arbitrary sequence are used as primers for PCR amplification of random genomic DNA segments (see Figure 1). Up to about a dozen discrete ethidium bromide bands were observed using single primers per PCR reaction. Remarkably, the average number of bands is independent of genomic size in the range from *Escherichia coli* to corn (over 1000-fold). The pattern is specific for each primer and is sensitive to single base changes in the primer (WILLIAMS *et al.* 1990). Thus when template DNA from two strains are used in parallel reactions, each band essentially tests 20 base pairs (10 at each end) for sequence polymorphisms. The polymorphic DNA is molecularly cloned by PCR amplification in the very act of polymorphism detection, thus facilitating subsequent cloning in conventional vectors, and only minute amounts of template DNA are required (25 ng per reaction).

Diverse inbred strains of *T. thermophila* were originally established based on natural polymorphisms at the mating type and surface antigen (serotype) loci (S. L. ALLEN, personal communication). Inbred *T. thermophila* strains B and C3 carry a wealth of DNA polymorphisms

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

Primer	Sequence	Primer	Sequence	Primer	Sequence
OPA-01	CAGGCCCTTC	OPA-12	TCGGCGATAG	OPB-12	CCTTGACGCA
OPA-02	TGCCGAGCTG	OPA-17	GACCGCTTGT	OPB-17	AGGGAACGAG
OPA-05	AGGGGTCTTG	OPA-19	CAAACGTCGG	OPB-20	GGACCCTTAC
OPA-06	GGTCCCTGAC	OPA-20	GTTGCGATCC	OPC-05	GATGACCGCC
OPA-09	GGGTAACGCC	OPB-04	GGACTGGAGT	OPC-06	GAACGGACTC

FIGURE 1.—List of primers used in this work. Primers (10-mers), primer names and sequences were obtained from Operon Technologies, Inc. OP prefix and leading 0 has been omitted elsewhere in the text.

(ALLEN et al. 1984; LARSON et al. 1986; LUEHRSEN 1986; LUEHRSEN et al. 1987, 1988; ENGBERG and NIELSEN 1990). The best characterized segments, the nontranscribed spacers of the B and C3 rDNAs, show an average of one DNA sequence polymorphism per 180 bp.

In this article we report the identification of a linked group of B, C3 RAPD DNA polymorphisms surrounding the *mat* locus. These polymorphisms were efficiently identified by a search specifically targeted to the *mat-Pmr* segment. We also provide the first crude estimates of the frequency of meiotic recombination as a function of nucleotide distance in *T. thermophila*.

MATERIALS AND METHODS

Strains and routine methods: The strains used are listed in Table 1. The history of inbred strains B and C3 is described in ALLEN et al. (1984). Nullisomic strains were a gift of Dr. PETER BRUNS, Cornell University. Meiotic segregant panels were obtained as described below.

Methods for long term maintenance of Tetrahymena stocks frozen under liquid nitrogen (FLACKS 1979), routine cell culture and crosses in Petri dishes (ORIAS and BRUNS 1975), or 96-well plates (BLEYMAN et al. 1992), mating type testing (ORIAS and BAUM 1984) and DNA minipreparations (LARSON et al. 1986) have been described.

Construction of meiotic segregant panels: Three panels of Tetrahymena meiotic segregant clones were constructed to test for Mendelian segregation and linkage, to determine genetic distances, and to target the search of RAPDs to the neighborhood of the *mat* locus. Construction involved three main steps, described below in more detail: (1) isolation of clones with independent segregant micronuclei but with a macronucleus retained from the F1 parent, (2) determining the micronuclear genotype of these segregants and (3) generating segregants expressing their micronuclear genotype.

Rationale: The rationale for each step is described below, and the genetic consequences of key steps are illustrated in Figure 2, A and B.

1. We started with B × C3 F1 progeny, heterozygous for numerous DNA sequence polymorphisms. They were induced to undergo meiosis in the context of a genomic exclusion cross (ALLEN 1967), by crossing them to strain A*. Conjugating pairs (round I of genomic exclusion) were isolated. During this meiosis the desired allele segregation and meiotic crossing over occurred. The micronucleus in each of the round I clones isolated is expected to be homozygous for the entire genome contained in a single haploid meiotic product nucleus. Micronuclei in different pairs are derived from independent meiotic events (Figure 2A). The round I clones are heterokaryons, however, as their macronuclei are retained from the B/C3 F1 parent.

2. The genotypes of round I clones were determined by appropriate test crosses. To determine *mat* genotypes, a test cross based on detecting mating types diagnostic of the *mat-2* allele (mating type IV or VII) or the *mat-3* allele (mating type I) was used. Nulli-*2L,4L* strain was used for the test cross. Since *mat* is located in micronuclear chromosome 2L (BLEYMAN et al. 1992), test cross progeny can only express mating types determined by the allele present in the round I clone.

3. To obtain panels of meiotic segregant clones that express the micronuclear germline genotype in their macronucleus, round I clones described above were individually crossed to A* in mass and allowed to undergo two rounds of genomic exclusion (see Figure 2B). Whenever possible, true round II progeny were selected in mass using drug resistance markers; otherwise, round II cultures derived from single pairs were screened on the basis of sexual immaturity. These round II cultures served as the source DNA minipreps for use in RAPD PCR reactions. Mass-selected round II cultures may contain up to hundreds or thousands of caryonidal clones (*i.e.*, clones with independently differentiated macronuclei) that started out with identical genotype. This was considered preferable *a priori* for reasons given in DISCUSSION.

Construction details: B/C3 heterozygous F1 clones SB990, SB983 and SB1804 (Table 1) were previously described (BLEYMAN et al. 1992). They were obtained by crossing a genetically marked inbred strain B derivative and C3-3685 (wild-type inbred strain C3). Phenotypically, the F1 clones are heterokaryons for two drug-resistance mutations, *Chx* and *Pmr*, which eventually allowed the mass selection of round II pairs. These F1 clones were crossed to strain A* to induce genomic exclusion. Round I pairs were isolated, and the exconjugant derived from the F1 parent was saved to avoid the micronuclear

TABLE 1

Strains used

Clone	Inbred strain	Genotype	Phenotype	Mating type	Source
A*	A	Defective	Wild type	III	WEINDRUCH and DOERDER (1975)
C3-3685	C3	*mat-3/mat-3*	Wild type	V	ORIAS and BRUNS (1975)
CU374	B	nulli-*2L, 4L, ChxA2/ChxA2*	cycl-S	IV	BLEYMAN et al. (1992)
SB983	B/C3	*mat-2/mat-3, ChxA2/Chx+ B-Pmr11/C3-Pmr+*	cycl-S, Pm-S	I	BLEYMAN et al. (1992)
SB990	B/C3	Same as SB983	Same as SB983	VII	BLEYMAN et al. (1992)
SB1804	B/C3	Same as SB983	Same as SB983	IV	BLEYMAN et al. (1992)

Only relevant genotypes and phenotypes are shown here. Mutant alleles at the *Pmr* and *ChxA* loci (see BRUNS and CASSIDY-HANLEY (1993) confer dominant resistance to paromomycin (pm) and cycloheximide (cycl), respectively. Wild-type alleles confer sensitivity. Most strains shown here are heterokaryons (BRUNS and BRUSSARD 1974); they have unlike micro- and macronuclear genotypes.

