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The Replication Advantage of a Free Linear rRNA Gene Is Restored by Somatic Recombination in *Tetrahymena thermophila*

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The autonomously replicating rRNA genes (rDNA) in the somatic nucleus of *Tetrahymena thermophila* are maintained at a copy number of approximately 10^4 per nucleus. A mutant in which the replication properties of this molecule were altered was isolated and characterized. This mutation of inbred strain C3, named *rmm4*, was shown to have the same effect on rDNA replication and to be associated with the same 1-base-pair (bp) deletion as the previously reported, independently derived *rmm1* mutation (D. L. Larson, E. H. Blackburn, P. C. Yaeger, and E. Orias, Cell 47:229–240, 1986). The rDNA of inbred strain B, which is at a replicational disadvantage compared with wild-type C3 rDNA, has a 42-bp deletion. This deletion is separated by 25 bp from the 1-bp deletion of *rmm4* or *rmm1*. Southern blot analysis and DNA sequencing revealed that during prolonged vegetative divisions of C3-*rmm4*/B-*rmm* heterozygotes, somatic recombination produced rDNAs lacking both the *rmm4*-associated deletion and the 42-bp deletion. In somatic nuclei in which this rare recombinational event had occurred, all 10^4 copies of nonrecombinant rDNA were eventually replaced by the recombinant rDNA. The results prove that each of the two deletions is the genetic determinant of the observed replication disadvantage. We propose that the analysis of somatically recombinant rDNAs can be used as a general method in locating other mutations which affect rDNA propagation in *T. thermophila*.

The rRNA genes (rDNA) in the ciliated protozoan *Tetrahymena thermophila* provide an excellent opportunity for studying DNA replication in a eucaryotic system. In the somatic nuclei of *T. thermophila*, the rDNA is a relatively short (21-kilobase pair [kb]), autonomously replicating molecule that is maintained at high copy number (about 10^4). A single copy per haploid genome is present in the germ line, making it amenable to genetic analysis (reviewed in reference 24). We have reported sequence variations among three different rDNAs with differing replication capabilities (8). In this paper we show that in cultures of cells having mixtures of two different types of rDNA, each disadvantaged in replication, infrequent somatic recombination produces recombinant rDNAs that are selectively propagated.

Each *T. thermophila* cell contains two nuclei: a transcriptionally quiescent micronucleus that serves as the germ line, and a transcriptionally active macronucleus that carries out somatic functions and determines the cell phenotype. New micro- and macronuclei develop at conjugation (12). When a pair of cells conjugate, the diploid micronucleus in each cell undergoes meiosis to generate haploid gamete nuclei. A zygote nucleus is formed in each cell by fusion of two gamete nuclei, one derived from each parent. The micro- and macronuclei differentiate from mitotic descendants of the zygote nucleus. The preexisting macronucleus is destroyed. Thus, the micronuclei in mating parental cells are the precursors of the macronuclei in their progeny.

Of the processes associated with macronuclear development, maturation of the rDNA is the most extensively studied (24). In the micronucleus, there is a single chromosomally integrated rDNA copy in each haploid genome. During macronuclear development, the rDNA is excised from the chromosome and converted into a 21-kb palindromic molecule. The palindrome is made up of two inversely oriented copies of the micronuclear rDNA sequence. The extrachromosomal rDNA acquires telomeres (1) and is amplified to a copy number of about 10^4 molecules per macronucleus. These maturation events are completed prior to the onset of vegetative growth, approximately 18 h after conjugation is initiated. The macronuclear rDNA is maintained at a high copy number throughout vegetative cell divisions.

Figure 1A diagrams one-half of an rDNA palindrome. The origin of replication is within the 5′ nontranscribed spacer (5′NTS), upstream from the transcription initiation site (Fig. 1A) (4). Two nuclease-hypersensitive regions within the 5′NTS (15), domains 1 and 2, have very similar sequences and contain evolutionarily conserved repeated sequence elements (5, 11). By mutational analysis, one of these repeated elements (type I) was implicated in the activation of rDNA replication (8).

We have previously shown that rDNA from a *T. thermophila* inbred strain C3 (C3 rDNA) has a replicative advantage over rDNA from inbred strain B (B rDNA), causing C3 rDNA to completely replace B rDNA in C3/B heterozygotes during vegetative growth (8). After mutagenesis of strain C3, we isolated a mutant bearing a mutation in the C3 rDNA, *rmm1*. We showed that C3 rDNA bearing the *rmm1* mutation (C3-*rmm1* rDNA) has a replicative disadvantage, causing the complete loss of C3-*rmm1* rDNA in C3-*rmm1*/B heterozygotes. DNA sequencing within the 5′NTS of C3, B, and C3-*rmm1* rDNAs revealed a 42-base-pair (bp) deletion in the B rDNA and a 1-bp deletion within the C3-*rmm1* rDNA compared with wild-type C3 rDNA. Both deletions affected the type I repeat element in domain 2 of the 5′NTS. The 1-bp

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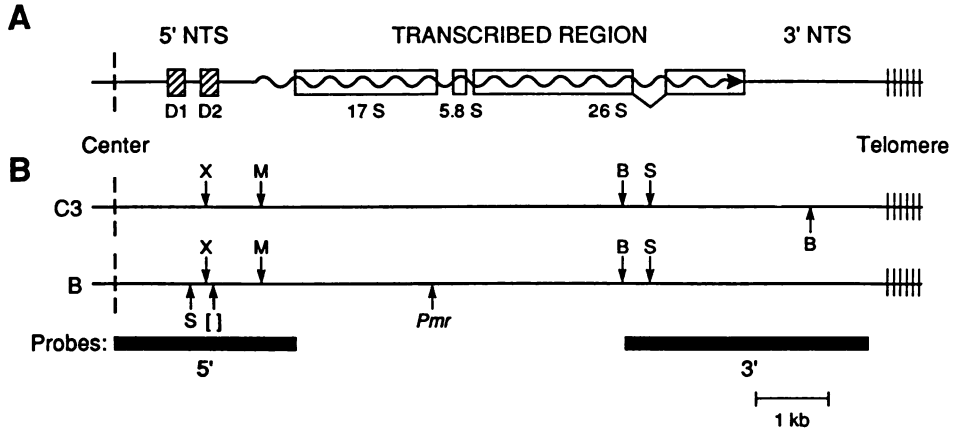

