Germline and Somatic Transformation of Mating *Tetrahymena thermophila* by Particle Bombardment

Donna Cassidy-Hanley,* Josephine Bowen,† John H. Lee,* Eric Cole,*,[1] Lynn A. VerPlank,† Jacek Gaertig,†,[2] Martin A. Gorovsky† and Peter J. Bruns*

**Section of Genetics and Development, Division of Biological Sciences, Cornell University, Ithaca, New York 14853 and †Department of Biology, University of Rochester, Rochester, New York 14627*

Manuscript received September 21, 1996
Accepted for publication February 13, 1997

ABSTRACT

Mating *Tetrahymena thermophila* were bombarded with ribosomal DNA-coated particles at various times in development. Both macronuclear and micronuclear transformants were recovered. Optimal developmental stages for transformation occurred during meiosis for the micronucleus and during anlagen formation for the macronucleus. Evidence is given for transient retention of the introduced plasmid. Genetic and molecular tests confirmed that sexually heritable transformation was associated with integration at the homologous site in the recipient micronuclear chromosome.

DNA-mediated genetic transformation has proven to be a powerful tool for the investigation of gene function and regulation in many different prokaryotic and eukaryotic systems. A variety of DNA transformation techniques have been successfully applied to a broad range of organisms. To date, two transformation techniques, microinjection (TONDRAVI and YAO 1986) and electroporation (GAERTIG and GOROVSKY 1992), have been successfully applied to the ciliated protozoan *Tetrahymena thermophila*. The development of nuclear transformation techniques in this organism is complicated by the fact that, like other ciliates, Tetrahymena separates somatic and germinal functions into two nuclei: the polyploid macronucleus that is solely responsible for the phenotype of the cell and the transcriptionally inactive diploid micronucleus that is responsible for the transfer of genotype to subsequent sexual generations (GOROVSKY 1980; BRUNS 1986). New macronuclei are formed from the micronuclear-derived germline during conjugation. Each of these nuclei must be considered as an independent target for DNA transformation, with specific advantages and disadvantages.

Macronuclear transformants can be detected directly following vegetative growth. However, since the somatic macronucleus is destroyed during sexual reproduction (conjugation), analysis of macronucleus transformants is limited to the vegetative stage of the life cycle, precluding additional genetic analysis of the transformed gene. Germ line (micronuclear) transformants can be studied genetically through successive generations, but since the micronucleus is transcriptionally inactive, the phenotypes of successful germline transformants can only be detected following conjugation and the production of a new macronucleus from the transformed micronucleus.

Thus far, transformation in Tetrahymena has been limited to the somatic macronucleus. Microinjection has been used to transform both the polyploid macronuclei of vegetative Tetrahymena and the newly formed macronuclear anlagen of conjugating cells. This technique has been extensively utilized in the analysis of gene amplification, chromosome fragmentation and DNA rearrangement (GODISKA and YAO 1990; YAO *et al.* 1990; YASUDA and YAO 1991). Electroporation of vegetatively growing cells yielded a very low level of macronuclear transformation (BRUNK and NAVAS 1988; ORIAS *et al.* 1988). However, high frequency macronuclear transformation was obtained when transformation vectors were introduced directly into macronuclear anlagen of conjugating cells (GAERTIG and GOROVSKY 1992). Conjugant electrotransformation (CET) yields efficient macronuclear transformation using high copy number vectors containing selectable markers and has been used to develop new vectors suitable for use in gene replacement experiments (GAERTIG *et al.* 1994). Integrative events using either microinjection or CET are frequent and occur mainly, if not exclusively, via homologous recombination.

Ribosomal genes (rDNA) have often been used to study transformation in *T. thermophila*. These genes exist as a single copy, integrated in chromosome 2 in the germline micronucleus (YAO and GALL 1977; BRUNS *et al.* 1985). During macronuclear differentiation, the micronuclear rDNA is excised, modified to form a unique 21-kb palindromic chromosome, and amplified

Corresponding author: Peter J. Bruns, Section of Genetics and Development, Cornell University, 169 Biotechnology Building, Ithaca, NY 14853-2703. E-mail: pjb4@cornell.edu

[1] *Present address:* Department of Biology, St. Olaf College, Northfield, MN 55057.

[2] *Present address:* Department of Biology, University of Georgia, Athens, GA 30602.

~10,000-fold (YAO 1986). Two naturally occurring variants of the rDNA locus that exhibit sequence polymorphisms at both the 5′ and the 3′ ends occur in *T. thermophila* strains B and C3. A variation in the C3 macronuclear chromosomal origin of replication also confers a replicative advantage in B/C3 heterozygotes such that, although both forms are initially present in the heterozygous macronucleus, the C3 variant completely outcompetes the B variety after ~30 fissions (LARSON *et al.* 1986). Several different forms of rDNA have been used as transforming vector, including the macronuclear rDNA chromosome (TONDRAVI and YAO 1986), a plasmid containing the micronuclear version of the rDNA gene plus the flanking sequences required for correct excision and amplification during macronuclear development (SWEENEY and YAO 1989; YAO and YAO 1989), and plasmids containing various modifications of the macronuclear rDNA sequence (KARRER and YAO 1988; YU *et al.* 1988; YU and BLACKBURN 1989; YU and BLACKBURN 1990).

In this paper, we describe a simple, efficient method for the mass transformation of the ribosomal genes in both the macronucleus and the micronucleus in *T. thermophila* using the Biolistic PDS-1000/He Particle Delivery System (Bio-Rad). This delivery system, which utilizes helium pressure to accelerate DNA-coated microparticles into target cells (SANFORD *et al.* 1991), causes relatively little cell damage. Biolistic transformation has been used to produce both transient and stable transformants in a wide variety of cells, tissues, and organelles (RASMUSSEN *et al.* 1994; CHRISTOU 1995; DIX and KAVANAGH 1995; GAINER *et al.* 1995; SOUTHGATE 1995). By bombarding mating cells at specific times during conjugation, we have generated macronuclear transformants at a rate equivalent to the transformation frequencies obtained using CET. More significantly, genetically stable micronuclear transformants have been produced using this system. Stable micronuclear transformants resulted from integration of the new sequences via homologous recombination leading to direct gene replacement or tandem duplication of the rDNA.

MATERIALS AND METHODS

Strains: All strains used in this study were derived from *T. thermophila* inbred strain B, except for A*III that was derived from inbred line A. The genotypes and phenotypes of all strains used in this study are shown in Table 1.

Cell growth: All cells were grown in modified Neff medium [0.25% proteose peptone, 0.25% yeast extract, 0.55% glucose, 33 μM $FeCl_3$, 250 μg/ml penicillin G and streptomycin sulfate, 1.25 μg/ml amphotericin B (Fungizone-GIBCO)] or enriched proteose peptone (GOROVSKY *et al.* 1975) at 30° on a platform shaker.

Mating: To induce conjugation, logarithmically growing cultures of two different mating types were counted in a Coulter Counter, centrifuged at 1100 × *g* in oil centrifuge tubes (Kimax brand, Kimble #45244-100) for 3 min, washed with 10 mM Tris-HCl (pH 7.5), and resuspended in fresh Tris at a concentration of 2×10^5 cells/ml for 12–18 hr on a 30° shaking platform incubator. After the starvation period, both cultures were readjusted to a final concentration of 2×10^5 cells/ml, and 25 ml of each were mixed together in a series of 500-ml Erlenmeyer flasks to yield 50-ml mating mixtures, which were maintained in a stationary 30° incubator. Individual 50-ml aliquots of conjugating cells were used for transformation at each specific time point. Matings prepared as described follow a well defined, fairly synchronous time course, insuring that the majority of pairs at each time point are at relatively similar stages of conjugation. The percentage cells pairing at the time of bombardment was determined by fixing a small sample from each flask and counting paired and unpaired cells. Matings that did not achieve at least 50% cells in pairs by 3.5 hr after mixing diverse mating types were not used.

Successful conjugation is followed by immaturity, during which the cells do not form pairs with cells of any mating type. Cells from crosses were verified as true progeny by being mixed with mature cells in mating conditions, and inability to form pairs scored.

Transformation vectors: Figure 1 presents both vectors used. Vector pD5H8 (YAO and YAO 1989) is a pUC19 derivative that contains a complete C3-derived micronuclear rDNA gene with a single base mutation in the 17S rDNA conferring resistance to paromomycin (SPANGLER and BLACKBURN 1985). Vector pD3/5-11 (YAO *et al.* 1990) is a modified version of pD5H8 that lacks both the 5′ and 3′ chromosome breakage sequences (Cbs) and adjacent micronucleus specific sequences as well as the final 500 bp of the 3′ nontranscribed region of the rDNA gene. Both linear and circular versions of pD5H8 were used as noted in individual experiments. The plasmid was linearized by digestion with *Xho*I and *Sal*I to remove the insert. The digestion was not complete, and the linearized form was not purified prior to use.

