Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4369–4373, June 1986
Genetics

Transformation of *Tetrahymena thermophila* by microinjection of ribosomal RNA genes

(ciliates/paromomycin resistance/rDNA minichromosome/foreign DNA/gene expression)

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Communicated by John R. Preer, Jr., January 10, 1986

ABSTRACT **The ribosomal RNA genes (rDNA) of *Tetrahymena thermophila* macronucleus exist as free linear 21-kilobase molecules that contain replication origins and telomeres. A mutation in this gene confers resistance to the antibiotic paromomycin. We have isolated rDNA from such a mutant (strain p2f), microinjected it into the macronucleus of a sensitive strain, and obtained drug-resistant cells at a frequency of 1–3%. The transformed cells have a distinct and stable phenotype. The rDNA of the transformants contains the expected sequences of the mutant rDNA as determined by oligonucleotide hybridization. rDNA from a different inbred line (C3-368), which contains heteromorphic restriction sites, has also been used for injection, and the results confirm the fact that the injected rDNA is indeed present in the transformants. Injection of rDNA from the C3 strains also increases the transformation frequency 5- to 10-fold and leads to the total replacement of the resident rDNA of the B-inbred strains. This is presumably due to the replication dominance of rDNA from the C3 strains over that of the B strains. Using this method, we have also been able to transform developing cells, at similar frequencies, by microinjecting into the macronuclear anlagen.**

Protocols for DNA-mediated transformation have been described for a number of eukaryotic organisms including yeast (1), *Drosophila* (2), *Neurospora* (3), *Dictyostelium* (4), and mammalian tissue culture cells (5). These methods have proven to be essential for the analysis of several different levels of gene regulation such as transcription, replication, and transposition. The ciliated protozoan *Tetrahymena thermophila* has been widely used in recent years for molecular genetical studies. In particular, it has provided unique insight into the problems of rRNA processing, rRNA gene amplification, DNA rearrangement, and telomere formation. To facilitate these and other studies, we have developed a transformation protocol for *Tetrahymena* by using the rRNA genes (rDNA) as the selectable marker. *T. thermophila* normally contains two nuclei—a micronucleus and a macronucleus. In vegetatively growing cells the macronucleus is polyploid and transcriptionally active, while the micronucleus is diploid and transcriptionally inactive (6). The micronucleus, however, is ultimately responsible for the genetic continuity of the organism, while the macronucleus is responsible for its phenotype. This dichotomy of nuclear functions allows extensive rearrangements to occur in the macronuclear genome without disrupting the genetic continuity of the organism. The most prominent example of gene rearrangement occurring during macronuclear development was discovered during the analysis of ribosomal RNA genes (reviewed in refs. 7 and 8). The micronucleus contains a single copy of rDNA per haploid genome (9, 10). The macronucleus contains 9000 copies of free linear palindromic dimers of rDNA. The generation of macronuclear rDNA involves chromosome fragmentation, telomere addition, conversion of a single copy gene to a palindromic one, and its amplification. All of these events appear to be precisely controlled during macronuclear development.

The presence of only a single copy of rDNA per haploid micronuclear genome has rendered the genes available to mutational analysis. J. Wilhelm and P. J. Bruns (personal communication) have isolated several antibiotic-resistance mutations, which have been mapped to the rDNA (11). Of interest here are the paromomycin-resistance mutations (*Pmr*) that in all three cases analyzed are the result of a single guanine to adenine base change in the 17S rRNA (12). The paromomycin-resistant rDNA (*rdnA2*) shows clear dominance over wild-type rDNA in appropriate genetic backgrounds—i.e., in crosses between the cells of the same inbred line (11). The presence of a dominant selectable marker taken together with the fact that the macronuclear rDNA is an autonomous chromosome of relatively small size have made rDNA an ideal molecule for the purpose of establishing transformation in *Tetrahymena*.

We demonstrate here a method for transformation of *Tetrahymena* by microinjecting the macronuclear rDNA that confers paromomycin resistance into the macronucleus of a drug-sensitive host. The transformation frequency is 1–3% when using paromomycin-resistant rDNA from a B-inbred line of *Tetrahymena*. The efficiency of transformation increases nearly 10-fold when the injected rDNA is from the C3-inbred line, which has a dominant property in replication (P. Yaeger, E. Orias, D. Larson, and E. H. Blackburn, personal communication). We find that relatively few copies of the C3 type of rDNA are needed to obtain transformants. The injected rDNAs are stably maintained as extrachromosomal molecules. Injection of rDNA into developing macronuclei of conjugating cells results in similar transformation frequencies.