FIG. 1. Map of macronuclear rDNA regions and polymorphisms relevant to this work. (A) One-half of the rDNA palindrome, with the center at the left (i) and the telomere at the right ( ). The transcribed region (〰) includes the coding region for the 17S, 5.8S, and 26S rRNAs and is flanked by the 5'NTS and 3'NTS. Domains 1 and 2 (D1 and D2, respectively) in the 5'NTS are shown (▨). (B) Relevant restriction sites *Xba*I (X), *Msp*I (M), *Bam*HI (B), and *Sph*I (S) are shown for the rDNA of *T. thermophila* C3 (top line) and B (bottom line). The 42-bp deletion in domain 2 ([]) and *Pmr* mutation of strain B are shown. Features specific to each type of rDNA are shown below their respective rDNA. The 5' and 3' probes used for the Southern blot analysis described in this paper are also shown (▬).

deletion was in a run of 11 A nucleotide residues, separated by only 14 to 25 nucleotides from the 42-bp deletion in B rDNA. The 1-bp deletion has a more pronounced effect than the 42-bp deletion, probably because it changes the critical spacing of conserved sequences within the type I repeat (8).

In this paper we report our analysis of *rmm4*, another of four rDNA maturation and/or maintenance (*rmm*) mutations isolated after selection for cells in which the replicative advantage of C3 rDNA over B rDNA was counteracted. We show that the independently derived *rmm4* mutation is associated with the same 1-bp deletion as was reported for the *rmm1* mutation. In our analysis of *rmm4*, we show that rare somatic events yield recombinant rDNAs capable of replacing the majority species of rDNA in a heterozygote population. The characterization of these recombinant rDNAs provides unequivocal proof that the 1-bp deletion (associated with *rmm1* and *rmm4*) and the 42-bp deletion (within B rDNA) are the determinants for the replication disadvantage of these rDNAs.

MATERIALS AND METHODS

Cell culture and *Tetrahymena* strains. All cultures were grown and maintained in PP210 medium (8), supplemented with drugs when specified. Matings and other routine genetic procedures were as previously described (13, 14). *Tetrahymena* strains used as parents in the matings are described in Table 1.

Isolation of the *rmm4* mutant. *rmm4* was isolated from a mutagenized culture of a wild-type strain C3, SB1934 (Table 1), by using procedures similar to that described for the isolation of *rmm1* from a previous mutagenesis (8). To prevent isolation of any *rmm* mutants that may have preexisted at a low frequency in the SB1934 stock culture, we inoculated approximately 20 cells from the culture into fresh medium prior to mutagenesis. These were grown to a dense culture, mutagenized with nitrosoguanidine, and subjected to selection and screening for *rmm* mutations by procedures similar to those previously described (8). The selection strategy imposes the requirement that an *rmm* mutation prevent C3 rDNA of the mutagenized strain from replacing B rDNA in C3/B heterozygotes (see Introduction). Screening was done to test for the germ line origin of the mutation and to get a preliminary indication that the rDNA had not been simply deleted from the germ line of the mutagenized strain. Of 4×10^4 cells surviving mutagenesis that were tested, 2 had *rmm* mutations. The mutations were named *rmm4*, which is the subject of this paper, and *rmm3*, which will be discussed elsewhere. Strains homozygous for *rmm4* were constructed from the initial isolates as described for the isolation of *rmm1* homozygotes. These were named SB841 and SB842 (Table 1).

Cell line experiments. Heterozygous cell lines were propagated by daily single-cell transfer as follows. A total of 10^6 cells of each parent strain (SB841 and SB1915 [Table 1]) were mixed in Dryl medium (14). The mated cells were refed 9 h after mixing. At 14 to 16 h, prior to the first cell division of exconjugants, 192 single cells were isolated from each mating into drops of growth medium. After approximately eight cell fissions, each cell line was transferred to a microdilution well and treated with 15 μg of cycloheximide per ml for 3 days to eliminate cells which had not mated. Each surviving cell line was transferred for a further 2 days to 12 ml of growth medium containing cycloheximide. Approximately 24 fissions after mating, a single cell from each cell

TABLE 1. rDNA types and other genetic markers[a] in parent strains

Strain	Micronucleus		Macronucleus				Mating type
	Type of rDNA[b]	Other markers	Type of rDNA	Phenotype			
				Pm[r]	Cycl[r]	6-Mp[r]	
SB841[c]	C3-*rmm4*	*Mpr*	B-*Pmr*	+	−	+	VI
SB842[c]	C3-*rmm4*	*Mpr*	B-*Pmr*	+	−	+	VI
SB805[c]	C3-*rmm1*	*Mpr*	B-*Pmr*	+	−	+	VII
SB1934	C3	*Mpr*	B	−	−	−	V
SB1917	B-*Pmr*		B	−	−	−	II
SB1915	B-*Pmr*	*ChxA2*	B	−	−	−	II
SB1914	B-*Pmr*	*ChxA2*	B	−	−	−	VII

[a] All strains are heterokaryons and are homozygous at all loci in the micronucleus. Locus names: *rmm4*, described in this paper; *rmm1*, rDNA maturation and/or maintenance impaired (8); *Pmr*, paromomycin resistance (2, 23); *ChxA2*, cycloheximide (Cycl) resistance (3, 19); *Mpr*, 6-methylpurine (6-Mp) resistance (3); r, resistant; s, sensitive.

[b] C3-type and B-type rDNAs are illustrated in Fig. 1.

[c] Derived from mutagenized strain SB1934.

line was inoculated into a fresh drop of nonselective medium for continued growth. After another 6 to 12 fissions, a single cell was again inoculated into fresh medium. This cycle of single-cell inoculations was repeated 30 times.

After each single-cell inoculation, the remaining cells in the drop were treated with 100 μg of paromomycin per ml to test for the presence of the *Pmr* allele. To verify that the cell lines were established from cross-fertilized progeny, samples of the cell lines at 24 fissions were tested for resistance to 15 μg of 6-methylpurine per ml.

