Tetrahymena thermophila Mutants Defective in the Developmentally Programmed Maturation and Maintenance of the rDNA Minichromosome

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ABSTRACT

The abundant rDNA minichromosome of *Tetrahymena thermophila* is generated by a series of developmentally controlled processing steps, termed rDNA maturation, during the formation of the new macronucleus in conjugating cells. rDNA maturation involves excision of a region encoding the single copy rRNA gene (rDNA) from its germline location, rearrangement of the rDNA into a palindromic minichromosome, *de novo* telomere addition, and amplification to approximately 10^4 copies. The rDNA is maintained at this high level in vegetatively growing cells. Using a previously developed genetic scheme for studying rDNA maturation and maintenance, we report the isolation of a new class of mutants defective for rDNA maturation. Several new rDNA maintenance mutants were also obtained. The maturation mutant, *rmm10*, is severely defective for the production of both monomeric and palindromic rDNA in the developing macronucleus. The *rmm10* mutation is recessive-lethal and *cis*-acting. None of the previously identified DNA sequence elements that control rDNA maturation or maintenance is mutated in *rmm10*. Therefore, additional *cis*-acting sequence elements must be required for rDNA maturation. Based on our current understanding of rDNA maturation processes, we suggest that the *rmm10* mutation affects rDNA excision rather than subsequent rDNA amplification/replication.

PROGRAMMED DNA rearrangement and gene amplification play important developmental roles in many organisms [reviewed in SCHATZ *et al.* (1992), HERSKOWITZ (1989) and STARK and WAHL (1984)]. In higher eukaryotes, spontaneous DNA rearrangement and gene amplification frequently lead to tumorigenesis and the resistance of tumors to chemotherapeutic agents [reviewed in STARK and WAHL (1984)]. In the ciliate *Tetrahymena thermophila*, DNA rearrangement and amplification are amenable to both molecular and genetic analysis [reviewed in YAO (1989) and KAPLER (1993)]. DNA rearrangement and gene amplification are obligatory steps in the developmental program that generates the abundant rDNA minichromosome, encoding the 17S, 5.8S and 26S ribosomal RNAs. The rDNA is subsequently replicated from a single origin (CECH and BREHM 1981), making this a useful model system for studying the regulation of eukaryotic DNA replication.

The developmentally programmed processing and amplification of the *T. thermophila* rDNA derives from the unique nuclear dimorphism typical of ciliated protozoa. *T. thermophila* contains two nuclei within a single cell: the transcriptionally silent germline micronucleus and the transcriptionally active somatic macronucleus [reviewed in YAO (1989)]. During conjugation a new germline micronucleus forms. The old macronucleus is destroyed and a new macronucleus is generated by the differentiation of a copy of the micronucleus. This process, termed macronuclear development, involves massive reorganization of the genome. Macronuclear chro-

mosomes are generated by site-specific fragmentation of the progenitor micronuclear chromosomes (YAO *et al.* 1987). Sequences within and adjacent to the macronuclear chromosomes are eliminated (GODISKA and YAO 1990) and telomeres are added *de novo* to the ends of these chromosomes (SPANGLER *et al.* 1988; YAO and YAO 1989; YU and BLACKBURN 1991). Macronuclear chromosomes attain an average copy number of about 45. The rDNA minichromosome is amplified further to approximately 10^4 copies (YAO *et al.* 1974). Fully processed macronuclear chromosomes are replicated on average once per cell cycle during subsequent vegetative growth of progeny (ENGBERG *et al.* 1972). However, these chromosomes segregate randomly and the macronucleus often divides unequally, so additional mechanisms must contribute to the control of macronuclear chromosome copy number (PREER and PREER 1979).

The processing steps which generate the amplified macronuclear rDNA are collectively termed rDNA maturation (Figure 1) (LARSON *et al.* 1986). In the developing macronucleus of conjugating cells, the single copy 10.3-kb rDNA region is excised from its germline location and rearranged into a 21-kb palindromic head-to-head minichromosome (KARRER and GALL 1976; ENGBERG 1983) whose termini are capped by telomeres (KING and YAO 1982). Monomeric 11-kb rDNA, a side product of rDNA maturation or a potential intermediate in palindrome formation, is also formed in the developing macronucleus (PAN and BLACKBURN 1981). Palindromic rDNA is amplified to approximately 10^4
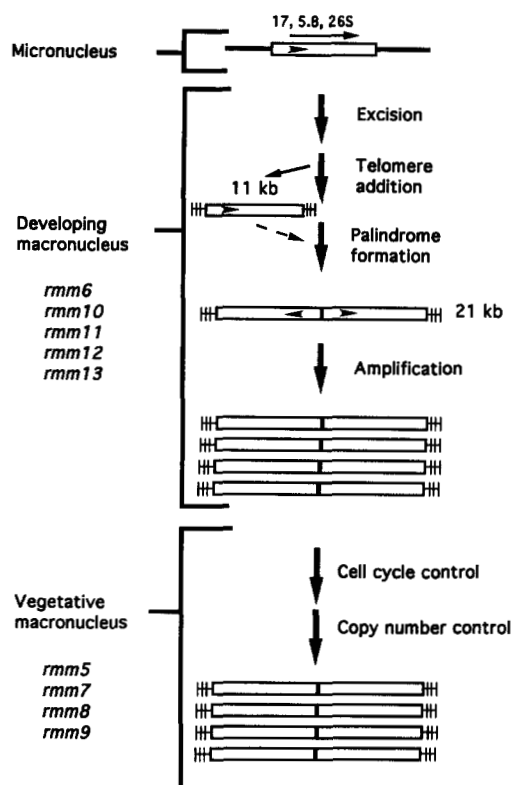
FIGURE 1.—Proposed pathway for rDNA maturation and maintenance. The micronuclear and macronuclear rDNA forms are diagrammed. The rDNA region (open box), encoding the 17S, 5.8S and 26S rRNAs (arrow), resides within a large chromosome in the germline micronucleus (flanking lines represent adjacent micronuclear DNA). In the developing macronucleus of mating cells, the 10.3-kb rDNA region is excised from its micronuclear position, telomeres are added *de novo*, and the rDNA is rearranged into a 21-kb head-to-head palindrome (arrow represents the direction of transcription and vertical bars at ends of the rDNA represent telomeres). The 21-kb palindromic rDNA is amplified to about 10^4 copies. Monomeric 11-kb rDNA, with telomeres on both rDNA ends is also formed in the developing macronucleus, but is lost during vegetative growth. Whether the 11-kb rDNA species serves as an intermediate in palindrome formation or is a side product of rDNA maturation is not known. In the vegetative macronucleus of progeny, palindromic rDNA is maintained at an amplified level through cell cycle control and copy number control mechanisms. The mutants *rmm5–rmm13*, described in this report, are listed on the left. Maturation mutants (*rmm6, 10, 11, 12* and *13*) show a defect during *C3* rDNA processing and amplification in the developing macronucleus; while maintenance mutants (*rmm5, 7, 8* and *9*) only appear to affect the *C3* rDNA during vegetative growth.

copies. The rDNA minichromosome is subsequently maintained at this level during vegetative fissions by cell cycle (ENGBERG *et al.* 1972) and copy number (LARSON *et al.* 1986) control mechanisms, collectively termed rDNA maintenance (Figure 1).

DNA transformation studies have identified *cis*-acting sequences required for rDNA excision and palindrome formation (YAO *et al.* 1987; YASUDA and YAO 1991). A classical genetic approach has also been developed for

studying the control of rDNA maturation and maintenance (LARSON *et al.* 1986; KAPLER and BLACKBURN 1994). Previously, this approach was successful for isolating rDNA maturation and maintenance (*rmm*) mutants with altered vegetative maintenance properties (LARSON *et al.* 1986; YAEGER *et al.* 1989; D. D. LARSON, personal communication). Here, we report the isolation of a new class of mutants defective for rDNA maturation. New rDNA maintenance mutants were also obtained. One mutant, *rmm10*, has a severe *cis*-acting mutation affecting rDNA maturation; however, none of the known *cis*-acting rDNA maturation or maintenance control elements are altered. Thus, the *rmm10* mutation will identify a new *cis*-acting regulatory element essential for rDNA maturation. We propose that this mutation affects the formation of macronuclear rDNA, probably at the excision step.

MATERIALS AND METHODS

Cell culture and Tetrahymena strains: Cultures were grown in 2% proteose peptone supplemented with 10 µm FeCl₃ and 250 µg/ml penicillin and streptomycin, 25 µg/ml amphotericin B. Standard genetic procedures have been described previously (ORIAS and BRUNS 1975; ORIAS and HAMILTON 1979). Relevant strains are listed in Table 1. Mutagenesis of the *C3-Pmr⁺* strain *SB1934* with nitrosoguanidine was performed as previously described (ROBERTS and ORIAS 1973). Mutagenesis of *SB1934* with ethyl methanesulfonate (EMS) was performed as for nitrosoguanidine, except that 10 µg/ml EMS was substituted, with an exposure time of 6 hr prior to mating.

