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Genetics

Circular ribosomal DNA plasmids transform *Tetrahymena thermophila* by homologous recombination with endogenous macronuclear ribosomal DNA

(ciliate/microinjection/gene replacement)

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ABSTRACT **We transformed the ciliate *Tetrahymena thermophila* by microinjection of circular plasmids containing the ribosomal RNA gene (rDNA). In the somatic macronucleus of *Tetrahymena*, the rDNA is in the form of linear palindromic molecules. The rDNA molecules from the C3 strain have a replication advantage over rDNA from both B strain and the C3 rDNA mutant *rmm1*. We constructed two circular plasmids carrying replication origin sequences from C3 rDNA and a point mutation (*Pmr*) in the 17S rRNA gene that confers resistance to the antibiotic paromomycin. One plasmid contained a single complete copy of the rRNA gene and its flanking sequences, while the other had an additional rDNA origin of replication. In all B or *rmm1 Tetrahymena* cell lines transformed with the plasmids, rDNA sequences from the plasmid were found in palindromic rDNA molecules. In one transformant line, a small amount of the plasmid was also retained in a form with the original circular restriction map. Our results show that the plasmids underwent homologous recombination with one arm of the endogenous rDNA to give heteropalindromic rDNA, or with both arms of the palindrome to form homopalindromic rDNA. The resulting recombinant molecules were able to replace the recipient's original rDNA completely, providing strong evidence that C3 rDNA sequences in the donor DNAs confer a replication advantage over recipient rDNA. Thus microinjection of circular plasmids provides a method for replacement of an endogenous gene or gene fragment with exogenous sequences.**

The ciliated protozoan *Tetrahymena thermophila* has been an advantageous system for the study of a variety of biological questions, and it has been amenable to combined molecular, biochemical, and genetic approaches. The potential of this system has recently been expanded by the demonstration that microinjection or electroporation of the free, linear, palindromic, autonomously replicating ribosomal RNA genes (rDNA) result in DNA-mediated transformation of the cells to drug resistance (1, 2). This was shown by introduction into the macronucleus of an rDNA allele carrying the dominant selectable marker *Pmr*, which confers paromomycin resistance on the recipient cells (3).

To realize the full molecular genetic potential of *Tetrahymena*, it would be useful to have a means of transforming *Tetrahymena* by using recombinant circular plasmids that can be manipulated easily in *Escherichia coli*. Use of the naturally occurring linear macronuclear rDNA molecules as vectors is complicated by their requirement for telomeres at their termini and by the fact that these rDNA molecules are palindromic, with two gene copies in inverted orientation on each molecule (4). We therefore constructed circular nonpalindromic plasmids that can be prepared and manipulated easily in *E. coli*, and we determined whether they could be used as vectors for introduction of rRNA genes into *Tetrahymena*. Here we describe results obtained with two plasmids, each carrying a single rRNA gene, that efficiently transformed *Tetrahymena* macronuclei when microinjected. In all transformants, the plasmids recombined, apparently by homologous recombination, with the endogenous palindromic rDNA molecules of the recipient cells, generating transformants containing rDNA in its natural palindromic form. All of the recipient rDNA in the transformants was eventually replaced by the recombinant palindromes carrying the introduced plasmid sequences. In addition, one transformed cell line also maintained a small amount of plasmid sequences with an unrearranged circular restriction map.

MATERIALS AND METHODS

Cell Strains and Culture. *T. thermophila* strains SB2120 (C3-*rmm1*, *Pmr*$^+$), SB1914 (B, *Pmr*$^+$), and AL11 (*Pmr*) were all generously provided by E. Orias (University of California, Santa Barbara). The descriptions in parentheses indicate the rDNA allele in the macronucleus in each strain. AL11 rDNA is a recombinant allele carrying the replication origin region from C3 rDNA and the *Pmr* marker and downstream region from B rDNA (5). Cells carrying only the wild-type *Pmr*$^+$ rDNA are sensitive to paromomycin at concentrations at or above 100 μg/ml. *Pmr* rDNA confers resistance to paromomycin up to 1000 μg/ml. All cells were grown at 30°C in 2% PPYS [2% proteose peptone (Difco)/0.2% yeast extract (Difco)/0.003% Sequestrine (CIBA–Geigy EDTA)] in flasks, with aeration by gentle swirling on a gyratory shaker, or in 20-ml cultures in 6-cm-diameter Petri dishes.