Time course experiments on mass cultures. A total of 4×10^6 cells of each parent strain were mixed in Dryl medium. The mated cells were refed 9 h after mixing. At 20 h, i.e., about the time of the first cell division of exconjugants, cells were diluted into 50 ml of growth medium supplemented with cycloheximide. In a series of dilutions, the cells were taken through nonselective medium from 5 to 10 fissions after mating, 6-methylpurine from 10 to 20 fissions, cycloheximide from 20 to 30 fissions, and nonselective medium thereafter. Then, 1×10^4 to 4×10^4 cells from cultures that had reached a density of 2.5×10^5 cells per ml were inoculated at each dilution into 50 ml of medium in 250-ml Erlenmeyer flasks. Cultures were maintained in logarithmic growth on a shaker at 110 rpm.

At various time points, between 1 and 300 fissions after conjugation, cells were harvested for DNA isolation. At the same time points, 96 single cells were isolated from the cultures into drops of nonselective medium and later replica plated to paramomycin to test for the presence of the *Pmr* mutation. Cells isolated from the cultures prior to completion of selection for cross-fertilized progeny (20 fissions) were also replica plated to cycloheximide and 6-methylpurine to determine the fraction of cells in the culture that resulted from cross-fertilization ($Cycl^r$, $6\text{-}Mp^r$). The non-cross-fertilized progeny, which include self-fertilized progeny (14) of the strain B parent and cells that retained parental macronuclei (Table 1), contribute only B rDNA to the culture. The fraction of B rDNA contributed from non-cross-fertilized progeny was taken into account when determining the relative amounts of different rDNA types in the heterozygote populations.

DNA isolation. Harvesting of cells and isolation of total cellular DNA for Southern blot analysis were done as previously described (8). When necessary, DNA preparations were desalted with Centricon-30 microconcentrators (Amicon Corp.) prior to digestion with restriction endonucleases. To isolate rDNA for cloning, we purified macronuclear rDNA by the procedure of Din and Engberg (6) with modifications described by Spangler and Blackburn (23).

Southern blot analysis. Because incomplete digestion of rDNA with *Bam*HI or *Sph*I might have led to inaccurate quantitations, digestion was monitored by observing the linearization of supercoiled M13mp19 DNA and pUC18 DNA included in *Bam*HI and *Sph*I digests, respectively. M13mp19 was included at 200 ng per well of the agarose gel and was visualized on photographs of ethidium bromide-stained gels after electrophoresis. pUC18, which hybridizes with the probes, was included at 2 ng per well and was visualized on autoradiographs of probed blots. After electrophoresis in agarose gels, DNA was transferred to nylon filters by standard procedures (10). *Sph*I digests were transferred in 0.4 M NaOH (18) to minimize intramolecular renaturation of the palindromic central rDNA fragments. Nick-translated ^{32}P-labeled probes were hybridized to filters by standard procedures (10). The probes consisted of pBR322, into which segments of the 5′ or 3′ rDNA NTSs shown in Fig. 1B had been inserted (pTtR1 and p6J, respectively) (5). Densitometric measurements of rDNA fragments were made on autoradiographs by using an LKB model 2222-010 Ultrascan XL laser densitometer.

Determination of the relative amounts of C3 and B rDNA-specific restriction fragments in heterozygotes. Cells of wild-type strains C3 and B were mixed in various proportions and harvested to yield control DNA preparations. In these preparations, the C3 rDNA made up 0, 1, 3, 10, 30, 50, 70, 90, 97, 99, and 100% of the total rDNA (C3 plus B rDNA), on the basis of the ratios of cells mixed and the lack of significant differences in total rDNA levels within cells of strains C3 and B (8). The control DNA preparations were digested with *Bam*HI and subjected to Southern blot analysis. Densitometric measurements were made on bands representing the C3 rDNA-specific 4-kb fragment and the B rDNA-specific 2.5-kb fragment (Fig. 1B). For most of the controls, this analysis was done on three or more separate blots. A broad range of exposures was analyzed in all cases. The intensity of bands from nearly all DNA preparations reported in this paper fell within the range of intensities analyzed from the controls. The range attached to the percentage of rDNA having the C3-specific *Bam*HI site (3′Bam-C3 rDNA) was calculated from the range of values obtained for densitometric measurements in repeated analyses of comparable controls.

Control DNA preparations were also used in a similar but less precise quantitative analysis of *Sph*I and *Xba*I-*Msp*I digests. *Sph*I digests were analyzed by visually comparing the relative intensities of a 14-kb C3 rDNA-specific band with the 2- and 6-kb B rDNA-specific bands on autoradiographs. *Xba*I-*Msp*I digests were analyzed by comparing the relative intensities of a 796-bp C3 rDNA-specific band with a 754-bp B rDNA-specific band, visually or by densitometric measurements (Fig. 1B).

Cloning and DNA sequence analysis. To obtain a source of pure C3-*rmm4* rDNA for cloning and sequence analysis, we crossed strain SB841 with strain CU374, which lacks the left arm of chromosome 2 in the micronucleus and therefore has no micronuclear form of the rDNA. This cross produced hemizygous cells containing only C3-*rmm4* rDNA in their macronuclei. Macronuclear rDNA was purified from these cells and prepared for cloning into pUC119 by restriction digestion with either *Xba*I alone or a combination of *Xba*I and *Taq*I. Recombinant clones containing each of three expected fragments of the 5′NTS were isolated: a 660-bp *Taq*I-*Xba*I fragment, a 430-bp *Xba*I-*Xba*I fragment, and an 820-bp *Xba*I-*Taq*I fragment (8). The 820-bp *Xba*I-*Taq*I fragment includes the domain 2 region and corresponds to the *Xba*I-*Msp*I fragment shown in Fig. 1B, but contains an additional 24 bp. Single-stranded DNA was produced from these clones by superinfection of the bacterial host strain MV1193 with helper bacteriophage M13KO7 (23a). Dideoxy sequencing was performed by previously described procedures (20).