Isolation of *rmm* mutants: Mutagenized *SB1934* cells (*C3-Pmr⁺*) were mated with *SB1917* (*B-Pmr1*). Progeny were refed by dilution to 1% proteose peptone at 24 or 48 hr after initiating mating (see below) and distributed into 96-well microtiter dishes at 200 cells/well. Mutagenized cells (2.4 × 10⁶) were analyzed in three experiments. Cross-fertilized F₁ progeny were selected sequentially with paromomycin (pm, 200 µg/ml) and 6-methylpurine (15 µg/ml), and then replica transferred in nonselective media for at least 10 passages (approximately 70 fissions). F₁ progeny that retained the *B* rDNA allele in the macronucleus were identified by replica transferring cells into pm after 10 passages. We obtained 2300 paromomycin-resistant (pm-r) F₁ progeny.

In an effort to identify temperature-sensitive and cold-sensitive maturation mutations, 1/3 of the cells in the F₁ cross described above were mixed at 30° and shifted to 35° from 8 to 24 hr after mixing to initiate mating, mixed at 25° and shifted to 20° from 8 to 48 hr after mixing, or incubated at 30° for the entire 24-hr mating period. Cells shifted to 35° were refed at 24 hr, whereas cells shifted to 20° were refed at 48 hr. Cultures were plated out as described above, subsequently grown at 30° and selected for pm-r progeny at 70 fissions. Although *rmm* mutants were isolated in these conditional mutant screens, their respective mutant phenotypes were not conditional.

To determine if pm-r F₁ progeny lines contained heritable mutations affecting C3 rDNA maturation or maintenance, heterozygous (*C3-Pmr⁺/B-Pmr1*) pm-r F₁ clones were individually mated with the homozygous *B-Pmr1* strain *SB1915*. Progeny from this secondary screen were selected en masse with cycloheximide, and eight progeny cells from each mating were individually isolated into media containing pm at 30–50 fissions. F₁ clones that produced only pm-resistant progeny at this time were analyzed further. (pm-r F₁ progeny that were phenocopies of the *rmm* mutant phenotype would have a *C3-*

TABLE 1

Genotype and phenotype of *T. thermophila* strains

Strain	Micronuclear genotype	Macronuclear phenotype	Mating type
SB1934	C3-rmm$^+$/C3-rmm$^+$, Mpr/Mpr	mp-s	V
SB1917	B-Pmr1/B-Pmr1	pm-s	II
SB1915	ChxA2/ChxA2, B-Pmr1/B-Pmr1	cy-s, pm-s	II
SB620	ChxA2/Chx$^+$, ts29/ts29	cy-s, temp-s	II
CU374	ChxA2/ChxA2, Mpr/Mpr nulli-2L,4L/nulli-2L,4L	cy-s, mp-r	IV
A*	Functional amicronucleate	Wild-type	III
SF102	C3/C3, rmm5/rmm5	mp-s, pm-s	III
SF126	C3-rmm6/C3-rmm6	mp-s, pm-s	III
SF106	C3-rmm7/C3-rmm7	mp-s, pm-s	III
SF110	C3-rmm8/C3-rmm8	mp-s, pm-s	III
SF114	C3/C3, rmm9/rmm9	mp-s, pm-s	III
SF118	C3-rmm10/C3-rmm10	mp-s, pm-s	III
SF122	C3-rmm11/C3-rmm11	mp-s, pm-s	III
SF130	C3-rmm12/C3-rmm12	mp-s, pm-s	III
SF134	C3-rmm13/C3-rmm13	mp-s, pm-s	III
SF137	C3/null, mono-2L,4L ChxA2/Chx$^+$, Mpr/Mpr$^+$	pm-s, cy-r mp-r	ND[a]
SF120	C3-rmm10/null, mono-2L,4L ChxA2/Chx$^+$, Mpr/Mpr$^+$	pm-s, cy-r mp-r	ND

Macronuclear phenotype designations: -r, resistant; -s, sensitive. Micronuclear genotypes are as follows: *C3*, wild-type *C3* rDNA (LARSON *et al.* 1986); *rmm*, rDNA maturation or maintenance mutation; *B-Pmr1*, paromomycin (pm) resistant B rDNA (BRUNS *et al.* 1985); *ChxA2* confers cycloheximide (cy) resistance (BYRNE *et al.* 1978); *Mpr*, confers 6-methylpurine (mp) resistance (BYRNE *et al.* 1978); *ts29* confers temperature sensitive (temp-s) growth (E. ORIAS and M. FLACKS, unpublished results); *nulli-2L,4L*, deleted for the left arm of chromosomes 2 and 4. *rmm* mutant alleles described in this paper were derived from mutagenized strain *SB1934* (see text). Unless otherwise stated, strains contain the *B-Pmr$^+$* rDNA allele in their micronucleus. The *C3*-specific *rmm5* and *rmm9* mutations have not been genetically or physically localized to the *C3* rDNA locus.

[a] Not determined.

Pmr$^+$/B-Pmr1, *rmm$^+$/rmm$^+$* F$_1$ genotype, and produce pm-sensitive (pm-s) progeny in this test cross. Note that this secondary screen is biased toward identifying *cis*-acting *rmm* mutations.) pm-r F$_1$ clones that were isolated in matings shifted to restrictive temperatures were mated in the secondary screen at both restrictive and permissive temperatures.

F$_1$ progeny that produced exclusively pm-r clones in the secondary screen were then mated with the *A** strain to generate whole-genome micronuclear homozygotes. The new diploid micronucleus that forms is derived from a single haploid meiotic pronucleus of the F$_1$ pm-r clone and is homozygous at all loci. Since these progeny retain the parental macronucleus of either the F$_1$ or *A** strains (ALLEN 1967), clonal lines carrying homozygous recessive-lethal mutations in their germline nucleus can be obtained. Test crosses with *B-Pmr$^+$* strains (*SB1969* and *SB1965*) identified *A** cross progeny that either inherited the *C3* rDNA or had deleted the *C3* rDNA in their micronucleus: they produced pm-s progeny in these matings. Crosses with strain *SB620*, which is homozygous for the recessive-lethal *ts29* mutation located on the left arm of chromosome *2*, identified lines that had deleted this locus, and most likely had also deleted the rDNA locus. Suspects that were not eliminated by these test crosses were remated with the homozygous *B-Pmr1* strain *SB1915* to confirm that they generated an *rmm* mutant phenotype. Nine lines were identified that reproducibly generated pm-r progeny at 50–70 fissions in this cross. Polymerase chain reaction (PCR) tests using primers that amplified only segments of the micronuclear copy of the *C3* rDNA showed that these regions were present in these lines, ruling out a gross deletion of the germline *C3* rDNA. These mutants were designated *rmm5–rmm13*. None of the mutants displayed a conditional maturation phenotype.

Developmental time courses: The wild-type *C3* strain (*SB1934*) or *rmm* mutants were mated with the *B-Pmr1* strain *SB1915*, and DNA was isolated from mating cultures at various times after pairing (hours) or during subsequent vegetative growth of progeny (fissions). Mated cells were refed 24 hr after

pairing. Cross-fertilized progeny were selected sequentially with cycloheximide and 6-methylpurine, and subsequently grown in nonselective media during vegetative fissions. All parental strains were heterokaryons, homozygous for the respective rDNA allele in their micronucleus, but containing only B rDNA in their parental macronucleus.

DNA isolation and Southern blotting analysis: Total genomic DNA was isolated as previously described (LARSON *et al.* 1986). For micronuclear DNA analyses, micronuclei were first purified by differential centrifugation of *n*-octanol-lysed cells as previously described (HOWARD and BLACKBURN 1985). Contaminating macronuclear rDNA was then separated from bulk genomic DNA by centrifugation in CsCl-Hoescht dye gradients (WILD and GALL 1979). Agarose gel electrophoresis and transfer to nylon membranes were performed according to the manufacturers recommendations (Nytran, Schleicher & Schuell; or Hybond N$^+$, Amersham Corp.). Cloned rDNA fragments were radiolabeled by random priming with [^{32}P]dATP and hybridized to Southern blots according to the manufacturers recommendations.