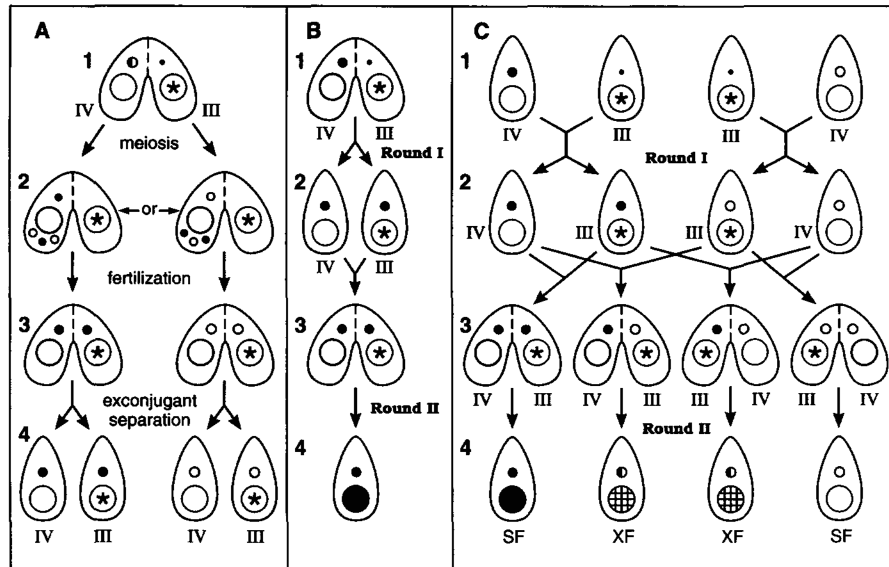

FIGURE 2.—Main nuclear events and genetic consequences of genomic exclusion crosses used in this work. Small circles, micronucleus; dot, A* micronucleus; large circles, macronucleus; starred, A* macronucleus. Shading represents genotype for one marker (*e.g., Chx*): solid, homozygous *Chx/Chx;* blank, *Chx+/Chx+*; cross hatched, *Chx/+*. Roman numerals indicate mating type. SF, genetic equivalent of self-fertilization; XF, genetic equivalent to cross-fertilization. (A) Round I of genomic exclusion, *e.g.,* as used to obtain round I progenitors of meiotic segregant panel members. Stages are as follows. 1, paired B/C3 heterozygote (left cell) and A* cell (right cell) ready to initiate nuclear events. 2, meiosis of the micronucleus has generated four haploid nuclei in the normal cell. Only the anterior meiotic product survives; the three others are destroyed. No meiotic product is formed in the A* cell. This is the meiosis where Mendelian segregation and crossing over, crucial to the linkage analysis, occur. 3, unidirectional fertilization has occurred. To reach this stage, the surviving meiotic product divided mitotically to generate two gamete nuclei, one of which was transferred to the A* cell. Both zygotic nuclei diploidized. 4, the exconjugants have separated. They retained their original macronucleus, therefore retaining their original phenotypes and mating types, and remain sexually mature. They are ready to resume vegetative multiplication or mate again, depending on nutritional conditions. Note the following consequences. (1) The two exconjugants are whole-genome homozygotes and have identical genotypes in their micronucleus, however, they still express parental phenotypes. (2) For a given heterozygous locus, the two homozygous genotypes segregate in 1:1 ratio in the micronucleus of round I progeny. (3) Meiotic events in different pairs are independent. (B) Two complete rounds of genomic exclusion, as used to generate meiotic segregant panel members or for certain testcrosses mentioned in the text. Stages are as follows: 1, round I exconjugant, obtained as in A, has paired with an A* cell and is ready to undergo round I of genomic exclusion. 2, exconjugants have separated (equivalent to stage 4, A). 3, round I exconjugants have paired again and will undergo round II of genomic exclusion. This second round involves normal conjugation events in both conjugants, including normal meiosis, reciprocal fertilization, and differentiation of new micro- and macronuclei. 4, round II products. Progeny have developed a new macronucleus, derived from a mitotic sister of the new micronucleus. Please note that since the original normal parent in this cross was a whole genome homozygote, the products of every round II pair are whole-genome homozygotes, genetically identical to one another and expressing their own micronuclear genotype in their macronucleus. (C) A three-way cross used to obtain progeny from two normal clones of identical mating type, as described by BRUNS *et al.* (1983). Stages are as follows: 1, mixture of two homozygous normal (nonstar) clones and A* clone in 1:1:2 stoichiometry, respectively. Cells pair in the two possible combinations shown. 2, cells of different mating type, after completing round I of genomic exclusion. 3, round I exconjugants have paired again in the four possible combinations shown and are ready to undergo round II of genomic exclusion (*i.e.,* normal conjugation). 4, round II products. SF, self-fertilization product; XF, cross-fertilization product. Note that the two middle final products are the full genetic equivalent of normal conjugation of the two original normal cells to one another, amd the outer final products are the genetic equivalent of self-fertilization of the corresponding original normal cells. If one of the parental normal cells is nullisomic (*e.g.,* strain CU374, used to determine *mat* genotypes), then the corresponding self-fertilization progeny immediately die.

deterioration that occurs in the A*-derived exconjugant (WEINDRUCH and DOERDER 1975). The micronucleus of each round I exconjugant clone should be homozygous for one of the four possible combinations of the two alleles at the *mat* and *Pmr* loci. These round I clones are heterokaryons, since their micro- and macronucleus are genetically different. An "H" suffix was appended to their name to distinguish them from the round II progeny derived from them (see below).