For DNA sequence analysis of rDNA molecules generated in C3-*rmm4*/B-*Pmr* and C3-*rmm1*/B-*Pmr* mass cultures, rDNA was isolated from the cultures after long-term vegetative growth and the 820-bp *Xba*I-*Taq*I fragment of the 5′NTS was cloned into pUC119. The DNA sequence of the critical region of this fragment, which includes the site of the 1-bp deletion in C3-*rmm1* rDNA and the 42-bp deletion in B rDNA, was verified by analysis of four independently cloned isolates from each recombinant rDNA preparation.

RESULTS

Isolation and initial characterization of the *rmm4* mutation. *rmm4* is one of four independently derived rDNA maturation and/or maintenance (*rmm*) mutations that were isolated and initially characterized by procedures similar to those described for *rmm1* (8). Induced in a *T. thermophila* strain homozygous in the germ line (micronucleus) for C3 rDNA, *rmm4* is tightly linked to the rDNA, and strains hemizygous for the mutation are viable and have a normal growth rate (data not shown).

rmm4 reverses the replication advantage of wild-type C3 rDNA (see Introduction), causing the loss of C3 rDNA in macronuclei initially heterozygous for C3 and B rDNA. This was shown by mating a *T. thermophila* strain homozygous in the germ line for C3 rDNA and *rmm4* (C3-*rmm4*) with a strain homozygous for B rDNA and *Pmr* (B-*Pmr*). *Pmr* is a dominant mutation within the rDNA that confers resistance to the antibiotic paromomycin (Fig. 1B) (2, 23). Twenty-one individual cell lines from the population of newly formed C3-*rmm4*/B-*Pmr* heterozygotes were established and maintained as described in Materials and Methods. All of the cell lines remained Pm^r throughout long-term vegetative growth (>240 cell divisions), demonstrating a persistence of the B-*Pmr* rDNA. A molecular analysis of the cell lines, using restriction fragment length polymorphisms between C3 and B rDNA in the 5'NTS and 3'NTS (discussed in more detail below), confirmed that the C3 rDNA had been replaced by B rDNA (data not shown). In contrast, most C3-*rmm*+/B-*Pmr* cell lines are Pm^s owing to the replication advantage of wild-type C3 rDNA over B rDNA (8).

All of the characteristics reported above for *rmm4* are the same as those reported for *rmm1* (8). Furthermore, we found that *rmm4* is associated with the same 1-bp deletion within the rDNA 5'NTS as that previously described for *rmm1* (see Introduction) (8).

Rare recombinational events lead to a prevalence of rDNAs with C3-*rmm* replication capabilities in C3-*rmm4*/B-*Pmr* mass cultures. The relative amounts of C3 and B rDNA in mass cultures of C3-*rmm4*/B-*Pmr* heterozygotes were determined by using a *Bam*HI restriction site polymorphism in the rDNA 3'NTS (Fig. 1). C3-*rmm4*/B-*Pmr* cultures were produced from three matings involving differing combinations of parental strains: SB841 × SB1915, SB842 × SB1915, and SB841 × SB1914 (Table 1). Cross-fertilized progeny were selected as described in Materials and Methods. At various time points throughout prolonged vegetative growth, DNA was isolated from the cultures, digested with *Bam*HI, and subjected to Southern blot analysis. rDNA yielding *Bam*HI fragments characteristic of the C3 and B rDNA 3'NTS will be referred to as 3'Bam-C3 rDNA and 3'Bam-B rDNA, respectively. At the same time points, 96 individual cells were isolated from each culture and tested for Pm^r. The results are shown in Fig. 2A.

At the earliest time point tested (ca. five fissions), both 3'-Bam-C3 and 3'Bam-B rDNAs were present, indicating that C3-*rmm4* as well as B rDNA had been amplified in the developing macronucleus. Over the next 80 fissions, the relative amount of 3'Bam-C3 rDNA gradually decreased. However, after 90 fissions, a reversal of this trend occurred, such that the 3'-Bam-C3 rDNA replaced nearly all of the 3'Bam-B rDNA in the population during the next 200 fissions. This pattern and timing were remarkably similar in the results from all three experiments. The data from the testing of single-cell isolates from these cultures for Pm^r were consistent with the data from the 3'Bam molecular analysis.

Figure 2B shows the results from two identical matings (SB805 × SB1915 [Table 1]) which produced C3-*rmm1*/B-*Pmr* cultures and one control mating (SB1934 × SB1915) which produced a C3-*rmm*+/B-*Pmr* culture. The results from the two C3-*rmm1*/B-*Pmr* (Fig. 2B) and the three C3-*rmm4*/B-*Pmr* (Fig. 2A) cultures are strikingly similar. The initial Pm^r phenotype and the early decline in relative amounts of 3'Bam-C3 rDNA in the mutant cultures show the replicative disadvantage of C3-*rmm4* and C3-*rmm1* rDNAs when in competition with B rDNA. The results from the C3-*rmm*+/B-*Pmr* control culture are consistent with the replication advantage of wild-type C3 rDNA over B rDNA: 3'Bam-C3 rDNA completely replaced the 3'Bam-B rDNA by 100 fissions, and all cells in the culture became Pm^s.

The resurgence of 3'Bam-C3 rDNA and Pm^s cells, observed after 90 fissions in the mutant mass cultures, was unexpected in view of the replication disadvantage of the mutated C3 rDNAs. The persistence of Pm^r in all of the mutant heterozygote cell lines (reported above) indicated that the resurgence of Pm^s cells in the mass cultures shown in Fig. 2 was due to the overgrowth of these cultures by a small subpopulation of cells (see Discussion). Nevertheless, the establishment of such rare cells having a prevalence of rDNA with C3 characteristics needed explanation. To investigate the possibility that somatic recombination between rDNA molecules was involved, we conducted a more complete molecular analysis on the DNA preparations selected from among the time points plotted in Fig. 2. The 5'NTS, as well as the 3'NTS, of the rDNA was investigated for the selected time points by using two other restriction fragment length differences between C3 and B rDNA. One difference is due to an *Sph*I site located 1 kb from the palindromic center in B but not C3 rDNA (Fig. 1B). The other difference, approximately 1.2 kb from the palindromic center, is due to the 42-bp deletion in B rDNA that is responsible for its replication defect. This deletion causes the B rDNA to have a perceptibly shorter *Xba*I-*Msp*I restriction fragment from that region (Fig. 1B).