MATERIALS AND METHODS

Cell Culture and Cloning. Strains p2f [*Pmr/Pmr, Mpr/Mpr* (paromomycin resistant, 6-methyl purine resistant, mating type VI)], CU427 [*Chx/Chx, pmr*$^+$*/pmr*$^+$ (cycloheximide sensitive, paromomycin sensitive, mating type VI)] and CU428 [*Mpr/Mpr, pmr*$^+$*/pmr*$^+$ (6-methyl purine sensitive, paromomycin sensitive, mating type VII)] were obtained from P. Bruns (Cornell University); the SB255 [*exo*$^-$*/exo*$^-$, *pmr*$^+$*/pmr*$^+$ (paromomycin sensitive, mucocystless, mating type IV)] (see ref. 13 for details) and C3-368 [*rdnA1/rdnA1*, *pmr*$^+$*/pmr*$^+$ (rDNA-A1, paromomycin sensitive, mating type V)] strains were obtained from E. Orias (University of California, Santa Barbara). Strains SB255, CU427, CU428, and p2f are derivatives of the B-inbred line. Strain C3-368 is from a different inbred line. All cultures were maintained in

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Abbreviation: rDNA, ribosomal RNA genes.

axenic media (14). Cell cloning was done by standard procedures (15).

Mutagenesis and Isolation of Paromomycin-Resistant Strains. To obtain *Pmr* mutations, C3-368 cells were exposed to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) according to the methods of Orias and Bruns (15). Briefly, 1.5×10^7 cells in 50 ml of growth medium were mutagenized with 0.5 mg of MNNG for 3 hr. The cells were washed several times and allowed to recover in growth medium for 16 hr. They were then starved in 10 mM Tris·HCl (pH 7.4) for 6 hr before being mated to strain CU427. The progeny were selected for resistance to paromomycin (100 μg/ml). One of the resistant cells (SL062) was cloned and used in this study.

Microinjection. The microinjection procedure is as described by Capecchi (16) with the following modifications for handling *Tetrahymena*. Appropriate strains were grown to mid-logarithmic phase or to various times in the mating schedule. For the injection of logarithmic-phase cells we preferred using the mucocystless strain SB255 since we found that release of mucocyst upon injection by other strains tended to clog our injection needle. The cells were concentrated 10-fold ($1–2 \times 10^6$ cells per ml) immediately prior to being deposited (under a dissecting microscope; ×40) as tiny droplets on a slide under mineral oil as described for cell cloning by Orias and Bruns (15). Upon reaching the slide, the droplet forms a fairly thick bubble in which the cells can swim quite freely. Next, we used a second micropipette to aspirate the excess liquid from each of the drops such that the cells appeared completely immobile under ×40 magnification. The slide was then transferred to an inverted microscope equipped with two micromanipulators, and the cells were injected. One of the micromanipulators (Narashige model MO-15) was used for the injections, while the second (Narashige MM-3) was used for feeding and allowing free movement of the cells immediately after injection. Generally, 70–90% of the cells remained viable after the injection. At the end of a microinjection experiment, the cells were cloned (15).

The solution of DNA was dissolved in a buffer containing 114 mM KCl, 20 mM NaCl, 3 mM NaH_2PO_4 (pH 7.4) (17) and injected into the cells by glass micropipettes having tip diameters of ≈0.3 μm. The glass micropipettes were pulled with a Brown and Flaming model 77 (Sutter Instruments) using 1.0 mm o.d./0.75 mm i.d. Omega Dot capillaries (Frederick Haer and Co.). Microinjections were monitored under direct visual control by using differential interference contrast optics (Zeiss Axiomat, ×250–400). The DNA solution was forced into the cells under constant pressure provided by a 200-μl Gilmont microburette. The amount of liquid injected into each cell was controlled by monitoring the changes in the refraction of the cells as the fluid entered the cells and by regulating the time that the micropipette remained in the cell. We estimate that on the average each cell receives an injection equivalent to 10% of its nuclear volume.

Purification of Macronuclear rDNA. rDNA from appropriate strains was purified by the hot phenol/cresol extraction procedure of Din and Enberg (18). The DNA obtained was ≈50% rDNA and was further purified by equilibrium centrifugation in CsCl gradients.