PCR assay for palindromic and monomeric macronuclear *C3* rDNA: PCR amplification was performed on genomic DNA isolated 20–24 hr after mixing the *rmm10* strain *SF118* with either the *B* rDNA strain *SB1915* (eight mating experiments) or the germline rDNA deletion strain *CU374* (three mating experiments). Primers 1 and 2 were used for PCR amplification of palindromic *C3* rDNA. The primer 1 region is deleted in the *B* rDNA allele (LARSON *et al.* 1986), and the primer 2 region corresponds to the nonpalindromic center of the rDNA palindrome. Primers 1 and 3 were used for PCR amplification of monomeric macronuclear 11-kb *C3* rDNA. Primer 3 hybridizes to the telomeric repeats present on the 5' end of the monomeric 11-kb rDNA species (PAN and BLACKBURN 1981; CHALLONER and BLACKBURN 1986). Control PCR reactions were performed on genomic DNA from the strain *SF137* which contains exclusively *C3* rDNA in its macronucleus. PCR amplification of C3 rDNA was not inhib-
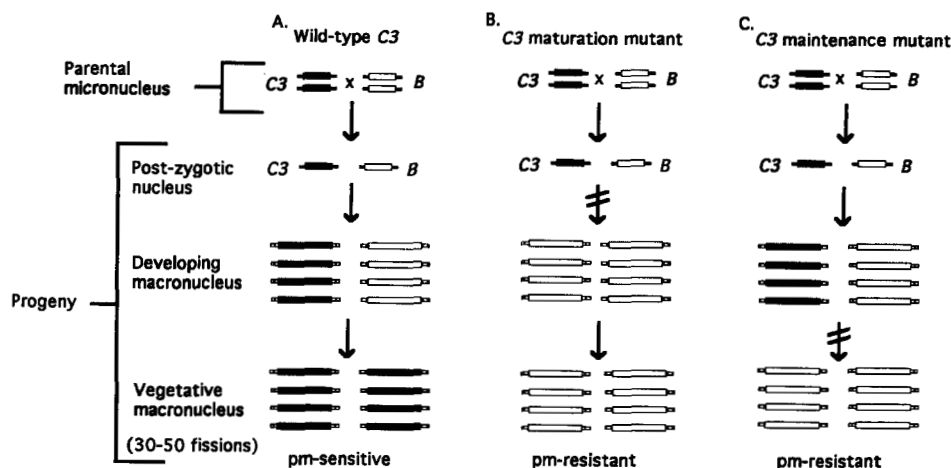
FIGURE 2.—Predicted fate of B and C3 rDNA in wild-type, rDNA maturation and rDNA maintenance mutants. Parental micronuclear rDNA alleles are depicted at the top (C3 rDNA, black box; B rDNA, open box; flanking lines represent adjacent micronuclear DNA). Macronuclear C3 rDNA confers sensitivity to pm, while the Pmr1 B rDNA allele carries a dominant mutation that confers resistance to pm. Totipotent post-zygotic nuclei of all progeny contain both the B and C3 rDNA alleles in their germline configuration. One of these nuclei becomes the germline micronucleus, while the other differentiates into the somatic macronucleus. (A) In the newly developing macronucleus of heterozygous wild-type C3/B progeny, both the B and C3 rDNA are excised from their germline chromosomes, forming palindromic rDNA that is amplified to a high level (hatched lines at the end of macronuclear rDNA represent telomeres). The maintenance disadvantage of the B rDNA causes it to be lost by 30–50 fissions during vegetative growth of progeny. Barring a rare somatic recombination event (YAEGER et al. 1989), progeny become sensitive to pm because they lose the pm-resistant B rDNA from their macronucleus. (B) In C3 rDNA maturation mutants, only B rDNA is present in the newly developed macronucleus, due to a block in one of the steps in C3 rDNA maturation (Fig. 1). Progeny are stably resistant to pm. (C) In C3 rDNA maintenance mutants, both B and C3 minichromosomes are formed and amplified in the developing macronucleus, but the C3 rDNA is lost during vegetative fissions. Barring rare somatic recombination, progeny are stably resistant to pm.

ited by a large excess of palindromic B rDNA. PCR reactions were initiated by the ampliwax-hot start method as recommended by the manufacturer (Perkin-Elmer Corp.), using the following cycle program: 95° for 2 min, 1 cycle; 94° for 1 min, 58° for 30 sec, 68° for 2 min, 40 cycles; 72° for 10 min, 1 cycle.

PCR amplification and sequencing of the rmm10 micronuclear rDNA region: Sequenced segments of the rmm10 micronuclear rDNA region consisted of the entire 1.9-kb 5' nontranscribed spacer (NTS) and 440 bp of flanking micronuclear DNA, and a 115-bp segment at the 3' end of the rDNA spanning the telomere addition site. 5' and 3' NTS segments were amplified by PCR using primers 4 and 5, or primers 6 and 7, respectively, as described elsewhere (KAPLER and BLACKBURN 1994). Cloned PCR products were sequenced completely on both DNA strands by the dideoxy method using rDNA-specific primers.

PCR primers: primer 1, CACGAAGTCTCAAAAGTTG; primer 2, AAAACTAACAACAAAAAGCAAAAAAAAC; primer 3, CCCCAACCCCAACCCCAACCCCA; primer 4, TCTTACTGAAGCTCAAATCGAGCTG; primer 5, CAGTTATAAAAATAGTGTTTCATG; primer 6, AATAACCAAAAATCAAAG; primer 7, CAATAATGTATTAAAAATATGCTACTTATGCATTATC.

RESULTS

Isolation of new rDNA maturation and maintenance (rmm) mutants: The genetic strategy previously developed for isolating mutants defective in rDNA maturation in the developing macronucleus and/or vegetative rDNA maintenance (rmm mutants) is based on the in vivo competition between the naturally occurring B and C3 rDNA alleles during vegetative growth (PAN et al. 1982; LARSON et al. 1986). In matings between wild-type B and C3 strains, both rDNA alleles are correctly processed and amplified in the developing heterozygous ma-

cronucleus of progeny cells. However, the B rDNA is rapidly lost from the macronucleus during subsequent vegetative growth of progeny, owing to a vegetative maintenance advantage of the C3 minichromosome (Figure 2A) (LARSON et al. 1986). Consequently, by 30–50 fissions after mating, only the C3 rDNA is present in the macronucleus. As the Pmr1 B rDNA allele (BRUNS et al. 1985) confers resistance to the antibiotic pm, progeny from a wild-type C3 × B-Pmr1 mating are initially resistant to pm, but become sensitive as the B rDNA is lost. This phenomenon provided the opportunity to isolate mutants that abrogate the C3 vegetative maintenance advantage. To obtain such mutants, a C3-Pmr⁺ strain was mutagenized and mated with a B-Pmr1 strain. Resistance to pm was used to select for mutant progeny that stably retained the B rDNA in the macronucleus during vegetative fissions. We predicted that mutants impaired for maturation of the C3 rDNA in the developing macronucleus (Figure 2B) and/or affected in the subsequent vegetative maintenance of the C3 minichromosome (Figure 2C) would be obtained. Previous mutant screens had identified four rmm mutants affected in vegetative maintenance of the C3 minichromosome (LARSON et al. 1986; YAEGER et al. 1989; D. D. LARSON, personal communication).