Plasmid Construction. Plasmid pTrel was constructed as described previously and consists of a 4 kilobase-pair (kb) *Bam*HI fragment containing what we define here as the 3′ end region of B rDNA, including the telomeric (CCCCAA)·(TTGGGG) repeats, inserted between the *Bam*HI and *Pvu* II sites of pBR322 (6). To construct plasmid prD1, native palindromic AL11 rDNA (7.5 μg/ml) was "snapped back" to form half molecules (4) by boiling for 1 min and chilling rapidly on ice. The rDNA "snapbacks" were incubated with endonuclease P1 in 20 mM sodium acetate, pH 6.5, for 10 min at 37°C. This treatment digested the single-stranded DNA, derived from the central nonpalindromic sequence (7), at what we define to be the 5′ end of the rDNA snapbacks. A *Bam*HI linker was then ligated to the resulting blunt end. The 6.7-kb *Bam*HI fragment from the 5′ end of the rDNA was inserted into the *Bam*HI site of pTrel. Plasmid prD1 had the

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Abbreviations: rDNA, ribosomal RNA gene; NTS, nontranscribed spacer.

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inserted fragment in the correct orientation and thus contained a single complete colinear copy of the rDNA (one half of the palindromic molecule) (Fig. 1). Plasmid prD2, a derivative of prD1, was constructed by partial *Bam*HI digestion of prD1 and insertion of the 1.8-kb *Sau*3AI fragment from the 5′ end of the rDNA insert in prD1 into the *Bam*HI site at the 5′ end of the rDNA in prD1 (Fig. 1).

Microinjection of *T. thermophila*. The microinjection procedure was essentially the same as described by Tondravi and Yao (1). Microinjections were performed by using a Zeiss model CZG micromanipulator attached to a Zeiss IM35 inverted microscope. DNA (1 mg/ml) in TE (10 mM Tris·HCl/1 mM EDTA, pH 7.5) was injected into macronuclei with an Eppendorf model 5242 microinjector. For each microinjection experiment, about 10–20 cells were injected in small groups. An estimated $\approx 10^3$ plasmid or palindromic rDNA molecules were injected per cell. The 10–20 cells were combined and transferred to a Petri dish containing 20 ml of 2% PPYS. Further growth was carried out as described below.

Selection of Paromomycin-Resistant Cells. Serial transfers (0.6 ml) were performed daily into 20-ml cultures in 6-cm Petri dishes. Each day an aliquot (5 ml) of the unselected culture in 2% PPYS was also transferred to selective medium (2% PPYS containing paromomycin at 100 μg/ml). When growth in paromomycin was observed, cells were serially transferred to 2% PPYS containing paromomycin at 100 μg/ml, followed by transfer to paromomycin at either 300 or 1000 μg/ml. Resistance was scored only if the cells grew in paromomycin at 1000 μg/ml with doubling times similar to those of known resistant strains.