To determine the micronuclear genotype of each round I clone at the *Chx, Pmr,* and *mat* loci, they were testcrossed in microtiter plates. To determine their *Chx* and *Pmr* genotype, the clones were crossed to A* and allowed to undergo two rounds of genomic exclusion in mass culture (see Figure 2B). The progeny were separately tested for survival in growth medium containing cycloheximide (cycl) and paromomycin (PM) medium by replication. The drug-resistant round II progeny generated by this cross were saved as panel members.

To determine the *mat* genotype of round I clones, they were testcrossed in microtiter plates to nulli-*2L,4L* strain CU374 (Table 1). Progeny, which necessarily had to carry the *2L* micronuclear chromosome (and *mat* locus) derived from the round I clone, were selected for cycloheximide resistance (derived from CU374) by addition of cycl-containing medium to the refed conjugating mixture and were replicated once to the same medium. Survivors, estimated to have undergone anywhere between 18–28 fissions, were used to initiate 16

TABLE 2

Source of strains and *mat-Pmr* segregations in the meiotic segregant panels

Panel	Clone name[a]	F1 source	Segregations[b]		Recombination fraction
			mat-2:mat-3	*Pmr:Pmr+*	
1	SB1841–1843	SB983	63:35	43:55	26/98
	SB1844–1846	SB990			
	SB1847–1849	SB1804			
2	SB2301–2368	SB983	76:73	99:63	23/145
	SB2369–2439	SB990			
	SB2440–2482	SB1804			
3	SB2600–2933	SB990	166:168	444:424	71/334
Total			305:276	586:542	120/577

[a] Individual round II strains in panel 2 bear the suffixes C, P or A, depending on their mode of selection (see MATERIALS AND METHODS section).

[b] Segregation ratios and recombinant fractions are based on all the round I pairs tested for particular alleles or allele combinations.

single-cell lines from each round I clone. The cell lines were serially replicated until they had undergone at least 100 fissions, subcloned and tested for mating type. The appearance of test-cross progeny expressing diagnostic mating types (I *vs.* IV or VII) was used to determine the genotype of each clone (*mat-3/mat-3* or *mat-2/mat-2*). Round I clones derived from SB1804 could not be directly testcrossed with CU374 because both express the same mating type. Instead we did a three-way cross (round I clone + CU374 + A*) and allowed two rounds of mating to occur (see Figure 2C); the rest of the procedure was identical to that just described.

Drug selection greatly reduces the amount of labor involved in isolating round II progeny. Since the *Chx* locus is in micronuclear chromosome *1*R, it segregates independently of *mat. Pmr,* on the other hand, is linked (~30 cM) to *mat.* Unlike selection for PM-R, isolation of a panel using cycl-R selection does not bias segregation ratios and distances in the *mat-Pmr* region, and thus introduces no complication. To counteract any bias in the panel due to clones selected on the basis of PM resistance, round II segregants from round I clones not known to be *Chx/Chx* or *Pmr/Pmr* were obtained by a modified procedure. The round I clones were crossed to A*. Six pairs (new round I) were isolated in separate drops of 2% bacterized peptone (ROBERTS and ORIAS 1973) and incubated at 30°. Two days later pairing (round II) was observed. Forty-eight pairs (eight from each drop) were isolated (see Figure 2B for genetic consequences). Samples of the progeny were tested for sexual maturity by mixing with mating type testers as soon as practicable (within 20–30 fissions). Sexually mature progeny with parental mating types were discarded, since they had almost certainly retained the parental macronucleus and thus had not completed the second round of genomic exclusion.

Composition of the panels: Details on the composition and method of selection of the panels are given below. The F1 source of individual panel members and segregation data for *Pmr* and *mat* loci in each panel are shown in Tables 2 and 3, respectively.

Meiotic segregant panel 1 consists of 25 round II strains (Table 2), all generated by cycloheximide selection. Panel 1 are the *mat-Pmr* recombinants among the progeny of cross 2, described in BLEYMAN *et al.* (1992), used to determine the *mat-Pmr* distance. These selected strains are expected to have undergone an odd number of genetic crossovers between the *mat* and *Pmr* loci. Pools of DNA from panel 1 members were used to initiate the targeted search. RAPDs located exactly half way between the two loci could have been missed in the targeted search. Markers unlinked to *mat, Pmr* and *Chx* are expected to segregate 1:1 in this panel.

Meiotic segregant panel 2 consists of 79 round II strains (Table 2). They were isolated after cycloheximide selection (30 clones, "C" suffix), paromomycin selection (36 clones, "P" suffix) or by the nonselective procedure described above (13 clones, "A" suffix). (The different suffixes quickly indicate how to regenerate the round II population from the round I parent if necessary. The suffixes also serve as a reminder of the method of selection of panel members, which, if overlooked, can bias distance measurements in the neighborhood of the selective markers.) Unlike cycloheximide- and paromomycin-selected panel members, each A strain was derived from a single pair. This panel was obtained after isolating 192 round I pairs, of which 182 were typed for *mat* and *Pmr* genotype. Any marker should show 1:1 segregation in the entire set of 182 fertile round I clones.

Meiotic segregant panel 3 (Table 2) was obtained entirely by cycloheximide selection of progeny in a cross between round I progeny and the nulli-*2L,4L* strain, CU374. It consists of 334 strains with known *mat, Pmr, Chx* and *Mpr* genotypes, out of 960 round I clones isolated. The first 105 members have so far been tested for RAPD genotypes. Only markers on chromosomes *2*L or *4*L are expected to segregate 1:1 in this panel.