For each time point investigated, samples of DNA were subjected to Southern blot analysis with the following combinations of probes (illustrated in Fig. 1B) and restriction endonuclease digests: (i) 5' probe of *Sph*I digests; (ii) 5' probe of *Msp*I and *Xba*I double digests; and (iii) 3' probe of *Bam*HI digests. Quantitations were done as described in Materials and Methods. We chose to examine one of the matings which produced C3-*rmm4*/B-*Pmr* cultures (SB841 × SB1914), the two identical matings that produced C3-*rmm1*/B-*Pmr* cultures, and the one mating that produced the C3-*rmm*+/B-*Pmr* culture. The results are shown in Table 2.

Throughout the first 89 fissions in the C3-*rmm4*/B-*Pmr* culture, there was general agreement among the four sites tested, with B rDNA being the major (i.e., prevalent) type of rDNA in the culture (Table 2). By 112 fissions, a recombinant rDNA appeared as a minor type; it became the major rDNA type by 177 fissions. This recombinant, called Rec in Table 2, was by far the major rDNA species at 295 fissions. At this time, no rDNA having the C3-specific *Sph*I sites was detected, while more than 90% of the rDNA had C3 rDNA characteristics at the other three sites tested. As an example, the Southern blot analysis of DNA isolated from one representative clone, established from the mass culture at 217 fissions, is shown in Fig. 3. The production of the prevalent recombinant rDNA had to involve a recombination between the B rDNA-specific *Sph*I site and the 42-bp deletion in the 5' *Xba*I-*Msp*I fragment, separated by only 209 bp (Fig. 4A).

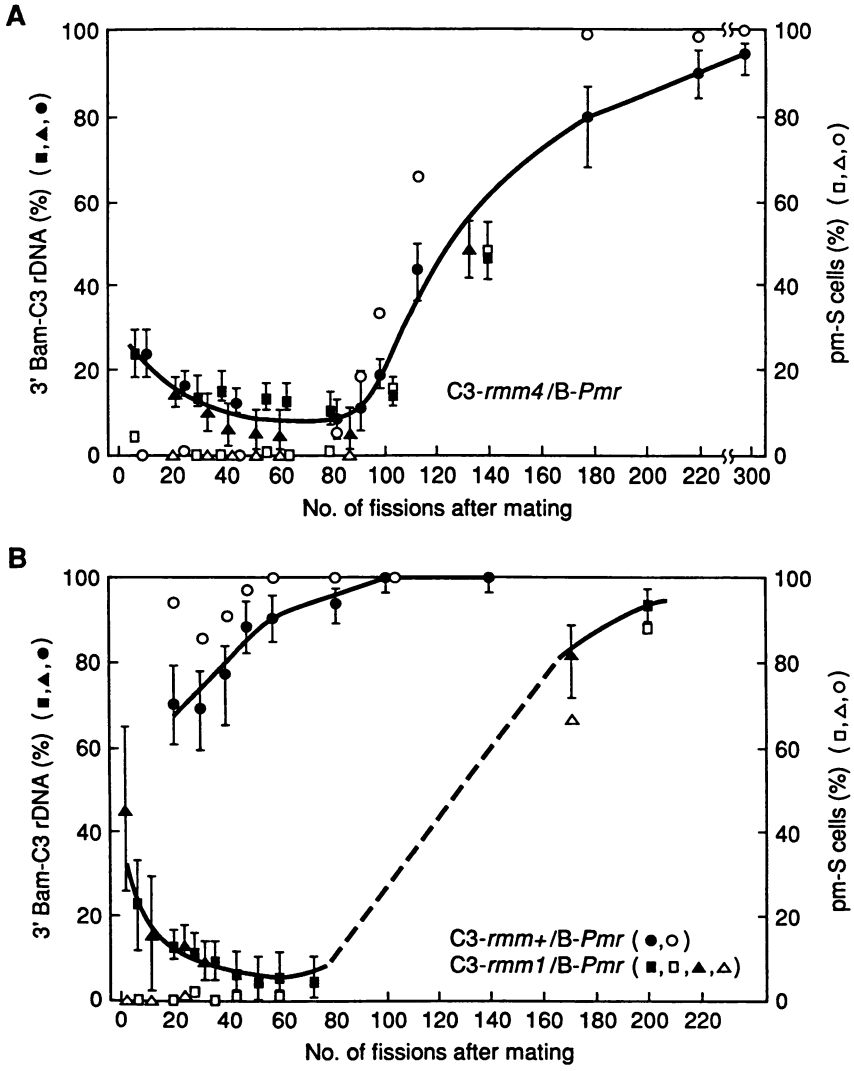

FIG. 2. Determination of 3'Bam-C3 rDNA and of Pm^s cell fractions in mass cultures as a function of the number of fissions after mating. Heterozygous progeny were maintained in the logarithmic phase of growth by periodic inoculations of 10^4 cells into fresh media. At the time points indicated on the abscissa, cells were harvested, and the percentage of total rDNA that was 3'Bam-C3 was determined by Southern blot analysis of *Bam*HI digests with the 3' probe (see Materials and Methods) (Fig. 1). At the same time points, 96 single cells were isolated from the mass cultures and tested for resistance to paromomycin. (A) C3-*rmm4*/B-*Pmr* progeny from three different crosses: SB841 × SB1915 (■, □), SB842 × SB1915 (▲, △), and SB841 × SB1914 (●, ○). Solid symbols denote the percentage of 3'Bam-C3 rDNA. Open symbols denote the percentage of Pm^s cells. The vertical lines show the possible ranges of the percent 3'Bam-C3 rDNA values which were determined as described in Materials and Methods. (B) C3-*rmm1*/B-*Pmr* progeny from two separate crosses of SB805 × SB1915 (■, □ and ▲, △) and C3-*rmm*+/B-*Pmr* progeny from a SB1934 × SB1915 cross (●, ○). Solid symbols, open symbols, and vertical lines are defined for panel A.

No recombinant rDNA molecules were detected in C3-*rmm1*/B-*Pmr* or C3-*rmm*+/B-*Pmr* cultures (Table 2).