DNA Isolation, Blotting, and Hybridization. Nuclear DNA isolation was done by described methods (19). Labeled DNA probes were made by the nick-translation method of Rigby *et al.* (20) using ^{32}P-labeled dCTP and dATP (3000 Ci/mM; 1 Ci = 37 GBq; Amersham). Restriction enzymes (New England Biolabs) were used according to the recommendations of the supplier. Southern blots were prepared by published procedures (9). Hybridization of the *Pmr* oligomer was carried out under the same conditions as the cloned DNA probes, except that the hybridization temperature was lowered to 25°C.

RESULTS

Injection of *rdnA2* Allele. Initial attempts to establish transformation in *Tetrahymena* involved injection of *rdnA2* (extrachromosomal rDNA purified from the paromomycin-resistant strain p2f; see ref. 11) into the macronucleus of strain SB255. Since rDNA is a 9000-copy gene in macronuclei, for selection we relied on the phenomenon of assortment, by which cells with homozygous phenotypes segregate during vegetative growth of heterozygous cell lines (details of assortment are reviewed in ref. 21). After injection, cells were cloned into growth medium and propagated for 10–20 generations without selection to allow time for assortment to occur, and then they were subcloned into medium containing paromomycin (100 μg/ml) to screen for drug resistance. Resistant transformants generally could be distinguished within 48 hr.

In three independent experiments, 318 cells were successfully injected with *rdnA2*, resulting in six paromomycin-resistant clones (see Table 1 for details). All these clones grow well at 100 μg of paromomycin per ml, and the two that have been tested (SL001 and SL002) grow well at 1 mg/ml. All resistant isolates exhibit full phenotypic stability. For example, after one transformant (SL051) had been propagated in the absence of paromomycin for >100 generations, 96 cells were subcloned into medium containing paromomycin; every one divided without any significant lag period.

The *Pmr* mutation in strain p2f is due to a guanine to adenine change at position 1707 of the 17S rRNA gene (12). This is the only known difference between the injected and the recipient cells' rDNA. We have used a synthetic 17-nucleotide oligomer with the *Pmr* sequence specificity to distinguish between the resident rDNA (*rdnA*$^+$) and the injected rDNA (*rdnA2*), and we determined that the transformants indeed contain the *rdnA2* allele. Whole-cell DNA was digested with *Hin*dIII, electrophoresed, blotted, and probed with the oligomer. Fig. 1*B* shows an example of the results. The oligomer hybridizes to a 2.2-kilobase band (12). From comparison of lanes 1, 9, and 10, it is clear that the probe hybridizes to both the wild-type *rdnA*$^+$ and the *rdnA2* alleles when the posthybridization washes are done in 1× SSPE (0.15 M NaCl/10 mM $Na_2H_2PO_4$/1 mM EDTA, pH 7.4) at 30°C, but it hybridizes only to the *rdnA2* allele when the stringency of washing is raised to 0.25× SSPE at 30°C.

These results provided evidence that the putative transformants contain *Pmr* rDNA. However, they did not permit us to rigorously exclude the possibility that the drug-resistant clones had all arisen by mutation rather than by transformation, since all three independent PM-R isolates studied thus far contain the same guanine to adenine base change detected here (12). To rule out this unlikely possibility, the experiments were repeated with injection of rDNA from a different strain (C3-368) that contains an extra *Bam*HI site (10, 22).

Injection of *rdnA1* Allele. Previous studies of rDNA from the C-inbred line of *Tetrahymena* (designated *rdnA1*; see ref. 10) have revealed two important and useful properties in comparison with the rDNA from the B-inbred line (both *rdnA*$^+$ and *rdnA2*): First, *rdnA1* contains an extra *Bam*HI site

Table 1. Injection of *rdnA2*

Exp.	No. of clones surviving injection	No. of PM-R clones (%)
1	118	3 (2.5)
2	170	2 (1.1)
3	30	1 (3.3)

rdnA2 is isolated from strain p2f, which is a paromomycin-resistant (PM-R) derivative of the B-inbred line. One milligram of rDNA per ml was used in the microinjections.