Particle delivery system: Cells were transformed using the DuPont Biolistic PDS-1000/He, Particle Delivery System (Bio-Rad). M10 (0.7–1.1 μm) tungsten bombardment particles (60 mg tungsten particles/ml sterile H_2O) were prepared according to the Large Batch DNA-Particle Coating Method (SANFORD *et al.* 1991). The tungsten particles were coated

TABLE 1

Description of the strains used in this study

Strains	Micronuclear genotype	Macronuclear phenotype (expressed)	Micronuclear rDNA
B*VII	*	Cys Mps Gals VII	*
A*III	*	Cys Mps Gals III	*
IA264	*galr/galr*	Cys Mps Gals II	B *Pms/Pms*
CU427	*Chx/Chx*	Cys Mps Gals VI	B *Pms/Pms*
CU428.1	*Mpr/Mpr*	Cys Mps Gals VII	B *Pms/Pms*

Chx and *Mpr* are dominant mutations conferring resistance to cycloheximide and 6-methylpurine, respectively. *gal* is a recessive mutation conferring resistance to 2-deoxygalactose (for references to mutations, see BRUNS and CASSIDY-HANLEY 1992). Drug-sensitive and -resistant phenotypes are indicated by s and r. Roman numerals indicate mating type. B indicates strain B rDNA and *Pms* indicates sensitivity to paromomycin. *, a "star" strain lacking a functional micronucleus. The decimal point in the designation of strain 428.1 indicates that the original strain CU428 was rejuvinated by passage through round I genomic exclusion (ALLEN 1967) and the mating type VII exconjugant clone retained after testing for fertility.

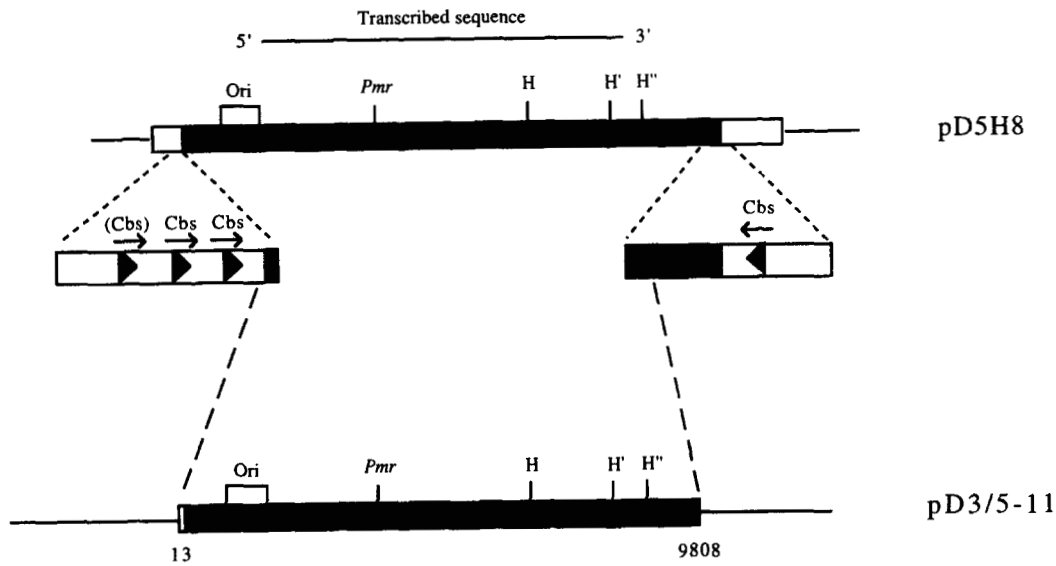


FIGURE 1.—Transformation vectors. Vector pD5H8 (YAO and YAO 1989) is a pUC19 derivative that contains a complete C3-derived micronuclear rDNA gene (wide black line) with a single base mutation in the 17S rDNA conferring resistance to paromomycin (*Pmr*). Vector pD3/5-11 (YAO *et al.* 1990) is a modified version of pD5H8 that lacks both the 5′ and 3′ chromosome breakage sequences (Cbs) and adjacent micronucleus specific sequences (wide white lines). The pD3/5-11 rDNA sequence begins at base 13 and terminates at base 9808, and is missing the final 500 bp of the 3′ nontranscribed region of the rDNA gene. Ori, origin of replication; H, *Bam*HI site present in both B and C3 rDNA; H′, *Bam*HI site introduced into the C3 rDNA in the pD5H8 vector; H″, *Bam*HI site normally present in C3 rDNA.

immediately before use with 20 μg DNA/100 μl particle suspension.

Preparation of cells for bombardment: At selected time intervals during conjugation, the 50-ml mating mixtures were centrifuged in oil centrifuge tubes at 1100 × *g* for 2–3 min, washed once with 25 ml 10 mM HEPES buffer (pH 7.5), and resuspended in 1 ml of HEPES buffer at a final concentration of ~1 × 10^7 cells/ml. The 1-ml cell sample was evenly spread on a sterile 9-cm circular filter paper (Whatman 50), prewet with 2 ml of 10 mM Hepes buffer and placed in a 100 × 15 mm sterile petri plate cover. Immediately after spreading the cells were bombarded with the DNA coated tungsten particles at 700 psi. Following bombardment, cells were immediately resuspended in Neff medium and treated according to the specific experimental designs described below.

Transformation in diploid by diploid matings: Aliquots of mating cells were bombarded at half hour intervals for various times after mixing with 1.0–1.2 mg tungsten particles, coated with 3.3–4.0 μg of DNA. Following bombardment, cells were resuspended in 50 ml of growth medium at a concentration of 2 × 10^5 cells/ml, and, if needed, serially diluted. For experiments with high yields of transformants (somatic transformation), aliquots of each dilution were distributed into five 96-well microtiter plates (100 μl/well). For experiments with a low yield of transformants (when vector pD3/5-11 was used), the cells were not diluted but plated directly into the microtiter plates. These first generation (G1) cells were maintained at 30° for 24 hr, and then 50 μl of 3× paromomycin in Neff medium (final concentration 100 μg/ml) was added directly to each well. Growth of paromomycin resistant cells was usually apparent after 2–3 days. Following 5 days in paromomycin, the number of wells containing paromomycin-resistant cells was counted and the number of transformants was calculated using the Poisson distribution. Dilutions that yielded growing paromomycin-resistant cells in approximately one-third of the total number of wells were used for the Poisson determination. Where appropriate, to ensure that the paromomycin-resistant cells were true progeny, all paromomycin-resistant lines were sequentially replicated into cycloheximide (25 μg/ml) and 6-methylpurine (15 μg/ml) to test for the expression of drug resistance markers present in the germline of the parental heterokaryons. A control bombardment using uncoated tungsten particles was carried out in the same way.

Transformation in diploid by star matings: Mating cultures of CU427 and A*III were bombarded with DNA-coated tungsten particles at half hour intervals from 2 to 6 hr after mixing. Following each bombardment, cells were resuspended in 100 ml of fresh Neff medium in 1000-ml Erlenmeyer flasks and allowed to complete round I mating at 30° for 24 hr to yield the initial transformed generation (G0). Next, 25 ml of G0 cells was adjusted to 2 × 10^5 cells/ml in 10 mM Tris and used in a mass mating with an equal volume and cell concentration of prestarved CU428. Pairing was checked after 8 hr and the cells were refed with 2× Neff medium after 24 hr. Refed cells were directly distributed into 96-well microtiter plates (100 μl per well) and allowed 24 hr growth at 30°. Fifty microliters of 75 μg/ml cycloheximide in Neff medium was added to each well (final concentration 25 μg/ml) and resistant cells were selected for 5 days at 30°. Cycloheximide-resistant G1 progeny cells were replicated into paromomycin and, following 5 days in drug, all paromomycin resistant lines were collected and subcloned for further analysis.

Molecular analysis of rDNA in transformed cells: Total genomic DNA from the progeny of micronuclear transformants was isolated (GAERTIG *et al.* 1994) and analyzed to determine the type of rDNA allele present in the macronuclei. PCR amplification and Southern blotting (SAMBROOK *et al.* 1989) were used to distinguish polymorphisms between C3 and B alleles at the 5′ and 3′ ends of the rDNA molecule, respectively, as described in Figure 7.