The rDNA of long-term C3-*rmm4*/B-*Pmr* cultures lacks the 1-bp deletion of C3-*rmm4* rDNA and the 42-bp deletion of B-*Pmr* rDNA. The prevalence of recombinant rDNA molecules in C3-*rmm4*/B-*Pmr* cultures raised the possibility that they had the replication capability of wild-type C3 rDNA. If so, the recombinant molecules could have a completely intact domain 2 region, lacking both the 1-bp deletion of C3-*rmm4* rDNA and the 42-bp deletion of B-*Pmr* rDNA. To test this possibility, we purified and cloned recombinant rDNA from a C3-*rmm4*/B-*Pmr* mass culture derived from the cross between strains SB841 and SB1914 (Fig. 2A) and examined the nucleotide sequences of four independently cloned isolates in the region containing domain 2, just downstream from the *Xba*I site (see Materials and Methods). This analysis showed that domain 2 of the recombinant molecules is in fact indistinguishable from that of wild-type C3 rDNA. Thus, the crossover event generating these rDNAs must have occurred within the 25-bp region that separates the 1-bp deletion of C3-*rmm4* rDNA and the 42-bp deletion of B-*Pmr* rDNA (Fig. 4B).

In the C3-*rmm1*/B-*Pmr* mass cultures, no recombinant rDNA molecules were detected by restriction analysis. However, the similarity in results of the C3-*rmm1*/B-*Pmr* and C3-*rmm4*/B-*Pmr* mass culture experiments shown in Fig. 2 suggested that rDNA molecules with wild-type C3 rDNA replication capabilities could have been generated in the C3-*rmm1*/B-*Pmr* cultures by a reversion or recombinational event that did not affect the *Sph*I site, but corrected the 1-bp deletion in C3-*rmm1* rDNA. Therefore, rDNAs from the C3-*rmm1*/B-*Pmr* mass culture derived from the

TABLE 2. Analysis of rDNA from mass cultures[a]

rDNA genotype of culture	No. of fissions after mating	% rDNA with indicated C3-type polymorphism			% Pm[s] cells	Inferred type of rDNA[b]	
		5′ NTS		3′ NTS (*Bam*HI)			
		*Sph*I	*Xba*I-*Msp*I			Major	Minor
C3-*rmm4*/B-*Pmr*	24	5–30	10–50	14–20	1	B	C3
	89	1–30[c]	5–20	6–20	19	B	C3
	112	5–30[c]	30–70	37–50	66	B	Rec, C3
	177	5–30[c]	70–100	69–87	99	Rec	C3
	217	10–30[c]	ND[d]	85–96	99	Rec	C3
	295	0–10[c]	90–100	91–98	100	Rec	None[c]
C3-*rmm1*/B-*Pmr*	20	10–30	ND	10–17	0	B	C3
	28[e]	0–30	ND	6–14	ND	B	C3
	43	3–30	ND	2–11	1	B	C3
	73	0–30	ND	1–10	ND	B	C3
	170[e]	50–90	ND	72–89	67	C3	B
	200	70–97	ND	90–98	89	C3	B
C3-*rmm*+/B-*Pmr*	20	70–90	60–90	60–78	C3	B	
	99	90–100	80–100	96–100	100	C3	None

[a] Selected time points from Fig. 3.
[b] Based on data in columns to the left. Rec, Recombinant rDNA (see text).
[c] Minor amounts of rDNA having the B-specific *Sph*I site in one-half of the rDNA palindrome, but not the other, were also detected.
[d] ND, Not determined.
[e] Time points for a duplicate experiment.

cross between SB805 and SB1915 shown in Fig. 2B were purified, and the nucleotide sequences of four cloned isolates were examined in the domain 2 region as described above. In contrast to the results obtained for the C3-*rmm4*/B-*Pmr* culture, rDNA from the C3-*rmm1*/B-*Pmr* culture still contained the 1-bp deletion characteristic of the mutant C3-*rmm1* rDNA. Examination of ca. 200 bp of the domain 2 region revealed no changes from the previously determined mutant C3-*rmm1* rDNA sequence. Thus, the resurgence of C3 rDNA in the C3-*rmm1*/B-*Pmr* mass culture cannot be explained simply on the basis of restoration of domain 2 sequences as in the C3-*rmm4*/B-*Pmr* cultures.

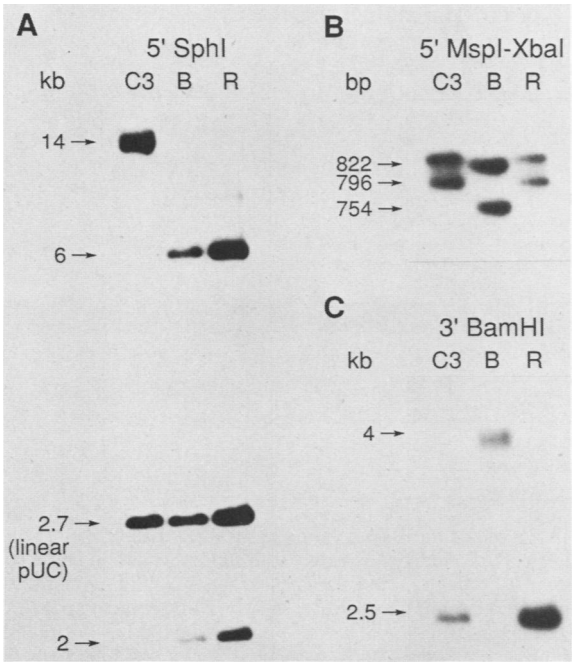

FIG. 3. Analysis of restriction fragment length polymorphisms in a clone having rDNA recombined within the 5′NTS. Samples of DNA from wild-type strain C3 (lanes C3), strain B (lanes B), and a cell clone established at 217 fissions from a C3-*rmm4*/B-*Pmr* culture (Fig. 2A) containing the recombined rDNA (lanes R) were digested with *Sph*I (panel A), both *Msp*I and *Xba*I (panel B), or *Bam*HI (panel C). The digested samples were separated on agarose gels, blotted to nylon filters, and hybridized to either the 5′ probe (panels A and B) or the 3′ probe (panel C) diagrammed in Fig. 1B. Supercoiled pUC18, which hybridizes to the pBR322-derived region of the probes, was added to each sample prior to digestion with *Sph*I (panel A) to monitor the extent of digestion (see Materials and Methods). The minor band in panel A, lane R, migrates as an 8-kb fragment, which is expected only from *Sph*I digestion of heteropalindromes. Note that the recombined rDNA has the *Sph*I restriction fragments of B rDNA and the *Msp*I-*Xba*I and *Bam*HI fragments of C3 rDNA, indicating recombination within the 5′NTS.