To identify mutants defective for rDNA maturation, the same genetic selection protocol was employed. In three independent experiments, SB1934 (C3-Pmr⁺) was mutagenized with nitrosoguanidine or EMS, and mated with SB1917 (B-Pmr1) (see Table 1 for all T.
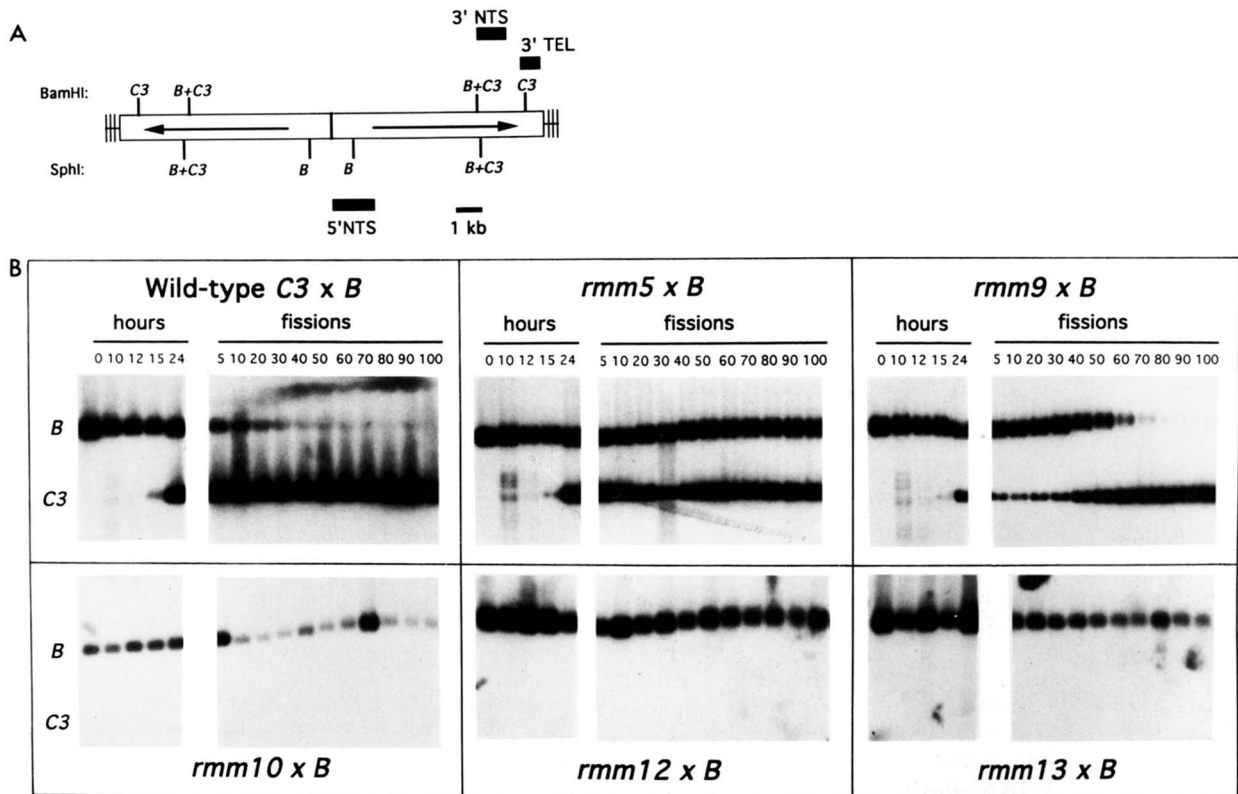
FIGURE 3.—*C3* rDNA levels during macronuclear development and vegetative fissions in wild-type and mutant *C3* × *B* matings. (A) Partial restriction map of palindromic macronuclear rDNA. The open boxes denote the palindromic rDNA, with arrows corresponding to the transcribed regions. *Bam*HI and *Sph*I restriction site polymorphisms used to distinguish between *B* and *C3* rDNA are shown above and below the rDNA map, respectively. *B* + *C3* restriction sites are common to both rDNA alleles. Allele-specific *Bam*HI fragments were detected with the 3′ NTS or 3′ TEL probes, and allele-specific *Sph*I fragments were detected with the 5′ NTS probe (probes, black boxes; 3′ NTS, 1.6-kb *Hind*III fragment; 3′ TEL, penultimate 1.2-kb *Hind*III fragment (CHALLONER *et al.* 1985); 5′ NTS, 1.9-kb fragment cloned into the *Bam*HI site of pUC119 (W. J. PAN and E. H. BLACKBURN, unpublished results)). (B) Southern blotting analysis of *C3* and *B* rDNA from matings of the *B* rDNA strain *SB1915* with the wild-type *C3* strain *SB1934*, rDNA maturation mutants *rmm10* (*SF118*), *rmm12* (*SF130*) and *rmm13* (*SF134*), or rDNA maintenance mutants *rmm5* (*SF102*) and *rmm9* (*SF114*). Genomic DNA was isolated from mating cultures at various times during conjugation (hours) and subsequent vegetative growth of progeny (fissions). DNA was digested with *Bam*HI and probed with the ^{32}P-labeled 3′ NTS probe depicted in panel A. Because the parental strains are heterokaryons with only *B* rDNA in their parental macronucleus, the production of *C3* rDNA in the newly developing macronucleus could be clearly assessed.

thermophila strains). Mating cells from this F_1 cross were distributed into microtiter dishes at a density of 200 cells/well (2.4×10^6 cells in total) and cross-fertilized F_1 progeny were selected as previously described (LARSON *et al.* 1986). F_1 progeny were then grown in the absence of drug, and tested for resistance to pm at 70 fissions, indicative of retention of the *B* rDNA in the macronucleus. We obtained 2300 pm-r F_1 lines.

A series of crosses determined which pm-r F_1 progeny harbored true germline *rmm* mutants (see MATERIALS AND METHODS; LARSON *et al.* 1986). Approximately 90% of pm-r F_1 lines were phenocopies of *rmm* mutations, generating pm-s progeny in a testcross with the *B-Pmr1* strain *SB1915* (frequency of this class: 10^{-3} of total F_1 progeny). These non-germline phenocopies are likely to have resulted from sporadic failures in *C3* rDNA maturation or from somatic recombination between *C3* and *B* rDNA in the macronucleus (YAEGER *et al.* 1989). Gross deletions of the left arm of chromosome 2, likely to in-

clude the entire rDNA locus, were also identified by crosses with the *ts29* strain *SB620* (frequency of this class: 10^{-4} of total F_1 progeny). Such F_1 lines produce temperature-sensitive progeny in the *SB620* cross, indicating hemizygosity at the recessive *ts29* locus; they were eliminated from further consideration. Nine new *rmm* mutants, *rmm5–rmm13*, were identified (Table 1) which met two criteria: 1) the ability to generate pm-r progeny reproducibly at 50 fissions when mated with the *B-Pmr1* strain *SB1915* and 2) physical evidence that the germline *C3* rDNA locus was not grossly deleted (see below). These mutants occurred at a frequency of 10^{-5} of total F_1 progeny.

New *rmm* mutants manifest both maturation and maintenance defects: To determine when each *rmm* mutation first affected *C3* rDNA production, the newly isolated *rmm* mutants were analyzed for the *C3* minichromosome in developing and vegetative macronuclei. Exploiting *C3*- and *B*-specific restriction site polymorphisms (Figure 3A), Southern blotting analysis was

used to assess *C3* rDNA production in matings between *rmm* mutants and the *B* rDNA strain *SB1915*. The parental strains were genetically constructed to be heterokaryons, homozygous for *C3* or *B* rDNA in their micronucleus, but contain only *B* rDNA in their parental macronucleus. Consequently, *C3* rDNA production could be monitored during new macronuclear development, as well as during vegetative growth of progeny. In matings between a wild-type *C3* and *B-Pmr1* strain, *C3* rDNA was amplified to a high level during macronuclear development of progeny cells (Figure 3B, 15 and 24 hr). In contrast, *C3* rDNA was not detected during macronuclear development in the progeny of matings between the *B-Pmr1* strain *SB1915* and five of the new *rmm* mutants: *rmm10*, *rmm12*, *rmm13* (Figure 3B, 15 and 24 hr), *rmm6* and *rmm11* (data not shown, KAPLER and BLACKBURN 1994), indicating that these mutations affect maturation of the *C3* rDNA in the developing macronucleus. The four remaining mutants, *rmm5* and *rmm9* (Figure 3B, 15 and 24 hr), *rmm7* and *rmm8* (data not shown), successfully generated *C3* rDNA during macronuclear development of progeny, and thus do not appear to be defective in *C3* rDNA maturation.

rmm maturation mutants differ in their ability to generate macronuclear rDNA during vegetative fissions: During vegetative fissions of wild-type *C3/B-Pmr1* progeny, the wild-type *C3* rDNA rapidly becomes the predominant species in the macronucleus (Figure 3B, 5–100 fissions) (LARSON *et al.* 1986). No *C3* rDNA was detected during vegetative growth of heterozygous *C3/B* progeny from matings of the maturation mutants *rmm10*, *rmm12* and *rmm13* (Figure 3B, 5–100 fissions). These *rmm* mutations might completely block *C3* rDNA maturation, or alternatively, affect a process required in both the developing and vegetative macronucleus, such as rDNA replication. In contrast, the maturation mutants *rmm6* and *rmm11* accumulated significant levels of *C3* rDNA during vegetative fissions, although they had undetectable *C3* levels at the completion of macronuclear development in these mass matings (KAPLER and BLACKBURN 1994). The 3′ chromosome breakage sequence, Cbs, (YAO *et al.* 1990) of *rmm6* and *rmm11* carries a mutation that has a variable penetrance for *C3* rDNA excision (KAPLER and BLACKBURN 1994). *rmm6* and *rmm11* progeny that correctly excise the *C3* rDNA replicate it to a high level during subsequent vegetative growth.

Unlike the mutations described above, the *rmm5*, *rmm7*, *rmm8* and *rmm9* mutations only affected vegetative maintenance of *C3* rDNA. In these mutants *C3* rDNA was amplified properly. However, both *C3* and *B* rDNA were stably retained in *rmm5*, *rmm7* and *rmm8/B* heterozygotes (*rmm5*, Figure 3B, fissions; *rmm7* and *rmm8*, data not shown). Hence, the *C3* rDNA had lost its vegetative maintenance advantage in these mutants. Southern blotting analysis to detect a 5′ NTS polymorphism showed that somatic recombination between the macronuclear *C3* and *B* rDNA

had occurred in *C3/B* progeny of *rmm5*, *rmm7* and *rmm8* (data not shown). Somatic recombination has been observed previously between *B* and *C3* rDNA derived from *rmm* maintenance mutants, and can confer a selective advantage to rDNA alleles that have recombined away deleterious mutations (YAEGER *et al.* 1989). As neither *C3*- nor *B*-specific polymorphisms within the 5′ and 3′ ends of the rDNA were lost in *rmm5*, *7*, *8/B* progeny, there is no evidence for a strong selection for or against a specific portion of the *C3* rDNA in these mutants. In *rmm9/B* heterozygotes, *C3* rDNA still predominated over the *B* rDNA during vegetative growth (Figure 3B); however, the rate of loss of the *B* rDNA was much slower than that for wild-type *C3/B* heterozygotes. In all of the new rDNA maintenance mutants, the *C3* rDNA maintenance phenotype was heritable, indicating that these lines contained true germline *rmm* mutations.