RAPD PCR methods: Reagents, concentrations and temperature cycling conditions used for RAPD PCR amplification were exactly according to WILLIAMS *et al.* (1990). Each 25-μl reaction was prepared by mixing 2.5 μl of 10× PCR buffer, 2.5 μl of 10 mM $MgCl_2$, 4 μl of dNTPs at 1.25 mM each, 5 μl of template DNA at 5 ng/μl, 1.25 μl of each of two primers at 4 μM and 0.125 μl of AmpliTaq DNA polymerase (Perkin-Elmer-Cetus) at 5 units/μl. Temperature cycling conditions wereas follows: 5 min at 94°, followed by 45 cycles of 1 min at 94°, 1 min at 36°, and 2 min at 72°, followed by a terminal extension period of 8 min at 72°. The primers (10-mers) are listed in Figure 1 and were purchased from Operon Technologies, Inc. The primer kits (20 primers each) were systematically tested in pairwise combinations in the following succession: A × A, B × B, A × B, C × C, A × C, and B × C. For targeting the search to the *mat* locus, reactions using primers up to A13,B20 were screened using meiotic segregant pools. Reactions using the rest of the primer combinations, beginning with A14,B1, were screened in the search targeted to chromosomes *2*L and *4*L. DNA from inbred strain B *vs.* DNA

from a mono-2L,4L culture were used as templates, as explained in RESULTS. PCR products were analyzed in 1.5% agarose gels stained with ethidium bromide. Repeatable polymorphisms were named according to the following nomenclature: a number to indicate the laboratory where the polymorphism was identified (number 1 for ours), the initials of the person that discovered the polymorphism, and a serial number for that person (*e.g.*, 1KN3).

Cloning polymorphic DNA in a plasmid vector: Desired RAPD products were cloned in a plasmid vector as follows. RAPD PCR products were size-fractionated by electrophoresis in a 1.5% agarose gel. Polymorphic bands were excised and the DNA was eluted from the gel slices by centrifugation through Whatman 3MM paper (WEICHENHAN 1991). The DNA was reamplified using the same PCR conditions and primer combination initially used to detect the polymorphism. Amplified fragments were cloned into pBluescript (Stratagene) that had been digested with *Eco*RV (New England Biolabs) and T-tailed as described by MARCHUK *et al.* (1990). Ligation and transformation were done as described in SAMBROOK *et al.* (1989). Isolation of the correct insert was confirmed by dot blots of RAPD PCR products.

Determining RAPD phenotypes using dot blot analysis: PCR reaction products were tested for the presence of a polymorphic band DNA by dot blots, using the following procedure. One-fifth of each PCR reaction was diluted in 6× SSC, boiled, and loaded onto a Minifold dot blot manifold (Schleicher & Schuell) according to manufacturer's instructions. Excised insert DNA from the clone of interest was labeled with ^{32}P-dATP by a random priming reaction (FEINBERG and VOGELSTEIN 1983). Filters were hybridized in 6× SSC, 5× Denhardts, 20 mM Tris, 0.1% SDS, 2 mM EDTA, 25 μg/ml salmon sperm DNA, and 10^6 cpm/ml probe for 16 hr at 65°. The filters were washed twice for 15 min in 3× SSC, 0.1% SDS, 5 mM EDTA at 55°, and once for 15 min in 0.3× SSC, 0.1% SDS, 5 mM EDTA at 55°. The filters were autoradiographed under X-OMAT AR X-ray film (Kodak).

Constructing a linkage map: After determining the genotype of meiotic segregants for each of the RAPD loci, the segregation data were used to test for genetic linkage and to make a genetic map using the MAPMAKER program (LANDER *et al.* 1987). This computer software uses the LANDER and GREEN (1987) algorithm to make simultaneous maximum likelihood estimates of genetic distance. Distances were expressed in cM and were corrected for undetected multiple crossovers using the Haldane equation,

$$RF' = -1/2 \ln(1 - 2RF),$$

where RF' and RF are the corrected and observed recombination fractions, respectively. We also used the MAPMAKER program to calculate the log likelihood (log odds or LOD) difference between the best (maximum likelihood) map order and all other possible map orders of the linkage group. A LOD value of 3.0 (1000:1 odds) was used throughout as the threshold of statistical significance, *i.e.*, for linkage detection and for determining map order.

RESULTS

Identification of DNA polymorphisms in Tetrahymena using the RAPD method: To test whether the RAPD method would work in Tetrahymena, we ran PCR reactions with different primers, either alone or in various combinations. As template, we used whole cell DNA preparations from inbred strains B and C3. The results are shown in Figure 3A. We concluded the following: (1) an adequate number of PCR bands were generated, regardless of whether 1, 2 or 3 primers were used; (2) the band pattern was specific for each primer combination; and (3) B and C3 DNA generated a fundamentally similar pattern when tested with the same primer combination, with some differences that represent potential polymorphisms (Figure 3A, lanes B A1/A3 and C3 A1/A3). We adopted the use of two primers per reaction, since the same collection of synthetic primers allows the testing of many more bands than if a single primer per reaction is used, as was originally done by WILLIAMS *et al.* (1990). The use of three primers per reaction did not seem to give bands in the useful size range not represented already in one of the two-primer combinations.

We have now identified and mapped some 30 RAPD polymorphisms. Some of these, listed in Table 3, were obtained using a search targeted to the *mat* locus, described below. The rest were obtained in an unbiased search and will be described in detail (J. H. BRICKNER, T. J. LYNCH, D. ZEILINGER and E. ORIAS, unpublished results). As shown in that paper, the polymorphisms are scattered over the five micronuclear chromosomes, so they appear to be useful general markers for genetic mapping in Tetrahymena.

The high copy number of the 21-kb macronuclear rDNA molecule (10^4 molecules per cell) gave us some *a priori* concern that it might template a significant proportion of the observed bands. To test this possibility, a Southern blot of a gel containing a total of 86 bands, obtained from nine reactions involving different primer combinations, was probed with labeled rDNA. Only four bands showed a hybridization signal (data not shown); the signal was weak and probably resulted from nonspecific hybridization. Thus the fraction of rDNA-templated bands is negligible. Subsequent work has failed to uncover a single rDNA-templated polymorphism, in spite of very favorable conditions, described below, for detecting polymorphisms linked to the *Pmr* (rDNA) locus.

Based on an early analysis of 80 different primer combinations, we found an average of 0.73 repeatable B+,C3− RAPDs per primer combination. These are polymorphisms in which a band is generated by B but not C3 DNA. We also found a smaller but less well characterized number for the reciprocal type of polymorphism (C3+,B− RAPDs). Based on the same analysis, we determined that ~60% of putative polymorphisms were observed repeatedly. This was reassuring, considering that many RAPD bands may well originate from slightly mismatched binding of primers to template DNA (WILLIAMS *et al.* 1990), and their final level of amplification must be strongly affected by stochastic "founder" effects.