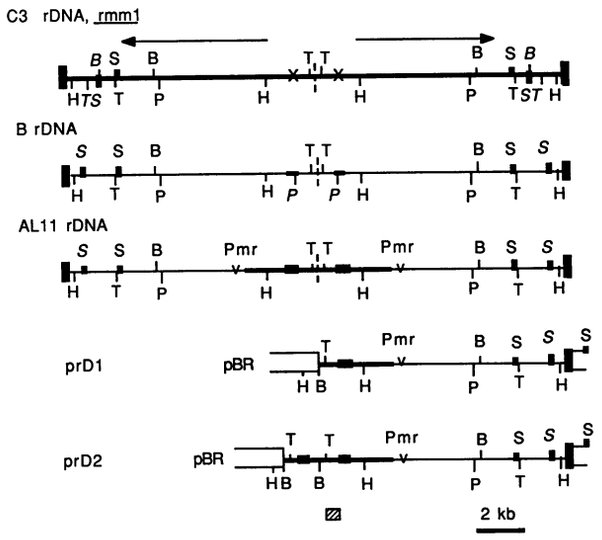

FIG. 1. Structures of donor DNAs and rDNAs of recipient cell strains used for transformation. Arrows, rRNA transcription units on the palindromic molecules. *Pmr*, dominant paromomycin resistance marker; vertical broken line, center of symmetry of the palindromic C3 *rmm1*, B, and AL11 rDNAs; vertical solid boxes, telomeric (CCCCAA)·(TTGGGG) repeats; thick lines, rDNA regions of the C3 allelic type; thin lines, B-type rDNA regions; open bars, pBR322 vector sequence in plasmids prD1 and prD2. AL11 rDNA is a recombinant allele with a replication origin region from C3 rDNA and the *Pmr* marker and 3′ spacer sequences from a B strain rDNA (ref. 5; see *Materials and Methods*); the junction point between C3 and B sequences is uncertain, but it is between domain 2 (see text) and *Pmr*. The region containing the origin of replication is shown as a thick bar for C3 rDNA and as a thinner bar for B rDNA. X, the *rmm1* mutation in the C3 domain 2. Restriction sites: B, *Bam*HI; H, *Hin*dIII; P, *Sph* I; S, *Sau*3AI; and T, *Taq* I. All *Bam*HI and *Sph* I sites are shown; only the *Hin*dIII, *Sau*3AI, and *Taq* I sites important for the analyses are shown. Sites that differ between C3 and B rDNA allelic types are italicized. Hatched bar, 5′ spacer probe.

DNA Isolation. Total cellular DNA was isolated (8) from 20-ml serial cultures of *T. thermophila* in selective medium. DNA preparations highly enriched in macronuclear rDNA were made from 2-liter cultures as described (3).

RESULTS

Microinjection of *T. thermophila* with rDNA and Plasmids. In the macronucleus of vegetatively growing *T. thermophila*, rDNA of the wild-type C3 allelic type has been shown to have a replication advantage over rDNA of both the B strain and the C3 mutant *rmm1* when both alleles are present in the same macronucleus (9). The origin of rDNA replication has been localized to a region in the 5′ NTS (nontranscribed spacer) encompassing two repeated DNA sequence elements called domains 1 and 2. DNA sequences in domain 2 have been shown by mutational and sequence analysis to be important for the replication advantage of C3 over B or *rmm1* (9). Therefore the circular plasmids prD1 and prD2 were constructed so that they contain C3-type 5′ spacers, the *Pmr* mutation, and B-type 3′ spacers (Fig. 1). Plasmid prD2 has a tandem repeat of the 1.8-kb *Sau*3AI fragment of prD1 that includes domains 1 and 2. Hence in prD2 the replication origin is present in two tandem copies. The recipient cells were either SB2120 (C3-*rmm1*, Pmr^+ rDNA in the macronucleus) or SB1914 (B, Pmr^+ rDNA in the macronucleus). The structures of the donor rDNAs and of the rDNAs of the two recipient cell lines used are shown in Fig. 1.

We carried out eight microinjection experiments, of which four were successful (Table 1). Microinjected cells were selected for paromomycin resistance by the selection scheme described in *Materials and Methods*. The number of cell generations in the transfers from injection to the first observation of a culture showing paromomycin resistance ranged from approximately 30 to 70 generations. After cells were selected in paromomycin at 100 μg/ml they were transferred to media containing higher concentrations of the drug. All the transformants were resistant to paromomycin at concentrations of up to 1 mg/ml.

Molecular Characterization of rDNA in Transformed Cells. DNA was isolated for analysis from the transformants obtained in experiments I–IV (Table 1) at different stages during the transfers after microinjection. For each experiment, four different DNA samples were prepared: sample 1, the first culture that showed resistance to paromomycin at 100 μg/ml, and the successive transfers of that culture to media containing paromomycin at 100 (sample 2), 300 (sample 3), and 1000 (sample 4) μg/ml. As three of these four samples are serial transfers (see *Materials and Methods*) they also represent a time course.

Southern blotting analysis of the DNA samples from each experiment showed that the rDNA in the transformants was all (experiments I, III, and IV), or mostly (experiment II), in the form of free linear palindromic molecules—that is, the

Table 1. Microinjection of *Tetrahymena* with rDNA and rDNA plasmids

Exp.	Donor DNA	Recipient	Days	Successful/ total
I	AL11 rDNA	SB2120	4	1/2
II	prD1	SB2120	10	1/3
III	prD2	SB2120	7	1/2
IV	prD1	SB1914	12	1/1

Donor DNAs and recipient strains are listed. The number of days before a culture showing resistance to paromomycin was observed is shown for each experiment. For each type of microinjection experiment the number of successful experiments out of the total experiments attempted is indicated.

normal macronuclear form. This was shown by digestion with *Bam*HI, *Sau*3AI, *Hin*dIII, and *Taq* I (Figs. 2–5 and data not shown) and *Xba* I (data not shown). All restriction enzymes produced the fragments expected for palindromic rDNA. For example, 13-kb, ≈4-kb, ≈4-kb, and 98-base-pair (bp) fragments, corresponding to the central *Bam*HI, *Sau*3AI, *Hin*dIII, and *Taq* I fragments respectively, are seen in Figs. 2–5.