In summary, the genetic approach we employed was successful in isolating a new class of mutants, those defective in *C3* rDNA maturation (Figure 1). Several new mutants of the maintenance class were also identified (Figure 1); they carry new mutations which will be described elsewhere (R. C. GALLAGHER, G. M. KAPLER and E. H. BLACKBURN, unpublished results). The remainder of this paper will focus on the new class of mutants defective for rDNA maturation, with particular emphasis on the mutant *rmm10*.

The rmm maturation mutations are cis-acting: Five *rmm* mutants were defective in *C3* rDNA maturation in the developing macronucleus of heterozygous *C3/B* progeny (Figure 1). PCR analyses of strains homozygous for these mutations in their micronucleus showed that the junctions between the 5′ and 3′ ends of rDNA region and flanking micronuclear DNA were intact in the respective germline genome (data not shown), ruling out the possibility that these mutants contained gross deletions of micronuclear copies of the *C3* rDNA. To determine whether their block in *C3* rDNA maturation was *cis*- or *trans*-acting, rDNA maturation mutants (*rmm/rmm*, *C3/C3*) were mated with the wild-type *C3* rDNA strain *SB1934* and analyzed for *C3* production during new macronuclear development. A *cis*-acting rDNA maturation mutation is predicted to block only the production of *C3* rDNA derived from the *rmm* mutant, whereas a dominant *trans*-acting mutation should block the production of *C3* rDNA derived from the wild-type *C3* parent as well. As expected, *C3* rDNA was detected in the developing progeny macronucleus in a control experiment in which the wild-type *C3* rDNA strain *SB1934* was mated with the *B* rDNA strain *SB1915* (Figure 4, *B*, 18 and 24 hr). Similarly, in control experiments in which the wild-type *C3* strain *SB1934* was mated with the *cis*-acting vegetative mutants *rmm7* and *rmm8* (R. C. GALLAGHER, G. M. KAPLER and E. H. BLACKBURN, unpublished results), or with the *cis*-acting maturation mutants *rmm6* and *rmm11* (KAPLER and BLACKBURN 1994), *C3* rDNA was also produced during macronuclear development (Figure 4, *rmm7* and *rmm11*, 18 and 24 hr; *rmm6* and
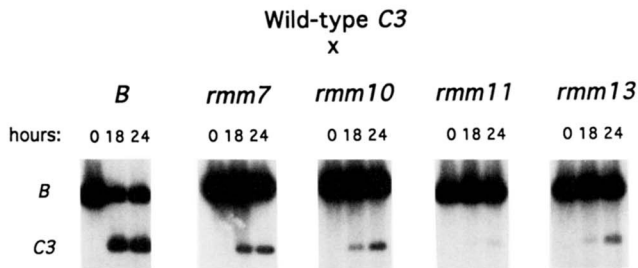
Wild-type C3
x

FIGURE 4.—*Cis-trans* test for macronuclear *C3* rDNA production in wild-type *C3* (*rmm*⁺, *C3*) × *rmm* mutant (*rmm*, *C3*) matings. Southern blotting analysis was performed on matings of the wild-type *C3* rDNA strain *SB1934* with the *B* rDNA strain *SB1915*, rDNA maintenance mutant *rmm7* (*SF106*) or rDNA maturation mutants *rmm10* (*SF118*), *rmm11* (*SF122*) and *rmm13* (*SF134*). Genomic DNA was isolated from mating cultures at various times during mating (hours). DNA was digested with *Bam*HI and blots were probed with the ³²P-labeled 3′ NTS probe (Figure 3A).

rmm8, data not shown). Likewise, the *C3* maturation mutants *rmm10*, *rmm12* and *rmm13* produced *C3* rDNA in the developing macronucleus of heterozygous *rmm/rmm*⁺, *C3/C3* progeny (Figure 4, *rmm10* and *rmm13*, 18 and 24 hr; *rmm12*, data not shown). In matings between the wild-type *C3* strain and *rmm10*, *rmm12* or *rmm13*, the observed levels of *C3* rDNA were somewhat lower than in wild-type *C3* matings with the *B* rDNA strain. However, this was also true for matings between the wild-type *C3* strain and known *cis*-acting mutants *rmm6*, *rmm7*, *rmm8* and *rmm11*. This discrepancy may derive from differences in the efficiency of successful progeny development between the *B* and *rmm* strains, either from mutagenesis of the *rmm* micronucleus or from differences in the parental macronuclei of homozygous *rmm* and *B* strains. The finding that *C3* rDNA was produced in matings of the *rmm* maturation mutants with the wild-type *C3* strain suggests that these mutations are *cis*-acting.

Linkage analysis was performed to determine whether the maturation mutations cosegregated with the micronuclear *C3* rDNA gene, as expected for *cis*-acting mutations. First, F₁ heterozygotes (*C3-Pmr*⁺/*B-Pmr1*, *rmm/rmm*⁺) were mated with the *A** strain to obtain whole genome homozygotes by round 1 genomic exclusion (see MATERIALS AND METHODS). Since the resulting progeny retain their old macronucleus (ALLEN 1967), recessive lethal mutations can be maintained in the homozygous state in the germline micronucleus. Whole genome homozygotes that were *Pmr*⁺ in their micronucleus were identified by test crossing round 1 progeny lines with various *B-Pmr*⁺ strains and screening for sensitivity to pm (LARSON *et al.* 1986) (see MATERIALS AND METHODS). The *Pmr*⁺ and *C3* maintenance advantage reside within the rDNA and are only rarely separated by meiotic recombination (frequency <0.1%). Micronuclear *Pmr*⁺ homozygotes were then mated with the *B-Pmr1* strain *SB1915* and progeny were assayed for sensitivity to pm at 50–70 fissions. All of the lines derived from *rmm6*, *10*, *11* or *12* produced progeny that were pm-resistant at 50–70 fissions, showing that they had retained the *B-Pmr1* minichromosome in their macronucleus. We

TABLE 2

Linkage analysis of rDNA maturation mutations

Maturation mutant	C3 rmm⁺ meiotic recombinants (observed) [a]	Frequency of recombination (%) [b]
rmm6	0/61	<3.0
rmm10	0/124	<1.6
rmm11	0/169	<1.2
rmm12	0/148	<1.4
rmm13	ND [c]	

[a] See MATERIALS AND METHODS for mutant strain constructions and diagnostic test crosses.
[b] *Observed events = 0/n*, frequency of recombination = <1/n/2.
[c] Not determined.

conclude that the *rmm6*, *10*, *11* and *12* mutations cosegregated with the *Pmr*⁺ *C3* rDNA allele (Table 2). Linkage analysis of *rmm13* was inconclusive. Although no meiotic recombinants between the *C3* rDNA and *rmm13* mutation were observed, clonal *rmm13* lines inheriting the *C3* rDNA allele often produced stably resistant pm-r progeny in matings with the *B-Pmr1* strain *SB1915*. However, this phenotype was not reproducible. The remainder of this paper focuses on the *rmm10* mutant.

rmm10 is recessive lethal, but shows no evidence for internal rDNA deletions or rearrangements: It was possible that the *rmm10* mutation caused a partial loss of function, resulting in the inability of *C3* rDNA to compete with *B* rDNA during rDNA maturation and possibly during subsequent vegetative rDNA maintenance as well. For example, the *cis*-acting vegetative maintenance mutation *rmm1* causes the loss of the *C3* rDNA during vegetative growth of *C3-rmm1/B* heterozygotes, yet *C3* rDNA is stably replicated in hemizygous *rmm1* progeny (LARSON *et al.* 1986). Thus, the *rmm1* mutation appears to affect only the *in vivo* competition between *B* and *C3* rDNA. Furthermore, PCR experiments suggest that there may be some competition between wild-type *C3* and *B* rDNA during macronuclear development, possibly at the level of amplification (ORIAS and BRADSHAW 1992).