Targeted search for RAPDs linked to the mating type locus: Conceptually, finding *mat*-linked RAPDs requires identifying B *vs.* C3 RAPDs and then testing them

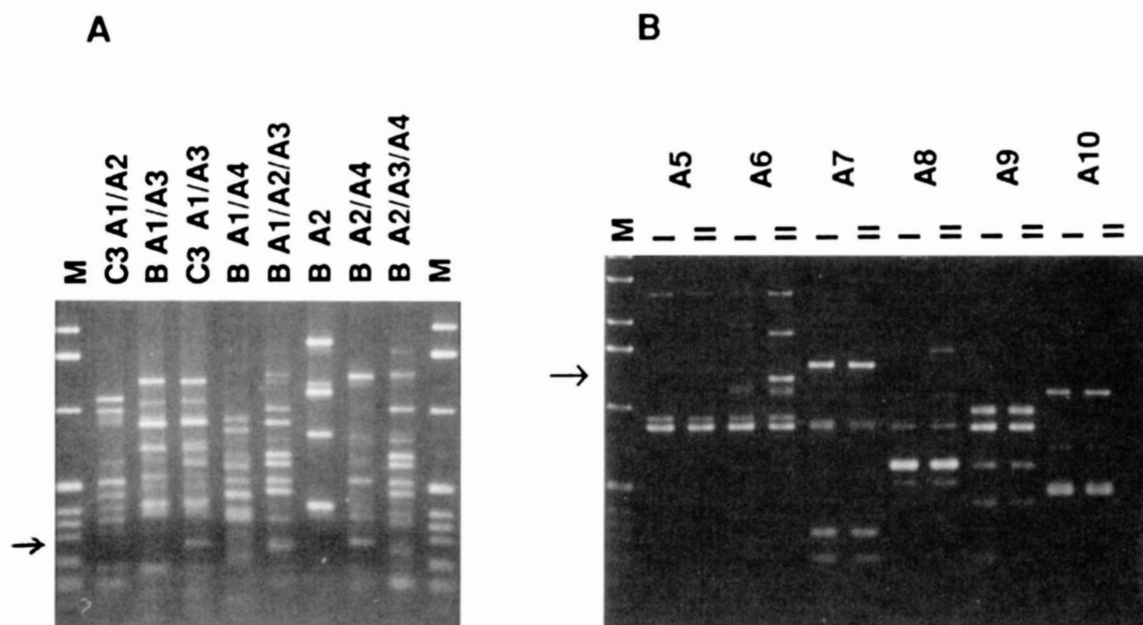

FIGURE 3.—Examples of RAPD PCR-amplified Tetrahymena DNA. (A) RAPD PCR products using DNA from inbred strains B and C3 tested with various primers used in single, double or triple combinations. Template DNA (C3 or B) and primer combinations used are indicated above each lane. Lane M, size markers (BRL 1-kb ladder; top band is 2 kb; lowest visible band is 154 bp.) Primer sequences are shown in Figure 1. The arrow shows a potential polymorphic band, produced with C3 DNA but not with B DNA, using the A1/A3 primers (third and fourth lanes). (B) Targeted RAPD screening, using pooled DNA from meiotic segregants, with various pairwise primer combinations. Pool I (consisting of a mixture of four *mat-3* panel 1 members) was used as template in odd-numbered lanes and pool II (mixture of seven *mat-2* panel 1 members) in even-numbered lanes. Primer A5 was used in every lane. DNA pools and second primers used are indicated above each lane. Lane M, BRL 1-kb ladder (top visible band is 4 kb size marker). Arrow points to the 1EO1 RAPD, *i.e.*, the upper member of a "doublet" seen in lane 3 (A6-I) but not in lane 4 (A6-II) at ~1.3 kb.

for genetic linkage using meiotic segregants. Based on a genome size of ~2.2×10^8 base pairs (see KARRER 1986), a collection of 2200 RAPDs should give polymorphisms with an average spacing of 100 kb. Assuming an average of about one B+,C3− or C3+,B− polymorphism per primer combination, we should thus screen at least 2200 primer combinations.

To speed up the search for *mat*-linked polymorphisms, we used the following shortcut that bypasses the detection and mapping of most unlinked RAPDs. Instead of directly comparing the parental inbred strain B and C3 DNA, we compared two DNA pools: one was derived from five homozygous *mat-3* panel 1 clones (pool I) and the other from five homozygous *mat-2* clones from the same panel (pool II). Most RAPDs unlinked to *mat* will segregate randomly and will be scrambled among the two pools; thus they will give a band in both lanes and they will thus escape detection (and additional work). RAPDs that are closely linked to *mat* and a minority of unlinked RAPDs, *i.e.*, those that by

TABLE 3

Allelic ratios of *mat-Pmr*-linked RAPDs among cycloheximide-selected meiotic segregants in panels 2 and 3

Loci:	1AS2	1CH1	1EO1	1EO3	1JB3	1JB10	1JB11	1KF2	1KN3	*mat*	1PM8	*Pmr*
Primers:	A12	A6	A5	A19	A1	A2	A2	A2	A2		B17	
	C5	B12	A6	B4	A9	A9	A20	C6	A17		B20	
Size:	1.0	1.3	1.3	0.4	1.0	1.2	0.45	0.6	0.3		0.5	
Search:	2L	mat	mat	2L	UB	UB	UB	2L	mat		mat	
band+	64	71	65	12	17	34	59	64	69	63	63	70
band−	61	54	63	13	13	28	59	64	61	67	64	60
Total	125	125	128	25	30	62	118	128	130	130	127	130

Abbreviations in RAPD names: AS, Anita Sucharczuk; CH, Christian Heid; EO, Eduardo Orias; JB, Jason Brickner; KF, Kenneth Ferguson; KN, Kathy J. Nakano; PM, Punam Mathur. Primer sequences are listed in Figure 1. Band DNA sizes are expressed in kb. Searches: mat and 2L were targeted to the *mat* locus and chromosome *2* L, respectively, as described in the text; UB, unbiased search (BRICKNER *et al.*, unpublished results). Alleles are designated according to parental inbred strain that contributed it. In every case the B allele is band+. None of the allelic ratios deviates significantly from the expected 1:1 ratios (probabilities of $\chi^2 > 0.05$).