DISCUSSION

The 1- and 42-bp deletions, in the 5′NTS of C3-*rmm4* (*rmm1*) and B rDNAs, respectively, confer a replication disadvantage. We have now isolated a second mutation, *rmm4*, indistinguishable in phenotype and DNA sequence alteration from the *rmm1* mutation, which was previously shown to affect rDNA replication (8). We are certain of the independent derivation of the two mutations because the *rmm4* mutation occurred at a frequency of 2.5×10^{-5} in a culture of the parental strain that was established (after the isolation of *rmm1*) with an inoculum of only 20 cells (see Materials and Methods). Two other *rmm* mutations still under investigation, *rmm2* and *rmm3*, do not have the same phenotype or DNA sequence alteration as *rmm1* and *rmm4*.

Our previous conclusion that the 1-bp deletion in the rDNA 5′NTS (Fig. 1) was the molecular basis of the *rmm1* mutation was based on its strategic location in a conserved repeat element near the rDNA origin of replication (8). It was concluded that the B rDNA replication disadvantage was due to the 42-bp deletion, because of its effect on the same repeat element. However, we could not rule out the

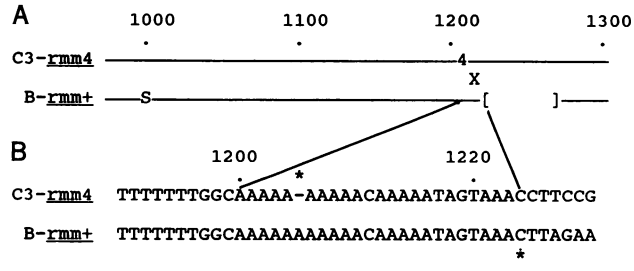

FIG. 4. Region of crossover between C3-*rmm4* and B-*rmm*+ rDNAs producing a wild-type C3 rDNA sequence within domain 2. (A) A region of the rDNA 5'NTS, approximately 1,000 to 1,300 bp from the rDNA palindromic center, is diagrammed. This includes much of domain 2 (approximately 1,100 to 1,350 bp [Fig. 1A]). Symbols: 4, 1-bp deletion in C3-*rmm4* rDNA; S, *Sph*I site specific to B rDNA; [ ], 42-bp deletion in B rDNA. A recombination anywhere within the 209-bp region between the *Sph*I site and the 42-bp deletion would produce the recombinant rDNAs detected by Southern blot analysis of the C3-*rmm4*/B-*Pmr* culture (see Table 2 and text). The X indicates the expected crossover location for production of recombinant rDNA having a replication advantage over either parental rDNA. (B) The 25-bp region in which the crossover occurred (positions 1200 to 1224), producing the wild-type C3 domain 2 sequence, is shown with flanking sequences. The numbers above the C3-*rmm4* rDNA refer to the corresponding positions of the respective nucleotides in wild-type C3 rDNA previously reported (8). The asterisks above the C3 rDNA and below the B rDNA show the respective locations of the 1- and 42-bp deletions. The 1-bp deletion from the run of A nucleotides is arbitrarily marked in the middle of the run. The sequences to the right of the 25-bp crossover region do not match, because of the 42-bp deletion in B rDNA.

possibility that other regions of the rDNA, not subjected to sequencing analysis, contained the determinants for the replication deficiencies in C3-*rmm1* and B rDNAs.

Our finding in the present study that *rmm4* is associated with the same 1-bp deletion associated with *rmm1* supports our previous conclusion. More importantly, we showed that a recombinant rDNA, having a completely intact domain 2 characteristic of wild-type C3 rDNA, replaced the nonrecombinant rDNA in a mass culture of C3-*rmm4*/B-*Pmr* heterozygotes. Production of these recombinant molecules had to involve a crossover within the 25 bp that separate the 1-bp deletion of C3-*rmm4* rDNA and the 42-bp deletion of B-*Pmr* rDNA. The emergence of rDNAs produced from this highly specific recombinational event precludes any doubt that the 1- and 42-bp deletions are the molecular determinants for the deficiencies in their respective rDNAs.

Pathways leading to the production of efficiently replicating recombinant homopalindromes and heteropalindromes. The recombinant rDNA molecules described above are homopalindromic; that is, the two halves of the palindrome are identical. This is evident from the homogeneity observed at each site tested for C3 or B rDNA characteristics (Table 2). However, formation of recombinant homopalindromes would have to involve heteropalindromic intermediates, unless recombination occurs prior to rDNA palindrome formation during macronuclear development. Figure 5 illustrates one possible pathway for the production of recombinant homopalindromes of the type that prevailed in the C3-*rmm4*/B-*Pmr* culture, that is, rDNAs that lack both the 1-bp deletion of C3-*rmm4* rDNA and the 42-bp deletion of B rDNA. In the pathway shown, each step produces a faster-replicating molecule.

Population replacement phenomena in mass cultures. The resurgence of rDNA with C3 characteristics seen in all five C3-*rmm4*/B-*Pmr* or C3-*rmm1*/B-*Pmr* mass cultures was unexpected (Fig. 2). The absence of such resurgence in the cell line experiments, maintained by serial single-cell transfers, leads to the conclusion that a minority of cells having C3 rDNA sequences take over in the mass cultures, which were maintained by periodic inoculations of 10^4 cells. A slight cell growth disadvantage (<5%) in the majority of cells (containing B-*Pmr* rDNA) can readily explain the observed replacement rate. This would also account for the persistence of B-*Pmr*+ rDNA (i.e., absence of C3-type rDNA resurgence) previously reported for C3-*rmm1*/B-*Pmr*+ mass cultures (8). The determinant of slow growth could well be the *Pmr* mutation itself. This seems plausible in view of the previous observation that cells having the *Hmr* mutation, conferring hygromycin resistance and located four nucleotides away from *Pmr* (23), have a lower growth rate (E. A. Spangler and E. H. Blackburn, unpublished observations).