For the microinjection experiments using SB2120 as the recipient (experiments I, II, and III), the donor DNA molecules (AL11 rDNA, prD1, and prD2) all contained B-type 3′ region sequences, while the rDNA sequences of the recipient SB2120 were of the C3 type [with the single base change of *rmmI* (9) in the 5′ spacer]. The 3′ regions of these donor and recipient rDNAs can be distinguished by their *Bam*HI and *Sau*3AI restriction patterns. *Bam*HI digestion of B rDNA gives rise to a 4-kb 3′ end fragment, while C3 rDNA has an extra *Bam*HI site near the telomere (see Fig. 1), producing

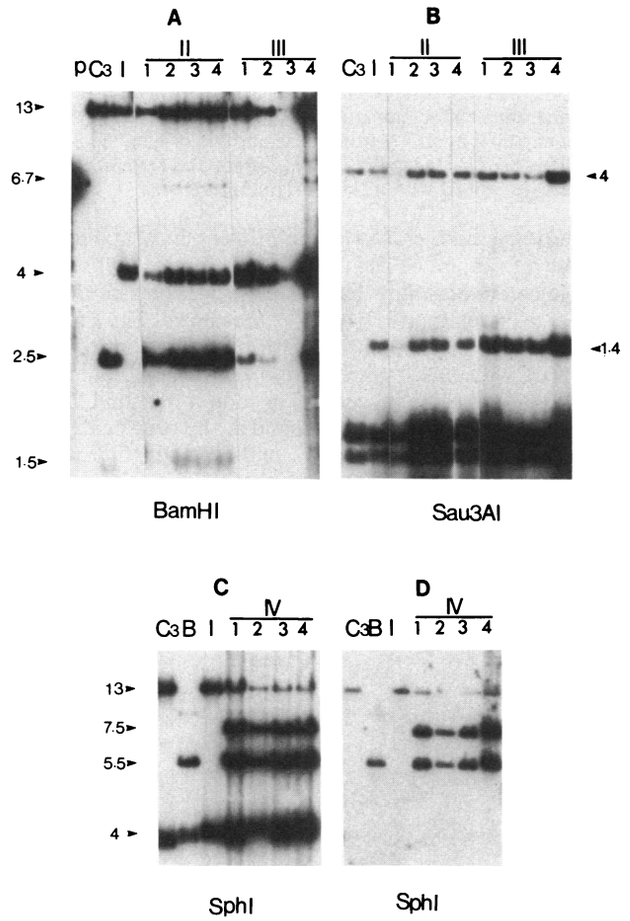

FIG. 2. Southern blotting analysis of rDNAs of transformants. Total cellular DNA of the transformants analyzed by Southern blotting and hybridized with either prD1 or the 5′ NTS probe. Lengths (in kb) of diagnostic bands are marked. (*A*) DNA of transformants from experiments I, II, and III (see Table 1) digested with *Bam*HI and probed with nick-translated entire prD1 plasmid. Lane p contains prD1 plasmid DNA, showing the single band consisting of the two 6.7-kb *Bam*HI fragments of prD1; lane C3 contains SB2120 recipient cellular DNA; lane I contains experiment I DNA 40 generations after microinjection; lanes 1–4 contain DNA samples isolated from transformant cell cultures as described in the text. (*B*) *Sau*3AI digests of the same DNA samples, probed with prD1 plasmid. (*C* and *D*) DNA of transformants from experiment IV. The DNA was digested with *Sph* I and the blot was probed with either a complete prD1 probe (*C*) or a probe specific for the 5′ NTS (see Fig. 1) (*D*). This probe hybridizes with a sequence known to be repeated on both sides of the *Sph* I site 1 kb from the center of the palindrome (9). Lanes C3 and B contain C3 and B rDNA controls; lanes 1–4 are marked as in *A*.

two bands, 1.5 and 2.5 kb, in Southern blots of *Bam*HI digests. An additional *Sau*3AI restriction site polymorphism in the 3′ spacer region (see Fig. 1) results in a 1.4-kb fragment characteristic of the B-type 3′ spacer. Southern blots of *Bam*HI and *Sau*3AI digests probed with nick-translated prD1 plasmid (Fig. 2 *A* and *B*) showed that the rDNA in the SB2120 transformants microinjected with either AL11 rDNA, prD1, or prD2 contained the B-type 3′ region. Hence, transformation to paromomycin resistance was attributable to replacement of (at least some of) the original recipient rDNA with sequences from the plasmid used in transformation.