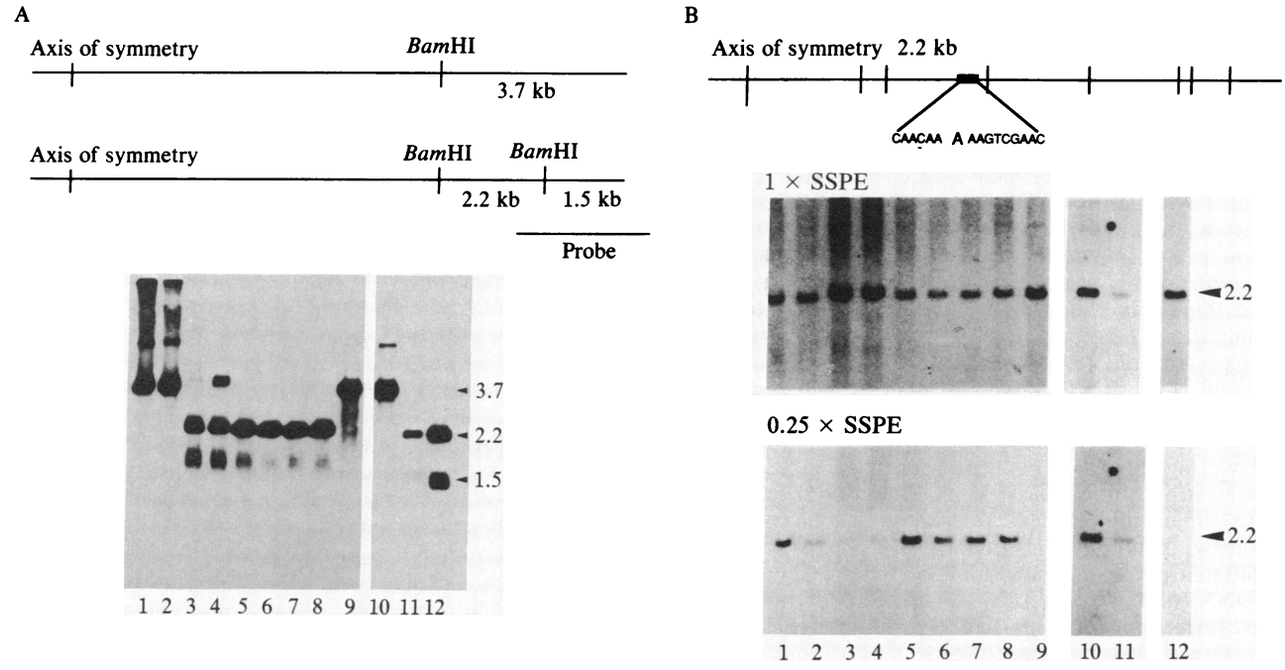

FIG. 1. Hybridization of DNA from representative transformed cells. (*A*) DNA from eight of our transformants was digested with *Bam*HI, size-fractionated on an agarose gel, blotted onto nitrocellulose, and hybridized with a telomeric probe. A map of the expected fragments is shown above the autoradiogram. DNA samples in each of the lanes are as follows: 1, DNA from logarithmic-phase SB255 cells transformed with *rdnA2*. 2, DNA from progeny of CU427 × CU428 mating cells transformed with *rdnA2*. 3 and 4, DNA from two p2f clones transformed with *rdnA1*. 5 and 6, DNA from two SB255 clones transformed with a solution of *rdnA3* (1 mg/ml). 7 and 8, DNA from two SB255 cells transformed with a solution of *rdnA3* (0.1 mg/ml). 9, DNA from CU427, which contains *rdnA*$^+$. 10, Purified *rdnA2*. 11, Purified *rdnA3*. 12, Purified *rdnA1*. All of the samples were analyzed in the same gel. (*B*) DNA from the same transformed cells as in *A* that were digested with *Hin*dIII and probed with a *pmr* probe. The probe is a synthetic 17-mer with specificity for the *rdnA2* mutation site. A *Hin*dIII map of rDNA is shown above the autoradiogram. In this map, the *Hin*dIII sites are represented by vertical lines; the sequence of the probe is also depicted with the large A indicating the mutation site. All the samples are arranged exactly as in *A*. In the first panel, the filter was washed in 1× SSPE at 30°C. Under this condition, the probe hybridizes to both the wild-type rDNA and the *Pmr* rDNA. In the second panel, the filter was washed in 0.25× SSPE at 30°C. At this stringency, the oligomer probe hybridizes only to the *Pmr* rDNA. All of the samples were from one gel. kb, Kilobases.

in the 3′ untranscribed region of the rDNA, permitting direct physical distinction between *rdnA1* and *rdnA*$^+$ (10). Second, macronuclear *rdnA1* has been shown to be dominant over *rdnA*$^+$ in replication or maintenance. In B/C3 heterozygotes the ratio of these two alleles in the macronucleus is initially 1:1, but by 50 generations after mating it has changed to >95% in favor of the *rdnA1* allele (P. Yaeger, E. Orias, D. Larson, and E. H. Blackburn, personal communication). However, *rdnA1* does not confer resistance to paromomycin.