RESULTS

Transformation in diploid by diploid matings: To target specific nuclei for transformation, we introduced

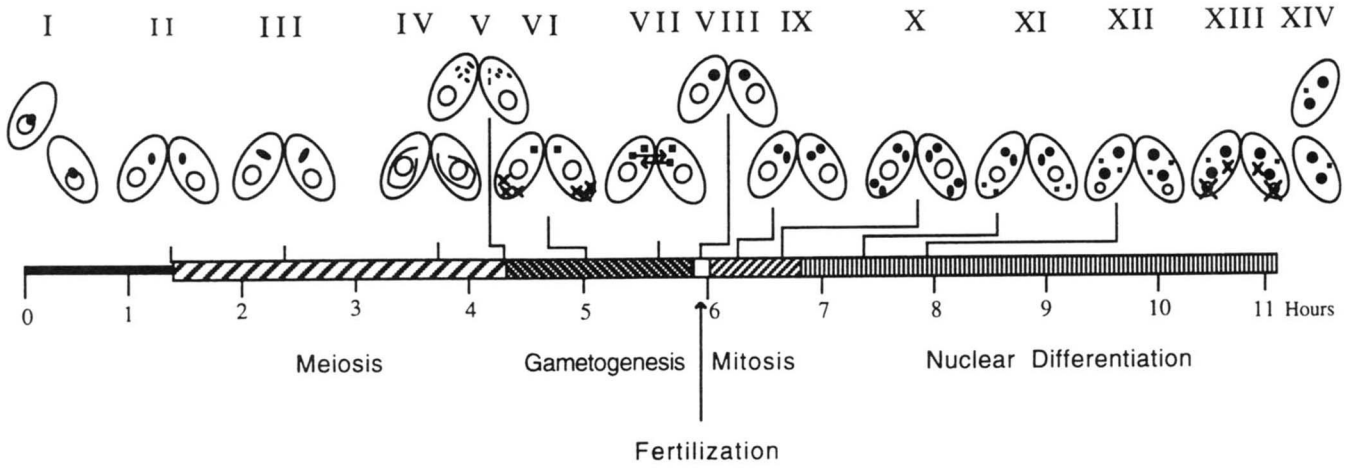


FIGURE 2.—Stages and timing of conjugation in a diploid by diploid mating at 30°. Prestarved cells of different mating types are mixed together in nonnutritive media (I). Pairing occurs (II) and the micronuclei in both cells undergo meiosis (III–V) that results in the formation of four haploid nuclei, three of which are lost (VI). The remaining pronucleus undergoes a single mitotic division followed by an exchange of haploid pronuclei between the two mating cells (VII). Cross fertilization of the migratory nucleus of one cell and the stationary nucleus of the second produces a diploid zygote nucleus (VIII) that immediately undergoes two postzygotic mitoses (IX and X). The products of the postzygotic divisions differentiate to form the new micronuclei and macronuclei of the immature daughter cells, the old macronucleus is eliminated, and the daughter cells separate (XI–XV).

plasmid pD5H8 DNA into cells at various times during conjugation. Figure 2 outlines the nuclear stages during conjugation; important events for transformation include those leading up to fertilization at hour six, and the subsequent development of new macronuclei from the new zygote genome from about hour eight onward. We expected macronuclear transformation to occur in the later times, as has already been observed with CET, since the transforming DNA contained all the information needed to be processed into rDNA macronuclear chromosomes: the vector has both a macronuclear chromosome origin of replication that outcompetes recipient macronuclear rDNA and a selectable mutation (paromomycin resistance). Figure 3 presents the number of paromomycin resistant clones recovered after bombardment at various times in conjugation.

A low level of transformation was observed at nearly every time point tested but a sharp peak occurred 10 hr after cell mixing at 30°, which is the time of development of new macronuclear anlagen in progeny cells. To determine the status of the micronuclei in cells showing the transformed phenotype, we performed crosses to a diploid strain. All 14 of the transformants produced by bombardment at times 3–5 hr of conjuga-

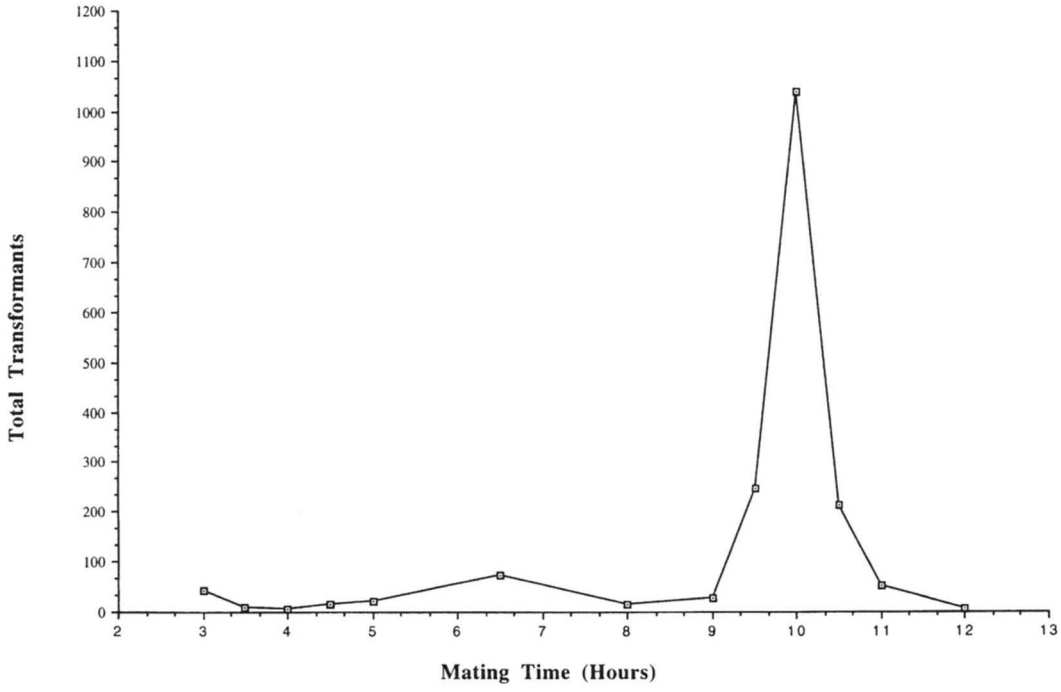


FIGURE 3.—Time course of transformation in a diploid by diploid mating. Aliquots of a mating of CU427 × CU428 were bombarded with pD5H8 at half-hour intervals from 3 to 12 hr after mixing at 30°. The number of wells containing paromomycin-resistant G1 cells were counted and the number of transformants was calculated using the Poisson distribution.

tion, and 14 transformants produced between 8 and 10 hr of conjugation, were mated with CU428.1. Forty-four individual progeny pairs from each mating were then isolated and assayed for immaturity to test for the completion of conjugation and for paromomycin resistance. All of the transformants arising from bombardment of cells in the latter half of conjugation and all but one of the transformants produced from cells bombarded in the first 6 hr of conjugation failed to yield paromomycin-resistant progeny cells, indicating that the mutation was solely in the macronucleus of the selected first generation progeny cells (G1).

A single 3-hr clone showed normal Mendelian segregation for a heterozygous paromomycin-resistance marker in the G2 (second generation) progeny: of 25 progeny clones examined, 11 were paromomycin resistant and 14 were paromomycin sensitive, a normal Mendelian ratio predicted following integration of the vector paromomycin-resistant allele into one of the micronuclear chromosomes of the paromomycin-sensitive transformed parent. Macronuclear rDNA isolated from the original G1 clone exhibited C3-specific polymorphisms at both the 5′ and 3′ ends of the rDNA molecule. However, two randomly selected paromomycin-resistant G2 progeny clones contained only B allele polymorphisms at both the 5′ and 3′ ends of the macronuclear rDNA molecule. G2 progeny containing paromomycin-resistant rDNA molecules that exhibit only the B allelic form at both the 5′ and 3′ ends of the rDNA molecule would be produced by recombination between the C3 vector carrying the paromomycin-resistant mutation and the paromomycin-sensitive B recipient rDNA at the homologous site in the micronucleus. This suggests that different transformation events were responsible for the resistance of the G1 and G2 clones. As discussed below, the C3-C3 molecules observed in the macronuclei of the original G1 clone probably originated from a vector that was processed into an rDNA chromosome in a developing macronuclear anlagen, whereas the resistance in the G2 came from an allele that was created by integration of the resistance mutation in the recipient micronucleus. A second instance of divergence between the allelic composition of the rDNA in G1- and G2-transformed macronuclei is discussed in the next section.