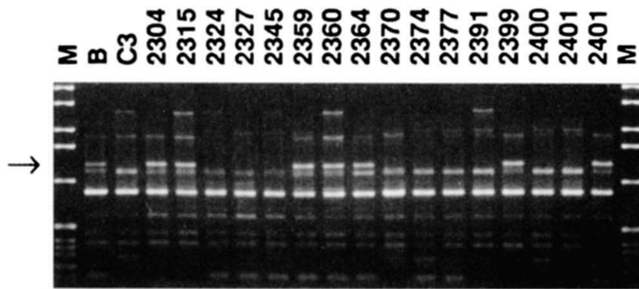

FIGURE 4.—Sample gel illustrating the linked segregation of RAPD 1EO1 and the *mat* locus. Lanes (template DNA sources) B and C3, parental inbred strains. Other lanes are individual panel 2 segregants. Panel members SB2304, SB2315, SB2359, SB2360, SB2364, SB2399 and SB2402 are *mat-2* homozygotes; the rest are *mat-3* homozygotes. (The SB prefix has been omitted from each name in the figure.) M, 1-kb ladder, BRL (top band, 4 kb; bottom band, 154 bp). PCR primers: A5 and A6. Arrow indicates RAPD 1EO1, the upper member of the doublet migrating at ~1.3 kb. Variability in other bands is due to marginally repeatable differences or to other segregating RAPDs. These and other tests (not shown) showed that six out of 203 panel members tested were *mat-1EO1* recombinants.

FIGURE 5.—Current maximum likelihood map of DNA polymorphisms in the *mat* linkage group. Distances are in cM, corrected for expected undetected cross-overs (see MATERIALS AND METHODS). LOD value for the order is 3.4, *i.e.*, 2400:1 odds. The scale is based on our preliminary estimate of 20 kb/cM in this region (see text).

chance segregated among the pool members like the *mat* locus, will result in at least one band+ segregant in pool I and five band− segregants in pool II, or vice versa; they will thus show a band with one pool and not the other, and thus will be detected (see Figure 3B). By reconstruction experiments (data not shown), we confirmed that the absence of a band means that none of the five clones has the band+ phenotype.

Polymorphic bands detected by the above screen were first tested for repeatability. To distinguish closely linked from unlinked polymorphisms, repeatable RAPDs were then tested with 25 individual meiotic segregants, as illustrated in Figure 4. RAPDs showing linkage to *mat* were then tested with up to 203 members of the meiotic segregant panels. Two of the polymorphic bands (JB11 and KN3) were also cloned and screened by dot blots, as described in MATERIALS AND METHODS, because the bands were occasionally faintly stained by EtBr and showed some variability in gels.

We have also successfully targeted the polymorphism search to the entire left arm of micronuclear chromosome *2*, by exploiting the ability of monosomic strains to uncover recessive alleles, such as the absence of a RAPD band. [An analogous approach was used to obtain chromosome-designated temperature sensitive mutations in *T.* THERMOPHILA (ALTSCHULER and BRUNS 1984)]. In this screen, we replaced the *mat-3* pool DNA with DNA from monosomic progeny derived from CU374. These progeny are hemizygous for C3-derived DNA in micronuclear chromosomes *2*L and *4*L, and heterozygous B/C3 elsewhere. Only RAPDs located on *2*L (and *4*L) should give a band with *mat-2* DNA pool and not with the mono-*2*L,*4*L DNA. Repeatable polymorphic bands were tested with a monosomic panel to distinguish *2*L from *4*L RAPDs, and with the meiotic segregant panels to test for linkage and measure genetic distance as above.

Using the above approaches, we detected 10 RAPDs linked to either *mat* or *Pmr.* As shown in Table 3, all markers segregated among meiotic segregants with ratios not significantly different statistically from the expected 1:1 Mendelian ratios. The results of tests with individual meiotic segregants were used to make the linkage map shown in Figure 5 with the aid of the MAPMAKER computer program (see MATERIALS AND METHODS). [A preliminary version of this map was communicated to BRUNS and CASSIDY-HANLEY (1993)]. The likelihood of obtaining the observed segregation among 203 members of the meiotic segregant panels based on the map in Figure 5 has a LOD of 3.4; *i.e.*, it is 3.4 log units higher (2400:1 odds) than that based on the second best map, in which the order of 1PM8 and *mat* is reversed. The statistical reliability of our map was improved by considering meiotic segregants from all three panels. For the distance calculation for Figure 5, however, only panel 2 and 3 members were used. Inclusion of data from the 25 panel 1 members would have slightly inflated certain distance measurements, as these clones had originally been chosen for being *mat-Pmr* recombinants. The expected location of *mat*-targeted, *mat*-linked RAPDs on the left arm of micronuclear chromosome *2* was confirmed by nullisomic mapping (J. H. BRICKNER, T. J. LYNCH, D. ZEILINGER and E. ORIAS, unpublished results).

The RAPD data we obtained also yielded a record of the cross-overs that occurred in the meiotic division that generated the genotype of every panel member tested (not shown). The observed distribution of cross-overs is shown in Table 4. No significant cross-over interference was detected: the observed distribution is not significantly different from the distribution expected if cross-overs are statistically independent of one another (probability of chi square > 0.10). This justifies the correction of raw recombination frequencies, using the Haldane equation (see MATERIALS AND METHODS), to obtain the cM values given in the map in Figure 5.

Frequency of meiotic recombination in the *mat-Pmr* neighborhood: The small fraction of Tetrahymena genetic markers that show linkage has led to the view that the frequency of meiotic recombination per unit of

TABLE 4

Distribution of crossovers among 178 meiotic segregants in panels 2 and 3

No. of xo	Incidence	
	Observed	Expected
0	113	104
1	43	56
2	15	15
3	6	3
4	0	0
5	1	0
Total	178	178

Numbers expected are based on Poisson distribution (mean number of crossovers per segregant is 0.54), *i.e.*, assuming that crossovers are statistically independent of one another. Classes 2–5 were pooled for purposes of the chi square test. Chi square sum, 4.61; d.f., 2; probability > 0.10. The 25 panel 1 members were excluded because they were selected for having an odd number of crossovers between *mat* and *Pmr* and would distort the above distribution. The actual mean number of crossover per segregants is likely to be higher, since a smaller number of segregants have been tested for the RAPDs farthest away from the *mat* locus, 1EO3 and 1JB3.

physical length is high (see BRUNS 1986). Our RAPD mapping allows us to make a rough estimate of the number of kilobase pairs per centiMorgan (kb/cM), at least for the *mat-Pmr* region. We found 10 RAPDs in a 187-cM segment (95 cM in the linkage group shown in Figure 5, plus at least 46 corrected cM on either side of it, where any RAPD would have shown linkage, *i.e.*, <30% recombinants, to the end RAPDs). The average genetic spacing between RAPDs is 187 cM/11 spacings or 17 cM. We estimate that we screened 645 B+,C3− RAPDs (884 primer combinations × 0.73 B+,C3− RAPDs per primer combination). If RAPDs have constant probability of occurrence per physical unit length (kb), then the average physical spacing expected between RAPDs is 2.2×10^5 kb/645 or 341 kb per RAPD. The average kb/cM then is 341 kb/17 cM or 20 kb/cM. The quality of this estimate is discussed below.