The following experiment was designed to unequivocally demonstrate the presence of injected DNA in transformed cells, as well as to assess the effects of replicon strength on transformation efficiency. Purified rDNA from strain C3-368 was injected into the PM-R B-inbred strain p2f. Fifty-two cells were successfully injected, cloned, and propagated. After ≈15 generations of unselected growth, each clone was subcloned into paromomycin (500 μg/ml) to screen for paromomycin sensitivity. Within 24 hr one clone, SL041, showed clear sensitivity. DNA was prepared from SL041 and 33 other randomly chosen clones, cut with *Bam*HI, electrophoresed, blotted, and probed with rDNA to screen for the *rdnA1* allele. The results confirmed the presence of *rdnA1* not only in clone SL041 but also (at reduced levels) in seven other clones. Fig. 1 (lane 9) shows the expected 3.5-kilobase band of the resident rDNA (*rdnA*$^+$), lane 12 shows the expected 2.2- and 1.5-kilobase bands of the injected rDNA (*rdnA1*), and lanes 3 and 4 show examples of two transformants.

It is clear that these clones contain *rdnA1* as well as some residual *rdnA*$^+$. Thus, the injected rDNA was maintained and propagated in some recipient cells, and in one case (SL041) the presence of the injected rDNA was coupled with phenotypic transformation. In other cases, the cell populations were found to be heterogeneous; e.g., subcloning of SL045 revealed the presence of both resistant and sensitive cells. The transformation frequency in this experiment, using the criterion of rDNA retention, was 20% of the injected cells compared to the 1–3% observed above. We believe that this higher frequency is related to the replicon strength of the *rdnA1* allele.

Injection of *rdnA3* Allele. The *rdnA1* allele simplifies molecular analysis of putative transformants and increases the transformation frequency, but *Pmr* rDNA permits positive selection. To combine these features, we mutagenized strain C3-368 and isolated a paromomycin-resistant clone, SL062, to be used as a donor strain. SL062 contains rDNA that hybridizes at high stringency with the oligomer specific for the mutation present in *rdnA2*, described above. This indicates that SL062 has the same guanine to adenine base change as all previously studied *Pmr* strains.

rDNA from strain SL062 (designated *rdnA3*) was purified and injected into SB255 cells. In one experiment, 52 cells were injected and cloned. Upon selection at 100 μg of paromomycin per ml, eight transformants were identified. The frequency of transformation to paromomycin resistance in this and other similar experiments was ≈15%—essentially the same as that detected by hybridization with unmutagenized *rdnA1*. Analysis of DNA from two transformants, SL081 and SL082, in parallel with purified *rdnA3* and *rdnA1* is presented in Fig. 1 (lanes 5, 6, 11, and 12, respectively). The results clearly show both the presence of the *Bam*HI fragments characteristic of *rdnA1*, and the *Hin*dIII fragment, which hybridizes with the 17-mer specific for the *Pmr* rDNA. A point to be stressed is that in these cases the injected rDNA (*rdnA3*) has totally replaced the resident rDNA (*rdnA*$^+$).

Longer exposures of Fig. 1 did not show any trace of the 3.5-kilobase *Bam*HI fragment characteristic of the *rdnA*$^+$ allele. Since the injected rDNA has replaced the resident rDNA, we expect all these transformants to have a stable phenotype during their entire asexual life.

Amount of Injected rDNA Necessary for Transformation. All rDNA injection experiments described thus far were performed by injection of rDNA at 1 mg/ml. If we assume an average macronuclear diameter of 10 μm, and that each injection is $\approx$0.1 nuclear volume, then each cell should receive $\approx$0.1 pl, or 3000 copies of rDNA by injection. This is $\approx$1/3rd the number of rDNA molecules in a mature G1 macronucleus. To determine the minimum number of rDNA molecules required to obtain the observed transformation frequency, we carried out experiments in which decreasing concentrations of *rdnA3* were injected into strain SB255, and the number of transformants was scored (Table 2). It is clear that lowering the DNA concentration by a factor of 250 (resulting in injection of as few as 12 copies of *rdnA3* per nucleus) did not affect the transformation frequency. Further dilution by another 1:5 (injection of 2–3 copies), however, resulted in lowering the transformation frequency by a factor of 5. DNA from two clones (SL084 and SL085) transformed at these lower concentrations has been analyzed (Fig. 1, lanes 8 and 9), and the results are identical with those reported above for clones SL081 and SL082.