One Transformant Contains Small Amounts of Unrearranged Donor Plasmid Sequences. In the transformant obtained by microinjection of SB2120 with prD1 (experiment II), a second rDNA species with a different restriction map from the palindromic rDNA was detected by hybridization of both rDNA and pBR322 to digested DNA, as shown in Figs. 2 and 3. The *Bam*HI, *Sau*3AI, and *Hin*dIII restriction fragments (Figs. 2*A* and 3) and the *Xba* I restriction fragments (data not shown) of this rDNA species were the same as in the original plasmid; thus this form of the rDNA retained its original circular restriction map. To determine whether a few cells had high amounts of plasmid with the circular restriction map, 22 single cells were isolated from a culture of this transformant cell line, grown for 20–25 generations, and analyzed by *Bam*HI and *Hin*dIII digestion and Southern blotting. In each of these cell sublines the fragments predicted by the circular map of prD1 were present in no more than 5% of the amounts of the palindromic fragments (data not shown). We have not determined whether this nonpalindromic form is the prD1 plasmid in its original circular form or some other form (see *Discussion*).

Palindromic rDNA of Transformants Can Be a Homopalindrome or a Heteropalindrome. In addition to differences between B and C3 rDNAs in the 3′ spacer, B rDNA has an *Sph* I site 1 kb from the center of the palindrome that is absent

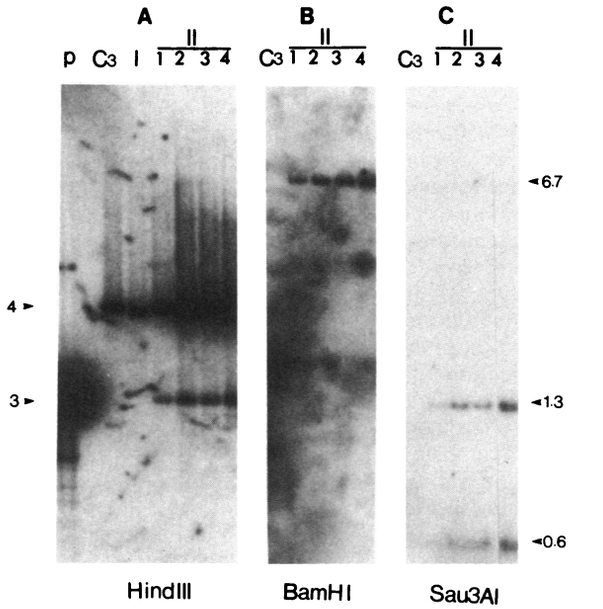

FIG. 3. Unrearranged plasmid sequences in one transformant microinjected with plasmid prD1. (*A*) Autoradiogram of *Hin*dIII digests probed with the 5′ NTS probe (see Fig. 1). The 4-kb fragment is the central fragment from palindromic rDNA. The 3-kb fragment is the same size as the band in prD1. Lane p contains plasmid prD1; lane C3 contains SB2120 (the recipient cell) DNA. (*B* and *C*) Duplicate blots of the same *Bam*HI and *Sau*3AI digests shown in Fig. 2 *A* and *B*, probed with nick-translated pBR322 plasmid. The 6.7-kb *Bam*HI fragment and the 1.3- and 0.6-kb *Sau*3AI fragments are identical in size to those of prD1.

from C3 rDNA (D. Larson and E.H.B. unpublished data), so that B and C3 homopalindromes have diagnostic 5.5- and 13-kb *Sph* I fragments, respectively (Fig. 1). In contrast, a heteropalindromic molecule with one C3- and one B-type 5′ spacer would have the 5.5-kb fragment plus a new 7.5-kb *Sph* I fragment spanning the center of the palindrome. This polymorphism was exploited in analysis of the transformants from experiment IV, in which B strain cells were microinjected with prD1 plasmid. Fig. 2 *C* and *D* shows that, in addition to the 5.5-kb *Sph* I fragment characteristic of the recipient cellular rDNA (lane B), in the transformants there is a 7.5-kb band containing 5′ spacer sequences. Because such a 7.5-kb fragment is the predicted result of replacement of only one arm of the recipient B-type rDNA palindrome with C3-type donor DNA, we conclude that the majority of the rDNA molecules in these transformants are heteropalindromes. In addition, a small amount of 13-kb fragment in these transformants indicated the presence of some C3 homopalindromes.