The *rmm10* strain *SF118* was tested for its ability to produce *C3* rDNA during macronuclear development when mated en masse with the germline rDNA deletion strain *CU374*. No *C3* rDNA derived from this mutant was detected by genomic Southern blotting (Figure 5, left panel, 18–36 hr). In contrast, high levels of *C3* rDNA were detected in matings between the rDNA deletion strain and wild-type *C3* strain (Figure 5, right panel, 18 and 36 hr). In three *SF118* × *CU374* mating experiments, each containing >5 × 10⁵ cells, only one hemizygous viable *rmm10* clone, *SF120*, was obtained. Smaller scale matings between *CU374* and independently derived lines homozygous for the *rmm10* mutation in the micronucleus revealed that inviability cosegregated with the *rmm10* rDNA allele (*n* = 124), suggesting that the *rmm10* mutation itself causes this inviability.

Southern blotting analysis of micronuclear DNA of *SF118* determined that the *rmm10* micronuclear rDNA

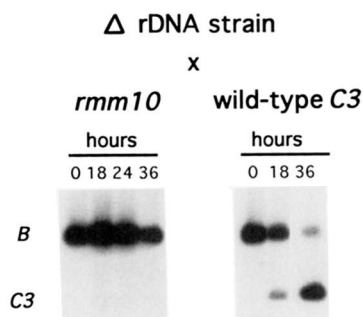Δ rDNA strain

x

rmm10   wild-type *C3*

FIGURE 5.—*C3* rDNA production in mass cultures of wild-type *C3* and *rmm10* hemizygotes. Mass matings between strain *CU374*, which carries a homozygous germline deletion of the rDNA (delta rDNA), with the *rmm10* strain *SF118* (left panel) or wild-type *C3* strain *SB1934* (right panel) were performed. Genomic DNA was isolated from these mass cultures during mating (hours), digested with *Bam*HI and probed with the ^{32}P-labeled 3′ NTS probe (Figure 3A).

copy and its flanking regions did not contain a large internal deletion or rearrangement. This was shown by digesting micronuclear DNA from homozygous wild-type *C3* and *rmm10* strains with *Hae*III or *Bgl*II and hybridizing Southern blots to 5′- or 3′-specific probes (Figure 6A). The hybridization probe for the 5′ end analysis consisted primarily of micronuclear sequences flanking the 5′ end of the rDNA (see map in Figure 6A). The 11-kb *Hae*III restriction fragment, diagnostic of the micronuclear rDNA copy, hybridized to this probe in DNA prepared from wild-type *C3* and *rmm10* strains (Figure 6A, left panel). The 5′ probe also hybridized to a 4-kb *Hae*III fragment from contaminating macronuclear rDNA (Figure 6A, left panel). The micronuclear-specific 3′ probe hybridized to a 5-kb *Bgl*II restriction fragment, corresponding to the micronuclear rDNA copy, in DNA isolated from both the wild-type *C3* and *rmm10* strains (Figure 6A, right panel). Thus, at this level of resolution, the *rmm10* micronuclear rDNA copy appears to be intact.

Macronuclear rDNA from the hemizygous viable *rmm10* clone *SF120* was also examined by Southern blotting and was shown to be palindromic (Figure 6B, 14-kb *Sph*I fragment), with a telomeric restriction fragment indistinguishable from wild-type *C3* rDNA (Figure 6B, 1.5-kb *Bam*HI fragment). Therefore, macronuclear *C3* rDNA from *SF120* does not appear to have been generated by grossly aberrant or incomplete processing of the germline *C3* rDNA. Since *SF120* encodes functional rRNA derived solely from the *rmm10* rDNA allele, the micronuclear *rmm10* rDNA cannot contain a gross internal deletion or rearrangement. Whether *SF120* had reverted the *rmm10* mutation was not determined.

The *rmm10* mutant phenotype has a high penetrance and is stable during prolonged vegetative growth: It was possible that *C3* rDNA was formed in a small number of *rmm10/B* progeny, but that those cells were lost during serial passaging. The *rmm11* maturation mutation, for example, does not completely block the appearance of

macronuclear *C3* rDNA in *rmm11/B* heterozygotes. Indeed, 25% of *rmm11/B* progeny successfully excise the *C3* rDNA; however, the *rmm11* mutation also affects the ability to amplify this correctly excised *C3* rDNA (KAPLER and BLACKBURN 1994). Since the *rmm11* mutation only manifests a phenotype during the formation of the new macronucleus, the underrepresented *C3* rDNA in these clonal lines eventually predominates during vegetative fissions, and hence they become pm-sensitive.

To assess whether *C3* rDNA was similarly formed in a subpopulation of *rmm10/B* progeny, these progeny were distributed into 200 microtiter wells at approximately 500 or 1 cell(s)/well and assessed for the ability to generate pm-s cells during prolonged vegetative growth in the absence of pm. At the high cell density used, pm-s progeny have been observed to overtake pm-r cells, presumably due to a growth advantage (YAEGER *et al.* 1989). However, by 100 fissions, no pm-s *rmm10/B-Pmr1* clones had appeared, indicating that the *rmm10* mutation has a high penetrance.

The *rmm10* mutation is not in a previously identified rDNA maturation or maintenance determinant: Several *cis*-acting elements involved in rDNA maturation and maintenance have been identified previously. The known maturation determinants consist of the Cbs elements immediately flanking the 5′ and 3′ termini of the rDNA which are required for rDNA excision (YAO *et al.* 1990; KAPLER and BLACKBURN 1994), and the inverted M repeat sequences required for palindrome formation (YASUDA and YAO 1991) (Figure 7). The vegetative replication origin has been localized in the vicinity of the 5′-most type I element (CECH and BREHM 1981). The known vegetative maintenance mutations map within or adjacent to the phylogenetically conserved type I elements repeated within the 5′ NTS (Figure 7) (LARSON *et al.* 1986; YAEGER *et al.* 1989; D. D. LARSON, personal communication, R. C. GALLAGHER, G. M. KAPLER and E. H. BLACKBURN, unpublished results).

As it is not known whether the vegetative replication origin is used to amplify the rDNA, and rDNA maintenance determinants have been implicated in the control of rDNA amplification as well (ORIAS and BRADSHAW 1992), segments of the *rmm10* germline rDNA region containing all of the known maturation and maintenance control elements were sequenced (Figure 7). The sequenced regions consisted of the entire 5′ NTS region and 0.4 kb of its flanking micronuclear rDNA, as well as a 115-bp 3′ region spanning the rDNA telomere addition site and the 3′ Cbs element. No base changes were identified in either of these regions. Thus, the *rmm10* mutation does not reside in any of the known elements that control rDNA excision, palindrome formation, telomere addition or vegetative rDNA maintenance. These results indicate that the *cis*-acting *rmm10* mutation must be in a novel determinant for rDNA maturation.

Monomeric and palindromic *C3* rDNA are rarely formed in matings with the *rmm10* strain: Monomeric or palindromic *C3* rDNA could not be detected by conventional Southern blotting analysis of matings of the