Nuclear vs. Cytoplasmic Injection. Although in the experiments described thus far we had intended to inject the nucleus, frequently the needle failed to enter the nucleus due to the thickness of the cells. Therefore, we carried out a series of experiments in which great care was taken to ensure that every cell that was permitted to survive had been injected in the nucleus. In four such experiments, logarithmic phase SB255 were injected with *rdnA2* or *rdnA3*; the results are summarized in Table 3. The transformation frequency in all cases was an unprecedented 28–47%, with all of the transformants displaying a stable phenotype.

In a complementary series of experiments, *rdnA2* and *rdnA3* alleles were deliberately injected into the cytoplasm (Table 3). With *rdnA3*, 2/96 transformants were obtained, and with *rdnA2*, there were 0/151. Thus, high efficiency transformation requires injection directly into the nucleus. It is interesting, however, that cytoplasmic injection does lead to any transformation.

Transformation of Conjugating Cells. *T. thermophila* is an ideal organism for studying genome rearrangements, since gene amplification, DNA elimination, and chromosome fragmentation all occur during specific stages of development. We have tested the transformation protocol in developing macronuclei. Cells of different mating types (CU427 and CU428) were mated in synchrony and injected with *rdnA2* or *rdnA3*. Only one of the two macronuclear anlagen in mating pairs with the expected nuclear configuration at a given time point was injected. Successful experiments have been carried out at various times in development, which span the known times of macronuclear genome rearrangements. The details are presented in Table 4. In Fig. 1 (lane 2), results of DNA analysis for a typical transformant (SL011) are presented. The following summarize the results. (*i*) The transformation frequency for *rdnA2* and *rdnA3* alleles in mating cells is the same as their respective frequencies for vegetative cells. (*ii*) All the transformants show a stable phenotype. (*iii*) As was the case with the vegetative cells, the transformed mating cells show resistance to paromomycin at 1 mg/ml.

Table 2. Injection of *rdnA3*

Concentration of *rdnA3* injected, mg/ml	Estimated* copies of rDNA	No. of clones surviving injection	No. of PM-R clones (%)
1	3000	52	8 (15)
0.1	300	60	7 (11)
0.1	300	68	7 (10)
0.02	60	53	12 (22)
0.004	12	57	10 (17)
0.004	12	39	5 (13)
0.0008	2–3	83	2 (2.4)

PM-R, paromomycin resistant.
*The estimate is based on the assumption that 0.1 pl of rDNA ($\approx$10% of the nuclear volume) is injected into each cell.

DISCUSSION

By microinjecting rDNA into the macronucleus, we are able to transform the ciliate *T. thermophila*. This conclusion is based primarily on three lines of evidence. First, when mutant rDNA is injected, the putative transformants contain the expected single base-pair change present in the donor strain. Although spontaneous mutation could produce the same result, mutation frequency under all other conditions is far too low to account for the 2–47% frequencies observed. Second, when restriction site heteromorphic rDNA is used for injection, such rDNA is recovered from putative transformants. Third, compared with nuclear injection, cytoplasmic injections lead to greatly reduced transformation frequencies. Furthermore, injection of pBR322 or other *Escherichia coli* plasmid DNA did not produce any transformants (data not shown). These observations clearly demonstrate that the phenotypic transformation is the result of the rDNA that has been injected.

The PM-R phenotypes of the transformants are indistinguishable from those of the rDNA donor cells. We attribute our success to the fact that the gene being used is from the same species, and that it normally exists and replicates as a minichromosome in the macronucleus. Assortment of the injected and the resident copies would be expected to occur, as it does for other macronuclear genes (ref. 21 and refs. therein), and should eventually lead to the formation of pure types, which should be (and in fact are) stable.

Direct DNA analysis supports the above interpretation. The bulk of the rDNA in the transformants is indistinguishable in structure from that of the donor cells. However, in the cases where the C3 types of rDNA (*rdnA1* and *rdnA3*) are injected into the B-inbred lines, the restriction fragments containing the telomeric sequences appear to lengthen slightly (Fig. 1*A*). Lengthening of telomeric sequences during vegetative growth has been observed in trypanosomes (23, 24), yeast (25, 26), and *Tetrahymena* (M.-C.Y., unpublished observations; ref. 27). What we have observed here may be a related phenomenon.