DISCUSSION

***mat-Pmr*-linked DNA polymorphisms:** The RAPD method, first used in plant molecular genetics (WILLIAMS *et al.* 1990), provides an efficient, general, relatively inexpensive and safe method to identify and map DNA polymorphisms in any organism. The Tetrahymena RAPDs we have identified and mapped in this work behave as *bona fide* nuclear genetic markers. Their 1:1 meiotic segregations (Table 3), *mat*-linkage (Figure 5) and 2L location (J. H. BRICKNER, T. J. LYNCH, D. ZEILINGER and E. ORIAS, unpublished results) lead us to rule out the possibility that the polymorphic bands of interest resulted from the amplification of contaminating DNA or other PCR artifacts.

With the aid of our targeted search, the RAPD approach has yielded 10 RAPDs detectably linked to the *mat* and *Pmr* loci (Figure 5). This density of genetic markers far surpasses any ever described in *T. thermophila*. Outside of the many tightly linked DNA polymorphisms known within the ribosomal RNA gene (see LARSON *et al.* 1986; ENGBERG and NIELSEN 1990), no other published Tetrahymena linkage group has included more than three markers. The present advance can be attributed to having screened the equivalent of close to 1000 DNA polymorphisms. The map on Figure 5 gives hints of RAPD clustering around *mat* relative to *Pmr*, even though our targeted search treated *mat* and *Pmr* symmetrically. However, it still may be too early to reach any statistically meaningful conclusion on this point.

The initial motivation for the search for *mat*-linked polymorphisms was to provide neighboring starting points for a chromosome walk to the *mat* locus. The RAPDs that flank the *mat* locus should be very useful for this purpose, as they could be ~80–130 kb away, based on our rough estimate of meiotic recombination frequency (discussed further below). Those RAPDs also place boundaries on the segment to be covered by the walk.

Frequency of meiotic recombination in Tetrahymena: We have estimated the average frequency of meiotic recombination for the *mat-Pmr* neighborhood to be 20 kb/cM. This estimate must be viewed as only very preliminary and could easily be wrong by a factor of two for the following reasons: (1) The genetic spacing estimate is based on 10 linked RAPDs; it is therefore subject to some sampling error. (2) The genetic spacing estimate assumes that we found all the repeatable DNA polymorphisms potentially demonstrable with the primers used. Based on an untargeted search that used a subset of the primers, we believe that the fraction we missed is small, probably much less than 1/2. In the kb/cM calculation (RESULTS), missed polymorphisms would shorten proportionally the average RAPD genetic spacing in the numerator. On the other hand, since most missed markers would be interstitial, their insertion would extend less than proportionally the total cM, and thus the genetic spacing between RAPDs, in the denominator. On balance, their absence leads to some underestimation of the kb/cM. (3) The physical spacing estimate assumes that B,C3 RAPDs are randomly distributed, *i.e.*, have constant probability of incidence per unit of physical chromosome length. The extent and scale of any possible clustering, if it exists, is still unknown.

Although rough, this is the first estimate of the frequency of meiotic recombination in Tetrahymena. Our estimate of 20 kb/cM is consistent with the view that the frequency of meiotic recombination per physical unit length of DNA is high in *T. thermophila* and accounts for the previous difficulty in finding linked genetic markers in this species. For example, according to our estimate,

the probability that two particular, randomly selected markers will fall within the same 40-cM interval, and thus show detectable linkage, would be ~0.4%.

Assuming that the meiotic recombination frequency in the *mat-Pmr* neighborhood is representative, the total genome length would be 2.2×10^5 kb/20 kb/cM, or 11,000 cM. However, local recombination frequencies can vary by as much as 10-fold compared to the genomic average (*e.g.*, tomato) (SEGAL *et al.* 1992). Our estimate is larger than the previous minimum estimate of 1500 cM (McCOY 1977), based on the linkage groups and the number of markers linked to no others known in 1977. Direct estimates of the frequency of meiotic recombination and its variability in Tetrahymena will require physical mapping of DNA polymorphisms.

RAPD markers and Tetrahymena genetics: RAPD polymorphisms are a new class of Tetrahymena genetic markers. All the advantages of the RAPD method listed in the Introduction have proven to be enormous time savers in this work. When compared to conventional genetic markers, *i.e.*, those that result in biological phenotypic differences, additional general advantages are noted as follows: universality of the method of phenotype detection, ability to test all the RAPD markers for linkage to one another by using a single panel of meiotic recombinants, absence of intrinsic differential growth or lethality conferred by either allele, and no loss of genotypic information due to interactions at the level of gene expression (*e.g.*, epistasis).

Our successful use of two-primer combinations in Tetrahymena appears to be an improvement over the use of single primers in the original method. In our preliminary work, two-primer reactions generally were richer in bands than, and showed few or no bands in common with, single-primer reactions. All our RAPDs so far mapped require two different primers, even though we screened all the corresponding single-primer reactions. The combinatorial use of primers greatly lowered the primer cost of our RAPD screen. This cost feature is particularly important for detecting linkage in *T. thermophila*, where the high frequency of meiotic recombination requires a higher physical density of markers than in many multicellular eukaryotes.

The nuclear dimorphism of Tetrahymena creates special opportunities for the application of the RAPD method, as well as generating special challenges. We discuss below how we have addressed them.

Use of whole cell DNA: Our mapping goal requires that the RAPD markers reside in the germline (micronuclear) DNA. However, an important time and labor saver has been the successful use of whole cell (rather than purified micronuclear) DNA preparations as template for PCR reactions. In exponentially growing cells the average micronucleus is essentially 4C and the average macronucleus is ~60C. Thus, ~90% of the DNA in such preps is macronuclear DNA and only ~7% is micronuclear DNA. Macronuclear rDNA (21 kb) and mitochondrial DNA (~40 kb) are high copy number species that together account for <2% of the total cell DNA amount and <0.03% of the total genomic complexity. The Mendelian segregations attest to the genetic specification of the RAPDs in the micronucleus. Given the preponderance of macronuclear DNA in our PCR reactions, however, it is likely that most (if not all) of our polymorphic RAPD bands physically used macronuclear DNA as the starting template.