Assuming that the peak at hour 10 represents mostly macronuclear transformants, we estimated the efficiency of this transformation as follows. At 10 hr, a single bombardment of 1×10^7 cells, or 5×10^6 possible pairs, yielded 1040 putative transformants. Since cytological observations indicated a pairing frequency of ~70%, the maximum possible number of transformants was actually 3.5×10^6 pairs, which gives a transformation frequency of 0.015% of the number of cells in pairs, or 315 transformants per mg DNA, which is comparable with results reported using CET (Gaertig and Gorovsky 1992). Control matings of CU427 and CU428 carried through the macronuclear transformation protocol and bombarded with "blank" particles (particles that had gone through the particle coating procedure in the absence of any vector DNA) yielded no paromomycin-resistant cells.

Micronuclear transformation in diploid by star matings: The results in the previous section suggest that if a competent vector is present in a developing macronucleus, it may be processed into a macronuclear chromosome. Interestingly, this happened in cells that were bombarded at early conjugation times, long before the anlagen were even present. Thus it is possible that the introduced vector DNA can persist through the several micronuclear mitotic divisions leading to anlagen during development. To further separate DNA introduction from anlagen develop-

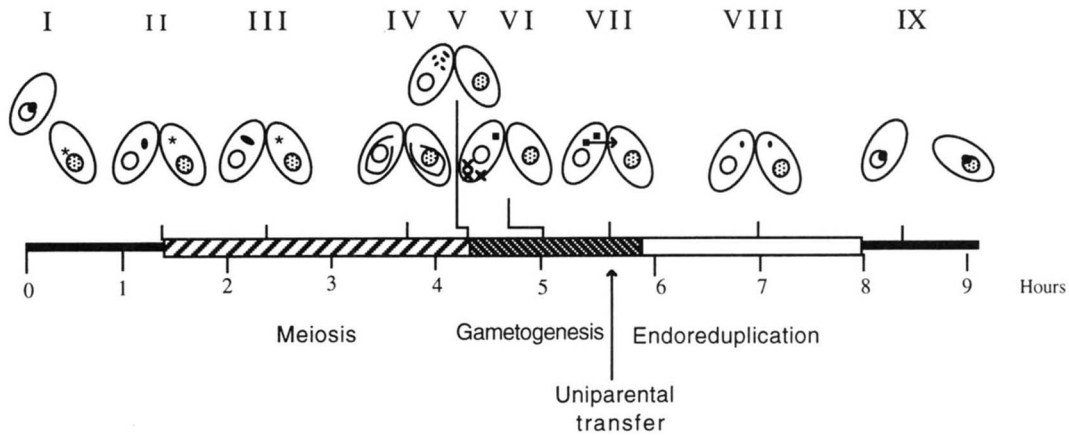


Figure 4.—Stages and timing of conjugation in a diploid by star mating at 30°. In the diploid partner, meiotic events proceed normally to the point of nuclear exchange (I–VI). The star cell lacks a functional micronucleus and does not produce a migratory nucleus. Unidirectional nuclear exchange occurs (VII), resulting in the production of identical, completely homozygous, diploid micronuclei in both mating cells (VIII). Conjugation aborts at this point without the formation of new macronuclei, mating cells separate (IX) and both mating partners retain their original phenotype, including the ability to mate (maturity) and mating type (Allen 1967).

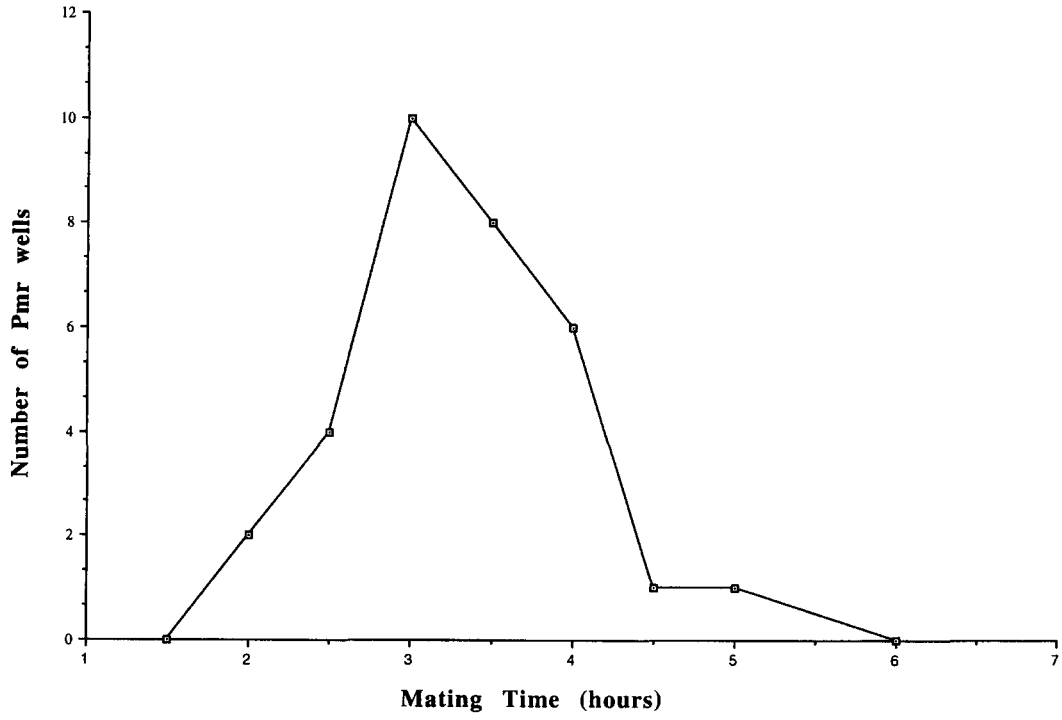


FIGURE 5.—Time course of micronuclear transformation in a diploid by star mating bombarded with pD5H8. Aliquots of a mating of A*III and CU427 were bombarded at half-hour intervals between 1-$^1/_2$ and 6 hr after cell mixing at 30°. The bombarded round I exconjugants (G0 generation) were mated en masse to CU428 and putative micronuclear transformants were identified as those cell lines expressing the paromomycin-resistance phenotype in drug selected first generation (G1) progeny. Because of the low frequency of heritable transformation, each well containing paromomycin-resistant cells was counted as a single transformation event.

ment, we bombarded cells in round I of a cross to a star strain, and then delayed round II mating to delay anlagen development. As Figure 4 outlines, in these matings the star strain lacks a functional micronucleus. Meiosis proceeds normally in the nonstar partner, but conjugation is aborted prematurely following unidirectional transfer of a prezygotic micronucleus from the nonstar to the star partner. Exconjugant cells derived from a star mating contain identical, completely homozygous diploid micronuclei, but re-

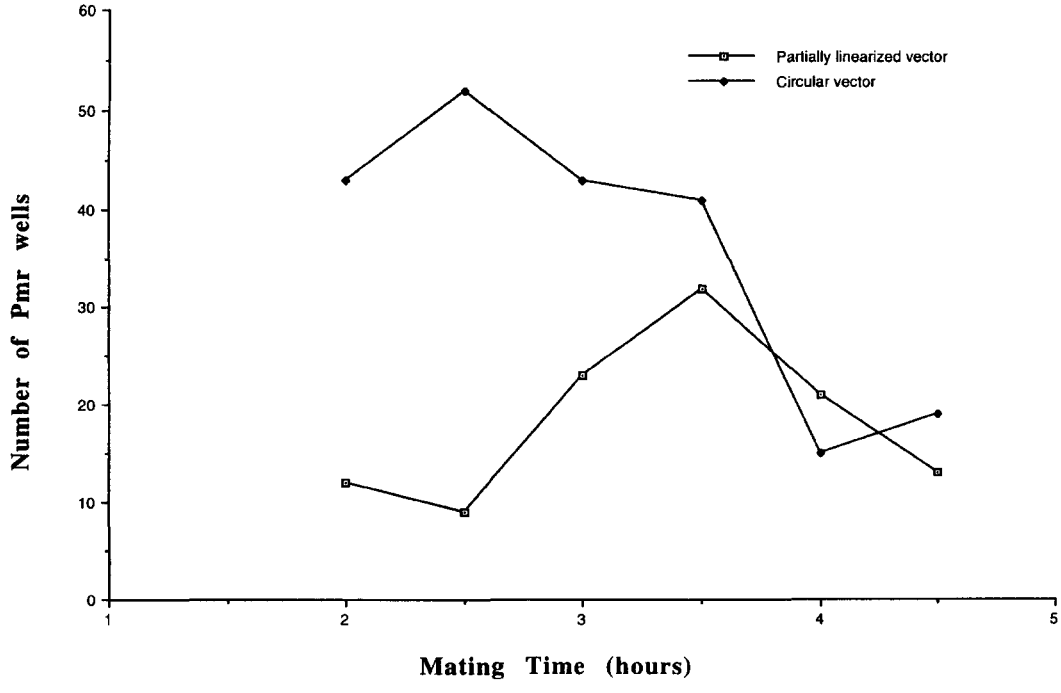


FIGURE 6.—Comparison of the transformation efficiency of circular and linear pD5H8. Aliquots of a single mating of A*III and CU427 were bombarded at the times shown with either circular pD5H8 or pD5H8 restricted with *Sal*I and *Xho*I to release the complete C3 rDNA insert. The restriction digest contained both circular and linearized molecules. Each well containing paromomycin-resistant cells was counted as a single transformation event.

tain their parental macronuclei. Since star mating exconjugants retain their parental phenotypes, including maturity and mating type, they can be grown and then remated to a diploid heterokaryon for a mating that leads to normal macronuclear development (round II). This two-step mating process allowed us to bombard cells at various times during round I, when no new macronuclei form, and then to create new macronuclei by a second mating only after the transformed cells were allowed to grow for two to three fissions.