Table 3. Comparison of cytoplasmic and nuclear injection

Exp.	DNA type*	Site of injection†	No. of clones surviving	No. of PM-R clones (%)
1	*rdnA2*	Nucleus	32	9 (28)
2	*rdnA2*	Cytoplasm	151	0 (0)
3	*rdnA3*	Nucleus	20	9 (45)
4	*rdnA3*	Nucleus	10	4 (40)
5	*rdnA3*	Nucleus	17	8 (47)
6	*rdnA3*	Cytoplasm	96	2 (2)

PM-R, paromomycin resistant.
*The *rdnA2* (B-inbred line, *Pmr*) concentration was 1 mg/ml; the *rdnA3* (C-inbred line, *Pmr*) concentration was 0.1 mg/ml.
†Only the cases in which the injection sites were clearly nuclear or cytoplasmic by visual inspection were scored in these experiments.

Table 4. Injection of developing macronuclei

Exp.	DNA type	Time of injection during mating, hr	No. of clones surviving	No. of PM-R clones (%)
1	*rdnA2*	12–15	60	3 (5.0)
2	*rdnA2*	13–17	133	2 (1.5)
3	*rdnA2*	17–19	73	2 (2.7)
4	*rdnA3*	11–12	50	9 (18.0)

PM-R, paromomycin resistant.
*The concentration of *rdnA2* (B-inbred line, *Pmr*) was 1 mg/ml; the concentration of *rdnA3* (C-inbred line, *Pmr*) was 0.1 mg/ml.

In our best experiments, as high as 45% of all injected cells were stably transformed. We are not aware of any other system in which a comparable rate of stable transformation is obtained. Injection of mammalian cells has been shown to cause high frequency of transformation (15), but the transformed phenotypes are mainly transient. Again, we attribute the high frequency of stable transformation observed here to the fact that the selectable marker used is a homospecific gene that exists in the form of a minichromosome. Although this is a rather special case, it nonetheless indicates that at least 45% of the cells in a growing population of *Tetrahymena*, and perhaps other eukaryotes, are susceptible to stable transformation by our method.

Each macronucleus contains ≈9000 copies of rDNA after division. We find that injection of as few as 12 copies of the rDNA is sufficient to cause a high frequency of transformation. Although this is only a rough estimate, the actual number is not likely to be much higher. We think this property is related to the preferential replication properties of the C3 types of rDNA (*rdnA1* and *rdnA3*). It should be interesting to find out what sequences in the C3 types of rDNA are responsible for their replication property. The transformation method established here provides a useful tool.

Transformation of a different group of ciliates, the hypotrich, has been reported (28). In this case, the *E. coli* gene that confers resistance to neomycin was used as a marker and the DNA was introduced into the cell as calcium phosphate precipitates. However, the transformed phenotypes are weak and the frequencies are low. Similar approaches have not yielded unambiguous results in *Tetrahymena*. It should now be feasible to modify our approach and establish a general method for *Tetrahymena* transformation. For instance, the *rdnA3* allele could be useful for constructing *E. coli–Tetrahymena* shuttle vectors. These vectors should facilitate the study of other genes, as well as the establishment of a mass transformation method. This kind of approach could also lead to the establishment of a method for the transformation of the germline (micronuclear) genome.

We have also carried out injections of the developing macronuclear anlagen and obtained transformation frequencies similar to those of the logarithmic-phase cells. These results make it possible to apply our method to the study of events that occur specifically during development, such as gene amplification and DNA elimination. For instance, one could inject cloned chromosomal copies of the rDNA into the macronuclear anlagen several hours before amplification normally takes place and select for transformants that would presumably have generated the extrachromosomal palindromic rDNA from the marked injected copies. Using this kind of approach one might be able to determine in the end the *cis*-acting sequences required for such events as chromosomal breakage, telomere addition, palindromic sequence formation, and preferential replication (29–31).

Studies of rDNAs in general have been hampered by the fact that the genes coding for them exist as multiple-copy sequences in the nucleus of all eukaryotes and in most prokaryotes known. It is thus difficult to generate mutants with altered rDNA sequences or to replace the rDNA with engineered sequences by transformation. Our findings provide a means to this end and should facilitate the study of the most abundant RNA in the cell.