A

Micronuclear rDNA structure

B
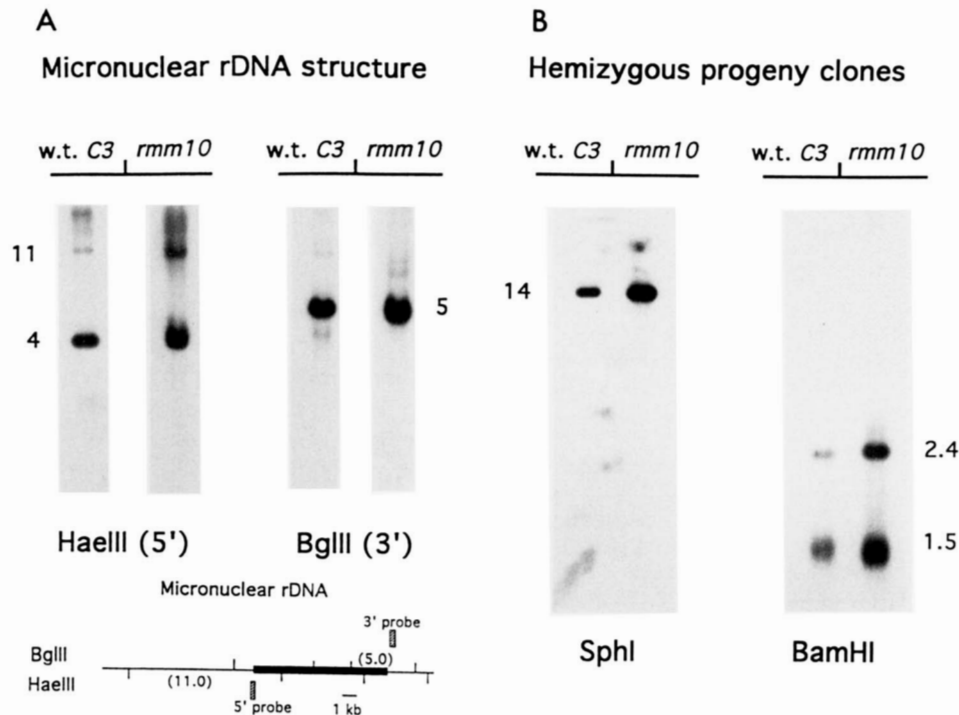
Hemizygous progeny clones

FIGURE 6.—Gene structure of the micronuclear and macronuclear *C3* rDNA copies derived from the *rmm10* mutant. (A) Micronuclear DNA isolated from the wild-type *C3* strain *SB1934* and the *rmm10* strain *SF118* was digested with *Hae*III or *Bgl*II and analyzed by Southern blotting with radiolabeled 5' (left panel) and 3' (right panel) fragments generated by PCR amplification (see map below). The 5' probe consisted of a 380-bp fragment, 310 bp of which are unique to the micronuclear region immediately flanking the rDNA. This probe hybridizes to an 11-kb micronuclear DNA fragment and 4-kb macronuclear DNA fragment. The 3' probe, which hybridizes only to a 4-kb micronuclear DNA fragment, spans a 600-bp region starting 100 bp downstream from the 3' end of the macronuclear rDNA chromosome (KING and YAO 1982). (B) Genomic DNA from the hemizygous wild-type *C3* strain *SF137* containing exclusively macronuclear *C3* rDNA, and the hemizygous viable *rmm10* clone *SF120* was digested with *Sph*I or *Bam*HI and hybridized to ^{32}P-labeled 5' NTS or 3' TEL probes (Figure 3A), respectively. The 14-kb *Sph*I fragment is diagnostic for palindromic macronuclear *C3* rDNA. The 2.4- and 1.5-kb *Bam*HI fragments are diagnostic for macronuclear *C3* rDNA; the latter fragment contains telomeric repeats of heterogeneous length.

rmm10 mutant *SF118*, both during macronuclear development (Figures 3B and 5) or subsequent vegetative growth of progeny (Figure 3B). For a more sensitive assessment of *C3* rDNA production in *rmm10* matings, a PCR assay was developed to determine how well the *rmm10* mutant formed monomeric or palindromic *C3* rDNA. To assay for the monomeric 11-kb *C3* rDNA formed in the newly formed macronucleus (PAN and BLACKBURN 1981; CHALLONER and BLACKBURN 1986), PCR primers 1 and 3 were used (Figure 8A). PCR primer 1 is specific to the *C3* rDNA, corresponding to a segment of the 5' NTS deleted in the *B* rDNA (LARSON *et al.* 1986), while primer 3 corresponds to the telomeric repeat sequence that is added to the 5' end of monomeric macronuclear rDNA. PCR analysis was performed on total cell DNA from matings between the *rmm10* strain *SF118* and either the *B-Pmr1* strain *SB1915* (eight matings) or the rDNA deletion strain *CU374* (three matings). The DNA was isolated 20–24 hr after initiating mating, when wild-type *C3* rDNA levels are maximal. Monomeric *C3* rDNA was not detected by this assay in either cross (data not shown). The sensitivity of this particular assay allowed us to conclude that there are, on av-

erage, fewer than 20 *C3* rDNA monomers per macronucleus (approximately 10% of maximal wild-type levels). These results indicate that the 11-kb *C3* rDNA monomer is at best present at a reduced level in the developing *rmm10* macronucleus, and possibly is not formed.

A more sensitive PCR assay assessed whether the *rmm10* mutant generated palindromic *C3* rDNA. This assay employed the *C3*-specific primer 1 in combination with primer 2, which corresponds to the nonpalindromic center of the rDNA, but is oriented so that only palindromic macronuclear *C3* rDNA can be amplified by PCR (Figure 8A). DNA from the hemizygous wild-type *C3* strain *SF137* was used to optimize the PCR assay for palindromic *C3* rDNA and assess its sensitivity. One picogram of input DNA, containing roughly 10^3 palindromic *C3* molecules, was sufficient to reproducibly generate a positive PCR signal detectable by Southern blotting (Figure 8B; 10^{-6} dilution). For PCR reactions on *rmm10* matings, 1 μg of input genomic DNA was used, equivalent to that of 10^5 cells. Under these conditions, fully amplified *C3* rDNA present in as few as 1 in 10^6 cells could be detected. Alternatively, this assay could detect a single *C3* palindrome per cell in as few
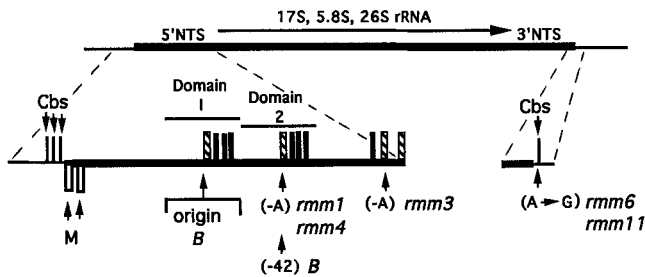
FIGURE 7.—DNA sequence analysis of the *rmm10* micronuclear rDNA region. The entire germline copy of the rDNA (black box) and flanking micronuclear DNA (solid lines) is shown at the top. The 5' NTS, 3' NTS and transcribed regions (arrow) of the rDNA are indicated. The sequenced regions of the *rmm10* (*SF118*) micronuclear rDNA locus are expanded below; they contain all of the known *cis*-acting rDNA maturation and maintenance determinants. Domains 1 and 2 are a 400-bp imperfect tandem duplication containing phylogenetically conserved type I (hatched bars) and type III (solid bars) sequences (CHALLONER *et al.* 1985). Type I elements are mutated in *rmm1*, *rmm4*, *rmm3* and *B* rDNA strains (LARSON *et al.* 1986; YAEGER *et al.* 1989; D. D. LARSON, personal communication). The *B* rDNA vegetative replication origin maps to domain 1 (CECH and BREHM 1981). Cbs elements (vertical lines) are involved in rDNA excision (YAO *et al.* 1990). M sequences (open bars) are involved in palindrome formation (YASUDA and YAO 1991). The 3' Cbs element is mutated in *rmm6* and *rmm11* (KAPLER and BLACKBURN 1994). The expanded 5' and 3' regions were sequenced in the *rmm10* mutant (*SF118*) and no base changes were identified.

as 1% of mating cells. This PCR assay failed to detect palindromic *C3* rDNA in 8 out of 11 independent mating experiments with the *rmm10* strain *SF118* (Figure 8, C and D). Given the level of sensitivity of the PCR assay, it is conceivable that rare reversion of the *rmm10* mutation could account for the low frequency of positive PCR reactions. We conclude that palindromic *C3* rDNA is rarely generated by the *rmm10* mutant. Thus, the *cis*-acting *rmm10* mutation causes a very severe defect in *C3* rDNA maturation, blocking the formation or propagation of both monomeric and palindromic *C3* rDNA.

DISCUSSION

Germline rDNA maturation mutants: We have exploited the *in vivo* competition between the naturally occurring *B* and *C3* rDNA alleles to isolate mutants defective for the developmentally regulated maturation and vegetative maintenance of the rDNA minichromosome of *T. thermophila*. Previous screens had only identified mutations affecting rDNA maintenance (LARSON *et al.* 1986; YAEGER *et al.* 1989; D. D. LARSON, personal communication). Four new rDNA maintenance mutants were identified (*rmm5*, *7*, *8* and *9*). As they carry novel mutations, additional *cis*-acting determinants must be involved in rDNA maintenance (R. C. GALLAGHER, G. M. KAPLER and E. H. BLACKBURN, unpublished results).

Five new mutants, *rmm6*, *10*, *11*, *12* and *13*, show a primary defect in rDNA maturation in the developing macronucleus. Thus, this approach can be used to study

rDNA maturation as well. All of the rDNA maturation mutations are *cis*-acting. The *rmm6/11* mutation resides in the 3' Cbs element (KAPLER and BLACKBURN 1994), whereas in *rmm10*, the previously identified maturation determinants are unchanged. Consequently, these germline mutations will identify new *cis*-acting maturation control elements, as well as help us study the mechanisms that regulate rDNA maturation. Since these *cis*-acting maturation mutations are recessive-lethal, we may be able to identify *trans*-acting factors that control rDNA maturation by extragenic suppression of the *rmm*-induced lethality.

rmm10-implications for maturation: The *rmm10* mutation causes a severe defect in *C3* rDNA maturation. This mutation has a high penetrance in hemizygous *rmm10* cells resulting in poor progeny viability. A single hemizygous viable line was obtained (frequency $<10^{-5}$). This line contained intact palindromic macronuclear *C3* rDNA derived from the *rmm10* rDNA allele, providing evidence that the *rmm10 C3* rDNA allele was not grossly rearranged or deleted in the germline. The severity of the *rmm10* mutation was also evident in *rmm10/B* heterozygotes; the *rmm10* mutation could not be "rescued" by high initial levels of macronuclear *B* rDNA in the newly formed macronucleus, since *C3* rDNA did not accumulate in the vegetative macronucleus after prolonged growth of *rmm10/B* progeny. PCR analyses revealed that macronuclear *C3* rDNA was rarely formed in the *rmm10* mutant. In contrast, the *rmm11/6* maturation mutation which is also hemizygous lethal shows a low penetrance in heterozygous progeny, excising and forming palindromic *C3* rDNA in at least 25% of *rmm/B* progeny cells (KAPLER and BLACKBURN 1994). In all of the maturation mutants examined, hemizygous lethality was linked to the *rmm* mutation (this work; KAPLER and BLACKBURN 1994). We suggest that these mutations cause this inviability.