Separate samples of an A*III and CU427 mating were bombarded with pD5H8-coated particles at half hour intervals between $1\,^1/_2$ and 6 hr after cell mixing at 30°. These G0 cultures were then refed and incubated for 24 hr at 30°. They were then each mated en masse to CU428 and plated in microtiter plates. Progeny G1 clones were selected with cycloheximide and were then tested with paromomycin; results are presented in Figure 5. Only cells bombarded between 2 and 5 hr after mixing produced paromomycin-resistant G1 clones, with the highest frequency of resistant lines occurring in cells shot between 3 and 4 hr.

The 32 paromomycin-resistant G1 progeny lines identified by this screen were subcloned, grown to maturity, and again mated to CU428 to test the stability of the micronuclear transformation. Individual mating pairs were isolated from each of the 32 crosses and the production of true progeny was determined for each pair by testing for cycloheximide resistance, 6-methylpurine resistance, paromomycin resistance, and immaturity. Of the 32 paromomycin-resistant G1 clones assayed, only one (clone D11) behaved like an integrated micronuclear transformant, exhibiting normal Mendelian segregation for the *Pmr* allele in the G2 generation.

PCR and Southern analysis of total genomic DNA from the G1 generation of clone D11 indicated C3-specific polymorphisms at both the 5′ and 3′ ends of the macronuclear rDNA molecule. However, analysis of total genomic DNA from four independent paromomycin-resistant G2 progeny clones showed that all four lines contained recombinant macronuclear rDNA: the 5′ end of the macronuclear rDNA from these four G2 lines contained only C3-specific polymorphic sequences, while the 3′ end exhibited B-specific polymorphic sequences. This result suggests that the initial paromomycin-resistant phenotype in the G1 was based on macronuclear transformation caused by the processing of a C3 vector molecule, and that the stable maintenance of the phenotype in the G2 resulted from a micronuclear transformation event involving recombination at the micronuclear rDNA locus. In this experiment, the vector must have persisted during the 24-hr growth of the round I culture (about four cell doublings) before it was subjected to round II mating and development of the new macronucleus.

Micronuclear transformation using restricted *vs.* circular pD5H8: To test factors that might affect stable integration of vector DNA into the genome, we compared the transformation efficiencies of partially linearized and circular pD5H8 on samples from different times of a single mating of A*III and CU427. Following the protocol described above, we identified a total of 213 initial G1 paromomycin-resistant transformants using circular pD5H8. In contrast, transformation using a plasmid that had been partially cut with *SalI* and *XhoI* to release the complete C3 rDNA insert generated 113 paromomycin-resistant G1 lines (see Figure 6). All 326 paromomycin-resistant G1 clones were grown to maturity and mated to strain IA264, and the resulting G2 progeny were selected by resistance to D-galactose and then assayed for paromomycin resistance. Of the 326 G1 lines tested, eight produced paromomycin-resistant G2 progeny.

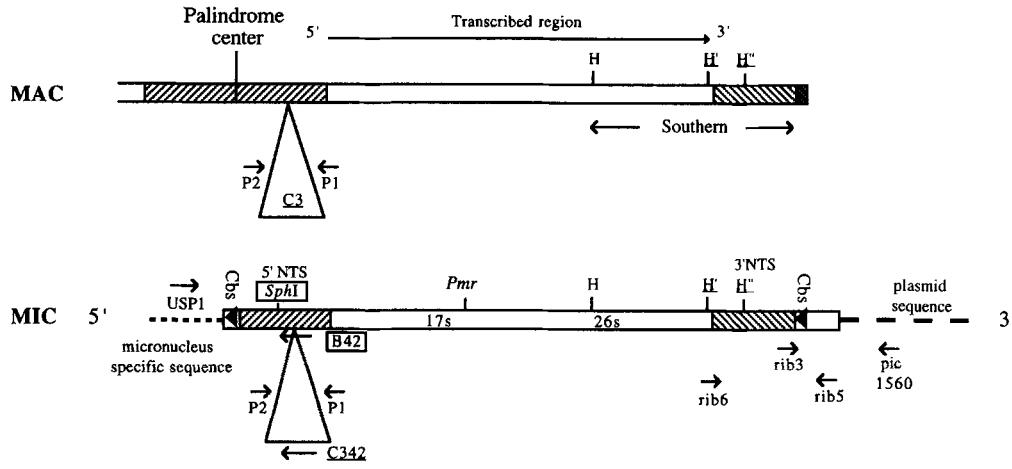
TABLE 2

Genetic analysis of micronuclear transformants

Clone	Vector form	G2 from xCU428		Round I genotype	
		Pmr	*Pms*	*Pmr*	*Pms*
1	Circular	0	35	0	15
2	Circular	24	12	3	5
3	Circular	17	15	8	3
4	Circular	25	12	3	2
5	Restricted	20	16	2	4
6	Restricted	15	17	2	1
7	Circular	19	14	7	5
8	Circular	16	17	3	3

Subclones of eight G1 clones originally showing paromomycin resistance in the G2 generation were analyzed. To quantitate the meiotic segregation of the *Pmr* allele in the G2, G1 progeny expressing paromomycin resistance were matured, subcloned, and crossed to a genetically marked heterokaryon. Individual G2 pairs were isolated and tested for paromomycin resistance and maturity. To further test the meiotic segregation of the *Pmr* allele, each clone was mated to B*VII, and individual round I pairs isolated. The genotype of each round I clone was determined by allowing individual round I pairs to undergo a second round of mating. Individual round II pairs were isolated and tested for paromomycin resistance and immaturity.

The eight G1 lines that had yielded paromomycin-resistant G2 cells were subcloned, mated to CU428.1, and individual G2 progeny were analyzed for immaturity and resistance to cycloheximide and paromomycin. Each G1 clone was also mated to B*VII and the genotype of isolated round I pairs was determined by allowing the exconjugants from each isolated pair to grow and mate with each other for round II and then analyzing for paromomycin resistance among round II progeny. As shown in Table 2, in both genetic tests seven G1 clones behaved as stably integrated heterozygous paromomycin-resistant transformants; one line failed to maintain paromomycin resistance following



Restriction Enzyme		Fragment Size (kb) B	C3	Specificity
MAC				
Southern Probe				
pD5H8 *Hin*dIII digest	*Bam*HI (genomic blot)	13.5, 3.8	13.5,1.9* 1.5, 0.4*	3' end - differentiates B and C3. * indicates vector derived restriction site.
PCR Primers				
P1-P2	None	0.5	0.55	5' end - differentiates B and C3.
MIC				
PCR Primers				
USP1-P1	*Sph*I	1.28, 0.35	1.6	5' end- differentiates B and C3. Requires integration in normal micronuclear location.
USP1-B425		1.5	None	5' end - B specific. Requires integration in normal micronuclear location
USP1-C342		None	1.5	5' end - C3 specific.Requires integration in normal micronuclear location.
rib6-rib5	*Bam*HI	1.6	1.26, 0.37	3' end - differentiates B and C3. Genomic location not specified.
rib3-pic1560		1.6	1.6	Indicates plasmid at 3' end of rDNA. Location not specified.
rib6-pic1560		3.1	3.1	Indicates plasmid at 3' end of rDNA. Location not specified.
rib6-P1		3.6, 6.3^	3.6, 6.3^	Indicates tandem arrangement. ^ indicates intervening plasmid sequence.
USP1-rib5				Whole fragment links 5' and 3' ends at normal genomic location.
	*Sph*I	6.0, 3.25 1.28	7.3, 3.25	5' end - differentiates B and C3 Specifies normal location.
	*Bam*HI	6.95 , 3.6	6.95, 1.9* 1.1, 0.4*	3' end - differentiates B and C3. Specifies normal location.* indicates vector derived restriction site.
USP1-pic1560		12.2	12.2	Indicates plasmid sequence at 3' end of normal location.