We are grateful to Dr. David Kirk for his advice and effort in setting up the microscope for microinjection. We also wish to thank Drs. D. Schlessinger for the use of his electrode puller, M. Altschuler for advice in the mutagenesis and cell manipulations, E. Slattery and D. Kirk for critical reviews of the manuscript, and Mrs. L. Wu for her technical assistance. This work was supported by a National Institutes of Health Biomedical Research Support Grant and by National Institutes of Health Grant GM26210. M.-C.Y. is the recipient of a National Institutes of Health Research Career Development Award. M.M.T. was supported by National Institutes of Health Predoctoral Training Grant GM07067.

1. Ilgen, C., Farabough, A., Walsh, J. M. & Fink, G. R. (1978) in *Genetic Engineering*, eds. Setlow, J. K. & Hollaender, A. (Plenum, New York), Vol. 1, pp. 117–132.
2. Rubin, G. M. & Spradling, A. C. (1982) *Science* **218**, 348–353.
3. Case, M., Schweizer, M., Kushner, S. R. & Giles, N. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5259–5263.
4. Hirth, K. P., Edwards, C. A. & Firtel, R. A. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 7356–7360.
5. Scangos, G. & Ruddle, F. H. (1981) *Gene* **14**, 1–10.
6. Gorovsky, M. A. (1980) *Annu. Rev. Genet.* **14**, 203–239.
7. Enberg, J. (1985) *Eur. J. Cell Biol.* **36**, 133–155.
8. Yao, M.-C. (1982) in *The Cell Nucleus*, eds. Busch, H. & Rothblum, L. (Academic, New York), Vol. 12, pp. 127–153.
9. Yao, M.-C. & Gall, J. G. (1977) *Cell* **12**, 121–132.
10. Pan, W.-C., Orias, E., Flacks, M. & Blackburn, E. H. (1982) *Cell* **28**, 595–604.
11. Bruns, P. J., Katzen, A. L., Martin, L. & Blackburn, E. H. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 2844–2846.
12. Spangler, E. A. & Blackburn, E. H. (1985) *J. Biol. Chem.* **260**, 6334–6340.
13. Orias, E., Flacks, M. & Satir, B. H. (1983) *J. Cell Sci.* **64**, 49–67.
14. Gorovsky, M. A., Yao, M.-C., Keevert, J. B. & Plegar, G. L. (1975) *Methods Cell Biol.* **9**, 311–327.
15. Orias, E. & Bruns, P. J. (1976) in *Methods in Cell Biology*, ed. Prescott, D. M. (Academic, New York), Vol. 13, pp. 247–282.
16. Capecchi, M. R. (1980) *Cell* **22**, 479–488.
17. Stacy, D. W. & Allfrey, V. G. (1976) *Cell* **9**, 725–732.
18. Din, N. & Enberg, J. (1979) *J. Mol. Biol.* **134**, 555–574.
19. Yao, M.-C. & Gorovsky, M. A. (1974) *Chromosoma* **48**, 1–18.
20. Rigby, P. W., Deickmann, M., Rhodes, C. & Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251.
21. Allen, S. & Gibson, I. (1973) in *The Biology of Tetrahymena*, ed. Elliot, A. M. (Dowden, Hutchinson & Ross, Stroudsburg, PA), pp. 289–336.
22. Pan, W.-C. & Blackburn, E. H. (1981) *Cell* **23**, 459–466.
23. Bernards, A., Michels, P. A. M., Lincke, C. R. & Borst, P. (1983) *Nature (London)* **303**, 592–597.
24. Pays, F., Laurent, M., Delint, K., Van Meirvenne, N. & Steinert, M. (1983) *Nucleic Acids Res.* **11**, 8137–8147.
25. Shampay, J., Szostak, J. W. & Blackburn, E. H. (1984) *Nature (London)* **310**, 154–157.
26. Pluta, A. F., Dani, G. M., Spear, B. B. & Zakian, V. A. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 1475–1479.
27. Larson, D., Spangler, E., Shampay, J. & Blackburn, E. (1985) *J. Cell Biol.* **101**, 73a (abstr.).
28. Wunning, I. U. & Lipps, H. J. (1983) *EMBO J.* **2**, 1753–1757.
29. Yao, M.-C., Zhu, S.-G. & Yao, C.-H. (1985) *Mol. Cell. Biol.* **5**, 1260–1267.
30. King, B. O. & Yao, M.-C. (1981) *Cell* **31**, 177–182.
31. Yao, M.-C. (1981) *Cell* **24**, 765–774.