The *rmm10* germline rDNA region is intact; however, palindromic *C3* minichromosomes are at best rarely formed. Based on our understanding of rDNA maturation and maintenance from DNA transformation and classical genetic studies, we propose that the *rmm10* mutation affects the formation of macronuclear *C3* rDNA rather than palindrome formation or subsequent amplification/replication.

Inverted repeated M sequences are required for palindrome formation; they may play a structural role in the formation of a hairpin structure in excised monomeric rDNA, this being a possible intermediate in palindrome formation (YASUDA and YAO 1991). Palindrome formation is not essential for rDNA replication, since monomeric *C3* rDNA is capable of overtaking *B* rDNA in the macronucleus during vegetative fissions (YAO *et al.* 1990). As monomeric *C3* rDNA derived from *rmm10* did not accumulate, either in the developing macronucleus or during subsequent vegetative fissions, the *rmm10* mutation must affect the formation or propagation of both monomeric and palindromic *C3* rDNA.

The 5' NTS region of the rDNA is sufficient for high copy number extrachromosomal replication of plasmids
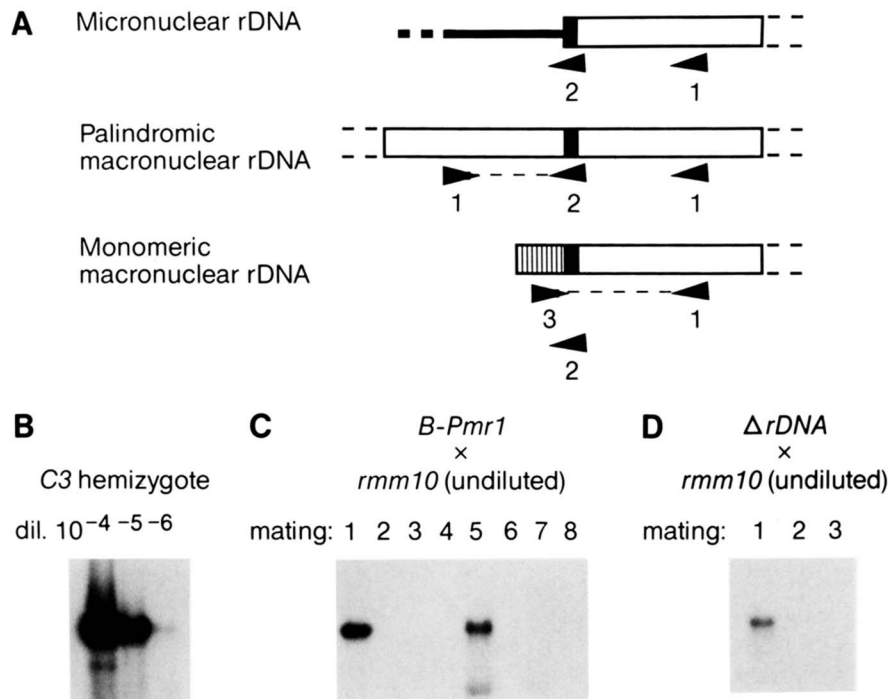
A Micronuclear rDNA

Palindromic
macronuclear rDNA

Monomeric
macronuclear rDNA

B

C3 hemizygote

dil. 10^{-4} $^{-5}$ $^{-6}$

C   *B-Pmr1*
                 ×
          rmm10 (undiluted)

mating: 1 2 3 4 5 6 7 8

D   Δ*rDNA*
                 ×
          rmm10 (undiluted)

mating: 1 2 3

FIGURE 8.—PCR analysis of palindromic *C3* rDNA derived from *rmm10* mutant. (A) Schematic of the 5′ NTS region of the micronuclear, palindromic macronuclear and monomeric macronuclear *C3* rDNA. The open box corresponds to the rDNA 5′ NTS upstream of the rRNA coding region (hatched box). The shaded portion (not to scale) corresponds to a 29-bp segment that becomes the nonpalindromic center of palindromic macronuclear rDNA. The thick black line in the micronuclear rDNA schematic represents flanking micronuclear DNA sequences. The hatched box (not to scale) at the 5′ end of the monomeric 11-kb macronuclear rDNA corresponds to the telomeric repeats that are added to this molecular species. The orientation of PCR primers 1–3 are indicated by the arrows, and the resulting PCR products are indicated by dashed lines connecting these primers (see MATERIALS AND METHODS for primer sequences). The primer 1 region is deleted in the *B* rDNA (LARSON *et al.* 1986). The orientation of primer 2 allows for PCR amplification of only palindromic macronuclear rDNA. Primer 3 can only PCR amplify monomeric macronuclear rDNA. (B–D) PCR reactions using primers 1 and 2 were analyzed by Southern blotting with the 5′ NTS probe (Figure 3A). (B) PCR assay for the *C3* rDNA palindrome in DNA derived from the macronuclear *C3* rDNA strain *SF137*. Tenfold serial dilutions of input DNA from *SF137* (undiluted = 1 μg) were subjected to PCR analysis to assess the sensitivity of this assay for detecting the *C3* rDNA palindrome. (C) PCR analysis of DNA (1 μg, undiluted) derived from eight separate mass matings between *rmm10* (*SF118*) and the *B* rDNA strain *SB1915*. The genomic DNA used in these assays was isolated at 20–24 hr after initiating mating. (D) PCR analysis of three separate mass matings between *rmm10* (*SF118*) and the germline rDNA deletion strain *CU374* was performed as described in (C).

introduced into vegetatively growing cells (W.-J. PAN and E. H. BLACKBURN, unpublished results). Since this region is not mutated in *rmm10*, we would expect that macronuclear *C3* rDNA would eventually predominate in the mutant if it was formed, but not amplified, in the developing macronucleus. This phenotype is observed in *rmm11/B* heterozygotes, which contain very low levels of the *C3* rDNA at the completion of macronuclear development, but replicate the *C3* rDNA to high levels during subsequent vegetative growth (KAPLER and BLACKBURN 1994). The absence of *C3* rDNA in the vegetative macronucleus of *rmm10/B* heterozygotes suggests that the *rmm10* mutation does not reside in a determinant that is required solely for amplification of the rDNA. As the 5′ NTS is not mutated in *rmm10*, it is unlikely that this mutation affects subsequent vegetative rDNA maintenance.

We propose that the *rmm10* mutation affects the formation of monomeric as well as palindromic macronuclear *C3* rDNA. However, the only known *cis*-acting determinant—the rDNA excision Cbs element (YAO *et al.* 1987; YU and BLACKBURN 1991), is not mutated in the *rmm10* germline.

Micronuclear sequences that flank Cbs elements are not required for chromosome breakage and subsequent telomere addition (YAO *et al.* 1990); however, the possibility that sequences within the rDNA play a role in the excision process cannot be ruled out. For example, accessibility of functional Cbs elements to processing enzymes could be regulated by DNA-protein interactions at other sites in the rDNA region, changes in chromatin structure or transcription activation. The selective effect of chromatin structure on site-specific chromosome breakage has been documented for the directional gene conversion regulating mating type determination in *S. cerevisiae* (KLAR *et al.* 1984). Furthermore, immunoglobulin κ locus rearrangement has been correlated with the activation of germline κ gene transcription (SCHLISSEL and BALTIMORE 1989). Alternatively, *cis*-acting sequences might prevent the rDNA from being degraded in the developing macronucleus. Approximately 10% of the micronuclear genome is not represented in the macronucleus, and it has been suggested that these micronuclear-specific sequences are actively degraded during new macronuclear development (BRUNK

and CONOVER 1985). Whether specific *cis*-acting sequences within the macronuclear-destined regions play an active role in their retention is not known. Identification of the *rmm10* base change should be informative for discriminating between these and other possibilities.

Severe maturation mutants such as *rmm10* may prove to be useful for identifying rDNA amplification determinants by a DNA transformation-based approach. Matings between *rmm10* and an rDNA deletion strain are inviable due to the severe rDNA maturation defect in the *rmm10* hemizygote. Transformation of *rmm10* hemizygotes with functional rDNA constructs should generate viable progeny that exclusively express the introduced rRNA genes, provided that the introduced rDNA construct is correctly processed and amplified. The minimal sequences required for rDNA maturation and maintenance, including determinants for rDNA amplification, may now be identifiable by this approach.

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