FIGURE 7.—Polymorphisms used to localize transforming DNA. A composite version of B and C3 micronuclear rDNA is shown. One half of the macronuclear palindrome, which is derived directly from the micronuclear gene, is shown above the micronuclear version. The location of strain-specific polymorphisms that were used to differentiate between the 5′ and the 3′ ends of B and C3 alleles and to establish orientation relative to the normal upstream micronuclear sequence are shown. Polymorphisms unique to C3 are underlined and those unique to B are boxed. Cbs (◀) indicate the location of the chromosome breakage sequences flanking the micronuclear rDNA region. The 5′ and 3′ nontranscribed sequences (NTS) are indicated by the light hatched regions. The telomere sequence on the macronuclear chromosome is indicated by the dark hatched region. *Pmr* is the single base mutation in the 17S rDNA that confers paromomycin resistance. H, H′ and H″ are *Bam*HI sites. H is present in both B and C3, H′ is a second *Bam*HI site artificially introduced into the vector C3 rDNA, and H″ is present only in C3. The position and direction of PCR primers used are indicated by short arrows. USP1 is a primer derived from micronuclear-specific sequence located 5′ of the end of the micronuclear rDNA. P1 and P2 are primers that flank a 42-bp region present in C3 but missing in B. The *Sph*I restriction site shown is present within the fragment defined by P1-P2 primers in B but not in C3. C342 is a primer derived from within the C3-specific 42-bp region and B42 is a B specific primer that spans the corresponding deletion in B. rib5 and rib6 are primers that flank the C3-specific H′ restriction site. rib3 is a primer located just upstream of the 3′ Cbs sequence. pic1560 is a primer derived from plasmid sequence in the pD5H8 vector that will only be present in the micronucleus if the plasmid sequence has become integrated into the micronuclear chromosome during transformation. The region used to

TABLE 3

Analysis of 5′ and 3′ polymorphisms in transformed lines

Specificity	Analysis		Transformant					
	PCR primers	Southern	1	2	3	4	5	6
Macronucleus								
5′ end of rDNA	P1-P2		C3	C3	C3	B	C3	C3
3′ end of rDNA		rDNA 3′ fragment	C3	C3	C3	B	B	B
Micronucleus								
5′ end of rDNA	USP1-C342		−	−	−	−	−	−
	USP1-B42		B	B	B	B	B	B
	USP1-P1		B	B	B	B	B	B
3′ end of rDNA	rib6-rib5		B	C3/B	C3/B	B	C3/B	C3/B
	rib3-pic1560		+	+	−	−	+	+
	rib6-pic1560		+	+	−	−	+	+
	rib6-P1		+	+	−*	−	+	+
across rDNA	USP1-rib5							
	5′ end		ND	B	ND	ND	B	B
	3′ end		ND	C3/B	ND	ND	C3/B	C3/B
	USP1-pic1560		ND	ND	ND	ND	ND	+

Six transformed lines initially expressing paromomycin resistance in the G2 were analyzed for the presence of B and/or C3 polymorphisms at the 5′ and 3′ end of both macronuclear and micronuclear rDNA. +, transformed lines producing the PCR fragments predicted based on a tandem arrangement of rDNA gene sequences separated by the vector plasmid sequence; −, no PCR product of the predicted size was produced; −*, no band of the predicted size was produced but a band corresponding to the appropriate size fragment minus the plasmid sequence was produced; ND, not done.

subcloning. Five of the stable transformants originated from the circular vector (one from the 3-hr time point, two from each of the $3^1/_2$- and $4^1/_2$-hr time points) and two from the partially linearized vector (4-hr time point).

G2 paromomycin-resistant progeny from six of the eight paromomycin-resistant G1 lines were analyzed to determine the allelic form of the 5′ and 3′ ends of the macronuclear rDNA. The strategies for this analysis are presented in Figure 7, and the results are tabulated in Table 3. A variety of recombinant rDNA forms were present in the macronuclei of these lines. Three lines, including transformant 1, which lost the paromomycin-resistance marker upon subsequent subcloning, contained macronuclear rDNA that exhibited C3 polymorphisms at both the 5′ and the 3′ ends of the molecule. Two lines contained recombinant rDNA molecules with C3 polymorphisms at the 5′ end but the B variant at the 3′ end. One stable paromomycin-resistant line showed only the B form at both the 5′ and the 3′ ends of the rDNA.

To further examine the question of site and mechanism of integration of the vector rDNA into the micronuclear chromosome, the DNA from these six G2 transformants was also tested using the PCR primer sets described in Figure 7. Again the results are found in Table 3. Using as an anchor a primer (USP1) derived from a micronucleus specific sequence just upstream of the rDNA chromosome breakage sequence, but not present in the plasmid, it was possible to determine the micronuclear chromosomal location of the rDNA sequence acting as template for the observed PCR product. This combination of primers also allowed us to establish the allelic origin of the 5′ and 3′ ends of the rDNA at the correct site, and the presence of integrated plasmid sequence in some of the transformants. Multiple sets of primers allowed us to confirm the allelic identity of the 5′ and 3′ ends of the chromosomal rDNA and to determine the tandem orientation of duplicate copies of the rDNA at the normal chromosomal location.

Figure 8 summarizes the configuration of the rDNA in the five stably transformed clones. In all cases the vector rDNA is integrated in the micronucleus at its homologous location. In three transformants (2, 5, 6) a tandem arrangement of two copies of the rDNA separated by the plasmid sequence has been generated at the homologous site. In each case the 5′ end of the resident B allele is located adjacent to the normal upstream sequence and is associated with a C3 3′ terminus.

differentiate the 3′ ends of the B and C3 macronuclear rDNA alleles by Southern hybridization, using blots of genomic DNA restricted with *Bam*HI and probed with ^{32}P-labeled pD5H8, is indicated below the macronuclear chromosome. The specific primer sets used, the restriction enzyme, if any, that was used to cut the PCR product, or in the case of the Southern hybridization, to cut the genomic DNA, and the expected fragment sizes generated are shown in the table below the drawing. The ability of each primer set to differentiate B and C3 and, when applicable, to determine chromosomal location is also indicated.

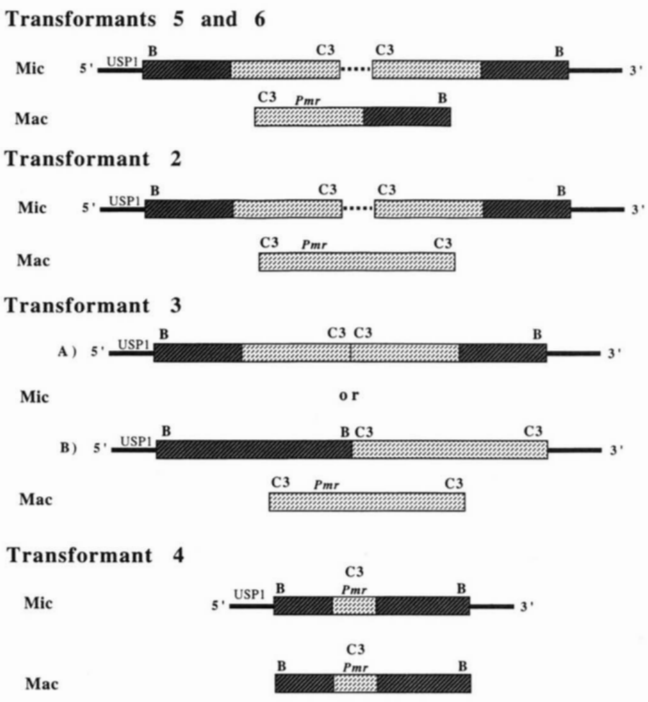


FIGURE 8.—Integration of transforming sequences into micronuclear chromosomes. A schematic overveiw of chromosomal arrangements suggested by the analysis in Table 3 is shown for each of the five stable transformants analyzed. Both the micronuclear (mic) and macronuclear (mac) versions are shown. B and C3 indicate the allelic form identified at the 5′ and 3′ ends of the molecules. —, micronuclear sequences adjacent to the rDNA locus; ---, plasmid sequences from the transformation vector integrated into the micronuclear chromosome; and USP1 indicates the location of the micronuclear-specific primer used to localize the insert. *Pmr* indicates the paromomycin resistance mutation carried on the C3 vector.

The second, tandem gene is separated from the first copy by the plasmid sequence and contains the 5′ C3 and 3′ B sequences. In transformants 5 and 6 the paromomycin-resistant macronuclear rDNA directly reflects the 5′ C3-3′ B arrangement of the second copy of the gene in the micronucleus. However, in transformant 2, the macronuclear version is C3-C3 while the micronuclear genes are B-C3 and C3-B, respectively. If the C3-C3 version were simply the product of residual vector, both C3-C3 and C3-B types of molecules should be present in the macronucleus unless the C3-B rDNA was outcompeted by C3-C3 molecules. The lack of C3-B in the macronucleus could be due to processing of both retained vector and chromosomal rDNA followed by phenotypic assortment (MERRIAM and BRUNS 1988), giving rise to a uniquely C3-C3 macronucleus in the subclone examined. This phenotype could also be a consequence of a somatic crossover early in the differentiation of the macronuclear anlagen, giving rise to the C3-C3 form that would rapidly outcompete the concomitantly produced B-B form.