The overgrowth of the mutant cultures by rare subclones must be preceded by events that lead to a prevalence of C3-type rDNA in those subclones. However, this event need not involve the restoration of domain 2 sequences discussed above. For example, the eventual overgrowth by Pm^s cells containing C3 rDNA in C3-*rmm1*/B-*Pmr* mass cultures (Fig.

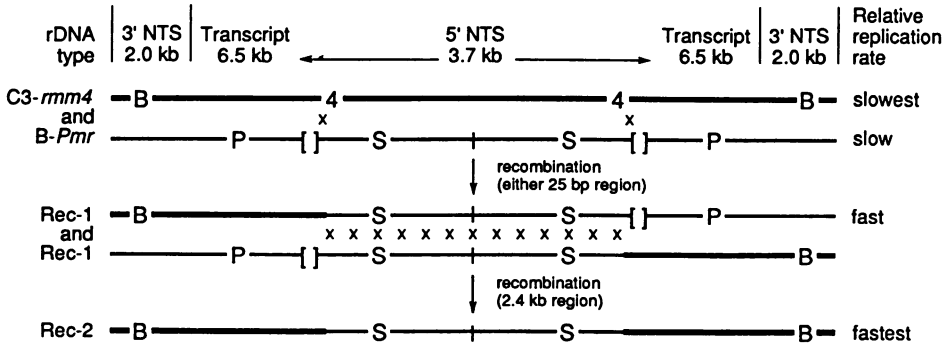

FIG. 5. Possible recombination pathway leading to the formation of rDNA devoid of the 1-bp deletion associated with C3-*rmm4* and the 42-bp deletion associated with B rDNA. Symbols: ▬, C3 rDNA; ——, B rDNA; | , palindromic center; B, *Bam*HI site of C3 rDNA 3'NTS; P, *Pmr* in B rDNA 17S coding region; S, *Sph*I site in B rDNA 5'NTS; 4, *rmm4* 1-bp deletion in C3-*rmm4* rDNA 5'NTS; [], 42-bp deletion in B rDNA 5'NTS; X, crossover sites that would generate the product shown. Distances are not to scale; the 5'NTS has been expanded with respect to the rest of the molecule. The Rec-2 product should have the replication capabilities of a wild-type C3 rDNA, having neither the *rmm4* mutation nor the 42-bp deletion. Rec-2 has all of the characteristics of the recombinant rDNA molecules shown in Table 2 and Fig. 3.

2) was not accompanied by any recombination detectable by our analysis (Table 2). Furthermore, DNA sequencing showed that the C3 rDNAs still had the 1-bp deletion of *rmm1*. One possible explanation is that rare cells, having exclusively C3-*rmm1* rDNA, arose as a result of the failure of the B-*Pmr* rDNA to mature in the developing macronuclei at conjugation. In the absence of competition from B rDNA, C3-*rmm1* rDNA would be maintained at normal copy number (*rmm1* hemizygotes are viable and show no noticeable growth defects [8]). An analogous phenomenon, i.e., the appearance of clones maintaining exclusively B rDNA in wild-type C3/B heterozygotes, has been described (9). Other, more complex explanations for the persistence of C3-*rmm1* in rare cells have not been ruled out.

Although the events leading to a prevalence of C3-type rDNA differed among the mutant cultures, their timing and frequency must have been similar; all five mutant cultures exhibited the same time course of takeover by cells containing C3-type rDNA (Fig. 2). This similarity suggests that the recombination within the 25-bp interval separating the deletions in C3-*rmm4* and B rDNAs also occurred during or soon after macronuclear development. A recombination frequency on the order of 1 per 10^3 cells would account for the timing with which cells having the recombinant rDNA overgrew the culture, assuming a constant 5% growth advantage of those cells. If recombination occurred in mature macronuclei (having 10^4 rDNA copies), the frequency of recombination between rDNA molecules in the 25-bp interval, prior to the loss of C3-*rmm4*, need not have been higher than 10^{-7} per molecule. The recombination frequency may be disproportionately high, because the interval is bound by a 42-bp deletion which may arrest branch migration and cause an accumulation of Holiday structure resolution events (Fig. 4B).

rDNA somatic recombination. The complex changes observed in the mass cultures call attention to the role of the *Tetrahymena* macronucleus in promoting somatic evolutionary adaptation. Infrequent macronuclear DNA recombination, coupled with the high ploidy of the macronucleus, provides the potential for an enormous degree of somatic genetic diversity, expressed and available for natural selection during asexual multiplication. This somatic mechanism does not replace but rather supplements the sexual (meiotic) mechanisms, common to *Tetrahymena* species and other eucaryotes, for generating diversity in the germ line.

Homologous somatic recombination has been implicated in a variety of molecular phenomena in eucaryotes (reviewed in reference 17). These include homozygosis of heterozygous loci (17), DNA amplification (21), tumorigenic expression of oncogenes (7), and homologous integration of transfected genes (22, 25). The fortuitous conjunction in *Tetrahymena* spp. of mutations that affect rDNA replication and cell growth, the multiplicity of rDNA polymorphisms, and the high copy number of rDNA in the macronucleus provide an excellent opportunity for molecular studies of somatic recombination. These advantages should also facilitate tests of pharmacological agents for their effect on recombination.

The analysis of recombinant rDNA can also be used to localize rDNA mutations. One example is provided here: the facile isolation of recombinants within a 25-bp region provides strong support for the importance of the type I repeat of domain 2 of the 5'NTS for rDNA replication. A similar approach has been used to localize the likely site of the *rmm3* mutation to the rDNA region distal to *Pmr* (P. Yaeger and E. Orias, unpublished observations). The same approach should facilitate the analysis of rDNA mutations affecting other essential functions, such as rRNA function, rDNA maturation during macronuclear development, and nucleolar organization.

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