We took two precautions in preparing DNA from members of the panels of meiotic segregants: we prepared DNA from a mixture of descendants from many conjugant pairs and after the minimum vegetative multiplication practicable. These precautions addressed several known or suspected phenomena: (1) the occurrence of programmed alternative DNA rearrangements (and other possible developmental accidents) in independently differentiated new macronuclei (YAO 1989), (2) the occurrence of macronuclear mutations, including deletions, after prolonged vegetative multiplication (ALLEN *et al.* 1985) and (3) the likely occurrence of RAPD polymorphisms in mitochondrial DNA from inbred strains B and C3. Whether due to our precautions or not, we have encountered no examples of any of the above problems so far.

Polymorphic strains: The educated choice of strains B and C3 was very rewarding. We found an average of about one B,C3 RAPD polymorphism per primer combination; 0.73 of these were of the B+,C3− type. It is too early to attribute meaning to this curious bias.

We can estimate the average frequency of B,C3 DNA sequence differences based on the frequency of one B,C3 RAPD per primer combination. An early analysis based on 14 different two-primer combinations yielded an average of 7.7 bands per RAPD PCR reaction (*i.e.*, per primer combination). Since the band pattern is sensitive to single base pair substitution (WILLIAMS *et al.* 1990) and the two 10-mers that prime each band test for the identity of 20 nucleotides, we estimate that 154 bp are tested by each primer combination. We estimate therefore a frequency of one B,C3 DNA sequence polymorphism per 154 bp. This estimate is in good agreement with the average frequency of one DNA sequence polymorphisms per 180 bp observed in the nontranscribed spacers of the B and C3 rDNA (see Table II in ENGBERG and NIELSEN 1990). At first sight this agreement is surprising. For reasons related to commercial availability and cost, we used primers that are GC rich (60 or 70% G or C residues). Yet, the average GC content of the *T. thermophila* genome is only 30%, and the bulk of the noncoding regions (including the rDNA nontranscribed spacers), which might have been expected to be more polymorphic, are only ~10% GC (KARRER 1986). We have no further insights into this apparent paradox.

Use of genomic exclusion to generate meiotic segregant panels: We used panels of meiotic segregants to target our

polymorphism search to the *mat-Pmr* neighborhood (discussed below) and to map the RAPDs based on the frequency of meiotic recombination. The meiotic segregant panels were extremely useful for mapping, because the mapping work increases as a linear, rather than square, function of the number of RAPDs and additional information is obtained from the complete pattern of cross-overs in every interval for every panel member.

The usefulness of our meiotic segregant panels was enhanced by exploiting genomic exclusion (ALLEN 1967) in their construction. This phenomenon allowed us to isolate meiotic segregants as "instant" whole-genome homozygotes, derived from the diploidization of single, independent, haploid meiotic products generated from a multiply heterozygous B/C3 F1. Such extreme homozygosity of segregant progeny is not readily attainable in other genetic model organisms with diploid germ lines. Homozygosity of the meiotic segregants resulted in several advantages for our analysis: (1) it circumvented the "dominance" of the band+ RAPD phenotype (WILLIAMS *et al.* 1990), (2) it resulted in clearer bands in the gel, due to the all-or-none nature of the band-generating DNA sequence, and (3) it prevented a potential source of confusion, special to Tetrahymena, namely the genetic homogenization of initially heterozygous macronuclei that results from random distribution of allelic copies during vegetative macro-nuclear division (phenotypic assortment) (see BRUNS 1986).

The retention of the parental macronucleus, which occurs in round I of genomic exclusion, allowed us to recover cells with a recombinant micronucleus as heterokaryons, covered by parental genetically comparable macronuclei (Figure 2A). This avoided segregation distortions caused by differential growth of recombinant progeny resulting from possible deleterious markers linked to a particular polymorphism. In addition, the round I heterokaryons were stored under liquid nitrogen, allowing us to regenerate the round II panels at will.

Targeting the polymorphism search: The use of meiotic segregant pools was our most direct approach to target our polymorphism search to the *mat* locus. It proved very successful; it yielded the closest RAPDs to the *mat* locus and saved us an estimated >90% of the work required to identify the linked polymorphisms if the primary screen had used the parental B and C3 strains instead.

The idea of a segregant pooling strategy for a targeted RAPD screening was independently developed by MICHELMORE *et al.* (1991), who discuss additional applications. Use of pools much larger than five clones, as done by MICHELMORE *et al.*, perhaps would have increased the probability of obtaining additional more distantly linked RAPDs. It also likely would have suppressed the detection of rare unlinked polymorphisms that by chance segregated as if linked to *mat-Pmr* in the pools, although this was only a minor burden.

We used a *2*L,*4*L monosomic strain as a second successful method of targeting our search to the *mat*-locus. As expected we obtained RAPDs not only in micronuclear chromosome *2*L, but also in *4*L (J. H. BRICKNER, T. J. LYNCH, D. ZEILINGER and E. ORIAS, unpublished results). The use of monosomics in a RAPD screen targeted to a particular chromosome or arm should be useful in a genome mapping attempt if, for example, RAPDs are rarer in a particular chromosome.

RAPD approach and its general usefulness for ciliate genetics: The above considerations indicate that the RAPD method is complemented well by special features of Tetrahymena genetics and promises a useful way to obtain a genetic map of the entire genome. Indeed, an untargeted search for RAPD polymorphisms (J. H. BRICKNER, T. J. LYNCH, D. ZEILINGER and E. ORIAS, unpublished results) has yielded RAPDs on all five micronuclear chromosomes. Such a global map will be useful for quickly finding the map location of new mutations or cloned genes, and for map-based DNA cloning in Tetrahymena. In related work (S. L. ALLEN, D. ZEILINGER and E. ORIAS, unpublished results), the *EstA* locus has been assigned to the *Chx* linkage group on micronuclear chromosome *1*R.

Since in effect the RAPD method reveals a seemingly inexhaustible source of "instant mutations", it should also be useful for studying basic genetic mechanisms in the micro- and macronucleus of other ciliates, and for constructing genetic maps in other ciliates and protozoa in which recessive mutations are difficult to obtain due to obligatory cross-fertilization. The main requirements would seem to be the ability to cross polymorphic strains and generate meiotic segregants in the laboratory and to harvest modest amounts of DNA from a culture.

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