Transformant 3 also contains tandem rDNA genes in the micronucleus, but the intervening plasmid sequence is missing (Figure 9). The 5′ end adjacent to the normal upstream sequences was again shown to be the resident B allele, but the associated 3′ end was not determined. The C3-C3 macronuclear configuration suggests that either the crossover event that generated the tandem configuration suggests that either the crossover event that generated the tandem configuration occurred near the 3′ end of the rDNA molecule, generating a B-*Pms*-B, C3-*Pmr*-C3 micronuclear configuration, or that a somatic recombination event occurred similar to that described above for transformant 2.

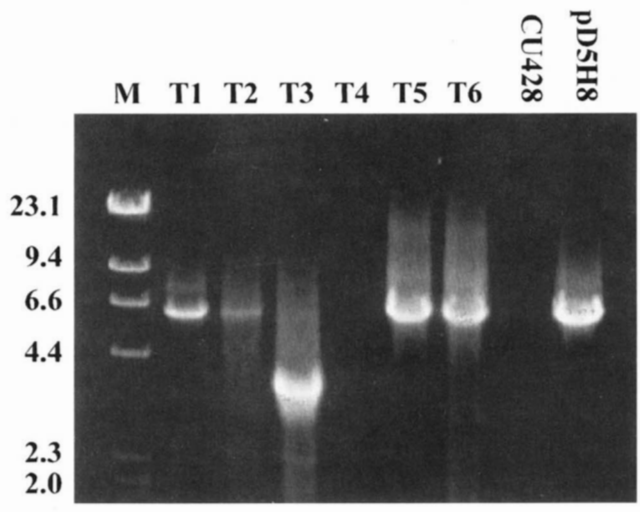


FIGURE 9.—PCR fragments produced by primers rib6 and P1. DNA was extracted from CU428 and six transformant lines using standard methods (GAERTIG *et al.* 1994). One hundred fifty nanograms of DNA were used for each PCR reaction. The positive control for the PCR reaction contained 39 pg of the transforming plasmid DNA (pD5H8). The PCR reactions were carried out with Elongase polymerase enzyme mix (GIBCO Life Technologies) and buffers, according to manufacturer's directions. Each reaction mix contained primers at a concentration of 200 μM each, 2.2 mM $MgCl_2$, and 200 μM dNTPs. Cycling conditions were as follows: one cycle at 94° for 1 min; followed by 40 cycles at 94° for 30 sec, 54° for 30 sec, 68° for 7.5 min; followed by one cycle at 68° for 15 min. PCR products were run on an 0.8% agarose gel in TAE buffer and stained with ethidium bromide. Marker lane contains *Hin*dIII cut λ.

In transformant 4, recombination with the homologous gene appears to have given rise to partial gene replacement. PCR analysis indicates a single *Pmr* rDNA gene in the micronucleus that is B-B in nature. This same B-B configuration was observed in the paromomycin-resistant macronuclear rDNA.

Transformant 1, which lost its paromomycin-resistant phenotype upon further subcloning, contained macronuclear rDNA that was exclusively C3 at both the 5′ and 3′ ends, and micronuclear rDNA that was completely B at both ends, suggesting that the paromomycin-resistant phenotype was probably generated by the processing of a transiently maintained plasmid rather than by an integration event. Maintained plasmid could account for the PCR results observed with this transformant.

TABLE 4

Micronuclear transformants recovered using vectors pD5H8 and pD3/5-11

	A pD5H8 Star mating		B pD3/5-11 Star mating		C pD3/5-11 Diploid mating	
Time of bombardment (hours)	Initial	Stable	Initial	Stable	Initial	Stable
2	8	0	0	0	3	3
$2^1/_2$	4	0	0	0	15	14
3	4	0	1	1	5	5
$3^1/_2$	12	0	0	0	6	4
4	6	0	1	1	3	3
$4^1/_2$	0	0	0	0	3	1
Total	34	0	2	2	35	30

Cells from either a "star" mating (A and B) or a diploid mating (C) were bombarded during conjugation at the times indicated. Initial transformants are lines expressing paromomycin resistance in G1 progeny. Stable transformants are those lines continuing to give paromomycin resistance in G2 progeny. Vector pD5H8 contains a complete copy of the C3 rDNA region, including a mutation conferring resistance to paromomycin. Vector pD3/5-11 is identical to pD5H8 except that it lacks both the 5′ and 3′ Cbs regions.

Micronuclear transformation using Cbs$^-$ vector pD3/5-11: In all of the preceding experiments, it is apparent that nonintegrated DNA that is competent to be processed into an rDNA macronuclear chromosome during anlagen development can give rise to macronuclear transformants in the absence of stable micronuclear transformation. To test this, we used the vector pD3/5-11, which is essentially the same as pD5H8 except that it is incompetent to be processed since it lacks the chromosome breakage sites on either side of the gene (see Figure 1). Transformation of a star mating was performed as above, except that separate aliquots of the same mating mixtures were transformed with either circular pD5H8 or pD3/5-11 to allow direct comparison of the transformation frequencies of each vector. In addition, a diploid by diploid mating was also used as the target for transformation by pD3/5-11. Paromomycin-resistant transformants were analyzed as described above. The number of transformants obtained in each of these experiments is shown in Table 4. The number of lines initially showing paromomycin resistance in the star mating is considerably lower when the vector missing the chromosome breakage sites is used for transformation. However, in both the star and diploid matings, a majority of the initially resistant clones (32/37) are stably transformed, showing normal Mendelian segregation through at least two subsequent generations (data not shown).

DISCUSSION

We have developed a technique to transform either the macro- or the micronuclei of conjugating *T. thermophila*. Using an rDNA vector containing a point mutation conferring paromomycin resistance, we have established conditions for biolistic transformation of conjugating cells.

Macronuclear transformants can be directly selected following biolistic bombardment of conjugating pairs and the formation of a new, transformed macronucleus. Optimal macronuclear transformation occurs during a narrow temporal window 10 hr following cell mixing, a time when the macronuclear anlagen are developing. This is the same time as that observed for macronuclear transformation using electroporation (GAERTIG and GOROVSKY 1992) and probably reflects the direct processing of introduced DNA into macronuclear chromosomes during conjugation.

We also observed macronuclear transformation in conjugants bombarded at conjugation times well prior to the point when anlagen formation occurs, especially in the star matings. This finding suggests that the transforming DNA introduced at one time could be maintained in a form that could be processed into macronuclear chromosomes at a later time. We do not know how or where the plasmid is maintained in these situations. It could merely persist in the cytoplasm or the germinal nucleus of the bombarded cells, possibly in sufficient numbers to insure its retention during cell division without plasmid DNA replication. On the other hand, it may be possible that macronuclear transformants can result from unstable micronuclear integration, although this seems highly unlikely.

Maintenance of nonintegrated vector DNA, is supported by experiments using an rDNA vector lacking both 5′ and 3′ Cbs regions. These experiments yielded far fewer initial transformants, but nearly all that were recovered were stable. Since Cbs sequences are required for macronuclear chromosome processing, and are found only in the micronuclear genome, the successful transformants must have become associated with these sequences by integrating into the germinal genome. The frequency of clones that do not transmit the new phenotype in subsequent crosses in this group

of transformants (5/37) may be an indication of the frequency with which an integrated vector secondarily recombines out of the recipient chromosome. Alternately, the rare somatic transformants in this group may have arisen in developing macronuclear anlagen by recombination between the amplifying B rDNA and nonintegrated, nonamplifying transforming DNA.

Optimal micronuclear transformation occurred within a narrow 2-hr window in conjugation: $2^1/_2$–$4^1/_2$ hr after cell mixing. During this period, the micronucleus moves away from a pocket in the macronucleus and enlarges, the chromatin lengthens into a premeiotic configuration known as the "crescent", and then begins to recondense into a metaphase chromosome configuration (MARTINDALE and BRUNS 1983). It is possible that the large size of the crescent may be responsible for the increased entry rate, by simply presenting a larger target for the DNA bearing particles. The same optimal entry time was seen in star crosses transformed with the fully competent pD5H8 vector; most of the transformants, selected after a second mating, were somatic but not germinal, again suggesting entry into the germinal nucleus but not integration. This suggests that the window between $2^1/_2$–$4^1/_2$ hr is not related to a recombinational hot spot that is necessary for integration, but rather is simply a time of heightened opportunity for entry into the nucleus itself.

A molecular analysis of the integrated sequences in the germinal transformants indicated that the vector C3 rDNA had physically recombined with the recipient B allele. Detailed analysis of five stable G2 micronuclear transformants using PCR involving a number of different primer sets indicated that in all cases stable transformation was accompanied by physical integration of the rDNA into the homologous site on the micronuclear chromosome. In four of the cases examined, a tandem duplication resulted; in the fifth instance, the product was a single, recombinant rDNA gene containing the C3 paromomycin-resistant mutation flanked on both sides by sequences of B origin.

We have shown that stable micronuclear transformants arising from homologous recombination can be produced in *T. thermophila* using biolistic transformation. This technique permits transformation of the germ line nucleus in this organism and should be of considerable use in a variety of genetic and molecular studies. We are currently examining the use of this technique with nonribosomal vectors.

We acknowledge the Plant Science Center at Cornell University for the use of their biolistic facility. The authors especially thank MENG-CHAO YAO for providing the clones used in this study. We also gratefully acknowledge VIRGINIA MERRIAM for her careful review of the manuscript. This work was funded in part by U.S. Department of Agriculture Cooperative Research Service Hatch project 186414 awarded to P.J.B. through the Cornell University Agricultural Experiment Station and in part by grant #2R01 GM21793-19 from the National Institute of General Medical Sciences to M.A.G. J.H.L. was supported by funds for undergraduate research from the Howard Hughes Medical Institute.

LITERATURE CITED

ALLEN, S. L., 1967 A rapid means for inducing homozygous diploid lines in *Tetrahymena pyriformis*. Science **155:** 575–577.

BRUNK, C. F., and P. NAVAS, 1988 Transformation of *Tetrahymena thermophila* by electroporation—parameters effecting cell survival. Exp. Cell Res. **162:** 525–532.

BRUNS, P. J., 1986 The genetic organization of *Tetrahymena,* pp. 27–44 in *The Molecular Biology of Ciliated Protozoa,* edited by J. GALL. Academic Press, New York.

BRUNS, P. J., and D. M. CASSIDY-HANLEY, 1992 *Tetrahymena thermophila,* pp. 2.175–2.179 in *Genetic Maps,* edited by S. J. O'BRIEN. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

BRUNS, P. J., A. L. KATZEN, L. MARTIN and E. H. BLACKBURN, 1985 A drug resistant mutation in the ribosomal DNA of *Tetrahymena.* Proc. Natl. Acad. Sci. USA **82:** 2844–2846.

CHRISTOU, P., 1995 Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. Euphytica **85:** 13–27.

DIX, P. J., and T. A. KAVANAGH, 1995 Transforming the plastome: genetic markers and DNA delivery systems. Euphytica **85:** 29–34.

GAERTIG, J., and M. GOROVSKY, 1992 Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants. Proc. Natl. Acad. Sci. USA **89:** 9196–9200.

GAERTIG, J., L. GU, B. HAI and M. A. GOROVSKY, 1994 High frequency vector-mediated transformation and gene replacement in *Tetrahymena.* Nucleic Acids Res. **22:** 5391–5398.

GAINER, A. L., G. S. KORBUTT, R. V. RAJOTTE, G. L. WARNOCK and J. F. ELLIOTT, 1995 Biolistic transformation of pancreatic islets. Fifth Congress of the International Pancreas and Islet Transplant Association: Cure of Diabetes by Transplantation, Miami Beach, Florida, USA, June 18–22, Transplantation Proceedings. **27:** 3278.

GODISKA, R., and M.-C. YAO, 1990 A programmed site specific DNA rearrangement in *Tetrahymena thermophila* requires flanking polypurine tracts. Cell **61:** 1237–1246.

GOROVSKY, M. A., 1980 Genome organization and reorganization in *Tetrahymena.* Annu. Rev. of Genetics **14:** 203–239.

GOROVSKY, M. A., M-C. YAO, J. B. KEEVERT and G. L. PLEGER, 1975 Isolation of micro- and macronuclei of *Tetrahymena pyriformis.* Methods Cell Biol. **9:** 311–327.

KARRER, K. M., and M.-C. YAO, 1988 Transformation of *Tetrahymena thermophila* with hypermethylated ribosomal RNA genes. Mol. Cell Biol. **8:** 1664–1669.

LARSON, D., E. BLACKBURN, P. YAEGER and E. ORIAS, 1986 Control of rDNA replication in *Tetrahymena* involves a Cis-acting upstream repeat of a promoter element. Cell **47:** 229–240.

MARTINDALE, D., and P. J. BRUNS, 1983 Cloning of abundant mRNA species present during conjugation of *Tetrahymena thermophila:* identification of mRNA species present exclusively during meiosis. Mol. Cell. Biol. **3:** 1857–1865.

MERRIAM, E. V., and P. J. BRUNS, 1988 Phenotypic assortment in *Tetrahymena thermophila:* assortment kinetics of antibiotic-resistance markers, TSA, Death, and the highly amplified ribosomal DNA locus. Genetics **120:** 389–396.

ORIAS, E., D. LARSON, Y-F. HU, G-L. YU, J. KARTTUNEN *et al.,* 1988 Replacement of the macronuclear ribosomal RNA genes of a mutant *Tetrahymena* using electroporation. Gene **17:** 295–301.

RASMUSSEN, J. L., J. R. KIKKEERT, M. K. ROY and J. C. SANFORD, 1994 Biolistic transformation of tobacco and maize suspension cells using bacterial cells as microprojectiles. Plant Cell Reports **13:** 212–217.

SAMBROOK, J., E. F. FRITSCH and T. MANIATIS, 1989 *Molecular Cloning: A Laboratory Handbook,* Ed. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SANFORD, J., M. J. DEVIT, J. A. RUSSELL, F. D. SMITH, P. R. HARPENDING *et al.,* 1991 An improved helium driven biolistic device. Technique **3:** 3–6.

SOUTHGATE, E. M., M. R. DAVEY, J. B. POWER and R. MARCHANT, 1995

Factors affecting the genetic engineering of plants by microprojectile bombardment. Biotechnol. Adv. **13:** 631–651.

SPANGLER, E. A., and E. H. BLACKBURN, 1985 The nucleotide sequence of the 17S ribosomal RNA gene of *Tetrahymena thermophila* and the identification of point mutations resulting in resistance to the antibiotics paromomycin and hygromycin. J. Biol. Chem. **260:** 6334–6340.

SWEENEY, R., and M-C. YAO, 1989 Identifying functional regions of the rRNA by insertion mutagenesis and complete gene replacement in *Tetrahymena thermophila.* EMBO J. **8:** 933–938.

TONDRAVI, M., and M-C. YAO, 1986 Transformation of *Tetrahymena thermophila* by microinjection of ribosomal RNA genes. Proc. Natl. Acad. Sci. USA **83:** 4369–4373.

YAO, M-C., 1986 Amplification of ribosomal genes pp. 179–201 in *The Molecular Biology of Ciliated Protozoa,* edited by J. GALL. Academic Press, New York.

YAO, M-C., and J. G. GALL, 1977 A single integrated gene for ribosomal RNA in a eukaryote, *Tetrahymena pyriformis.* Cell **12:** 121–132.

YAO, M-C., and C-H. YAO, 1989 Accurate processing and amplification of cloned germ line copies of ribosomal DNA injected into developing nuclei of *Tetrahymena thermophila.* Mol. Cell. Biol. **9:** 1092–1099.

YAO, M-C., C-H. YAO and B. MONKS, 1990 The controlling sequence for site-specific chromosome breakage in *Tetrahymena.* Cell **63:** 763–772.

YASUDA, L. F., and M-C. YAO, 1991 Short inverted repeats at a free end signal large palindromic DNA formation in *Tetrahymena.* Cell **67:** 505–516.

YU, G-L., and E. H. BLACKBURN, 1989 Transformation of *Tetrahymena thermophila* with a mutated circular ribosomal DNA plasmid vector. Proc. Natl. Acad. Sci. USA **86:** 8487–8491.

YU, G-L., and E. H. BLACKBURN, 1990 Amplification of tandemly repeated origin control sequences confers a replication advantage on rDNA replicons in *Tetrahymena thermophila.* Mol. Cell. Biol. **10:** 2070–2080.

YU, G-L., M. HASSON and E. BLACKBURN, 1988 Circular ribosomal DNA plasmids transform *Tetrahymena thermophila* by homologous recombination with the endogenous macronuclear ribosomal DNA. Proc. Natl. Acad. Sci. USA **85:** 5151–5155.

Communicating editor: S. L. ALLEN