The analyses described above were done on cultures of transformants that had undergone an estimated 40–80 vegetative fissions after microinjection. While in experiment IV the *Sph* I analysis enabled us to show that the transformant contained heteropalindromic molecules, it was possible that the transformants from experiments I, II, and III, in which SB2120 was transformed, contained mixtures of the original recipient rDNA molecules and molecules containing donor rDNA sequences. To test this possibility, the transformant cells from all four experiments were grown for additional periods in medium containing paromomycin at 100 μg/ml. In three serial transfers, the cells passed through an additional ≈60 generations of exponential growth, which should be sufficient to allow C3 rDNA to completely take over B rDNA in cells containing a mixture of B and C3 rDNA (9). Thus, after these transfers the transformant cell lines should contain only donor DNA or its derivatives.

DNA highly enriched for rDNA was prepared from these cells, digested with restriction endonucleases, and subjected to Southern analysis. In the transformant line from experiment IV, *Sph* I analysis showed that the C3 homopalindromes increased in amount compared with heteropalindromes during the additional ≈60 cell generations, and after a further ≈60 generations the homopalindromes became the major species (data not shown). In the transformants from microinjection of SB2120 cells, *Bam*HI and *Taq* I restriction fragment length polymorphisms in the rDNA 3′ spacer were used to identify B- and C3-type, and hence donor-derived and recipient-derived, rDNA sequences. An extra *Taq* I site in the C3-type 3′ spacer results in diagnostic 2.3- and 1.0-kb fragments in B and C3 rDNA, respectively (see Fig. 1). Fig. 4 shows that after ≈60 cell divisions the transformant from microinjection of SB2120 with prD1 (experiment II) still contained equivalent amounts of both B- and C3-type bands. This result is consistent with the rDNA molecules in this transformant being heteropalindromic. A small amount of the 6.7-kb *Bam*HI fragment from the nonpalindromic rDNA form is also seen in Fig. 4, lane II. In contrast, the rDNA in the transformant resulting from microinjection of SB2120 with prD2 (experiment III) contained only the B-type 3′ region (Fig. 4, lane III), indicating that these molecules are homopalindromes.

Transformed Cell Lines Result from Homologous Recombination of Microinjected rDNA. The finding that homopalindromic rDNAs were formed in experiments III and IV led us to investigate whether these transformant rDNAs were the product of processing of the plasmid to form a palindrome or whether homologous recombination between endogenous rDNA and the plasmid had occurred.

During the development of the macronucleus, a single rDNA copy is excised from the progenitor micronuclear chromosome and converted into free linear palindromic rDNA (ref. 10; reviewed in ref. 11). To test whether the microinjected plasmids had been processed in a similar manner in experiments III and IV, the central *Taq* I fragment of the rDNA of all four transformants was purified and compared with this fragment from the original palindromic rDNA of the recipient cells. The donor plasmid DNA is missing 44 bp of the sequence found at the very center of the palindromic rDNA (see Figs. 1 and 5*B* and *Materials and Methods*). Thus, we predicted that if the plasmid were processed to form a palindrome, the size and sequence of the

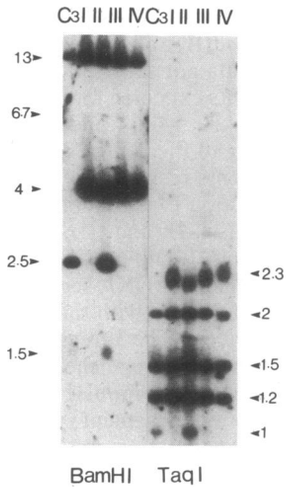

FIG. 4. Analysis of transformant rDNAs after prolonged vegetative growth. The transformant cells from experiments I–IV were grown in 2% PPYS with paromomycin at 100 μg/ml for an additional ≈60 generations after the analyses shown in Figs. 2 and 3. Preparations enriched in rDNA from these and the original SB2120 cells (lane C3) were digested with *Bam*HI (*Left*) or *Taq* I (*Right*), Southern blotted, and probed with nick-translated prD1.

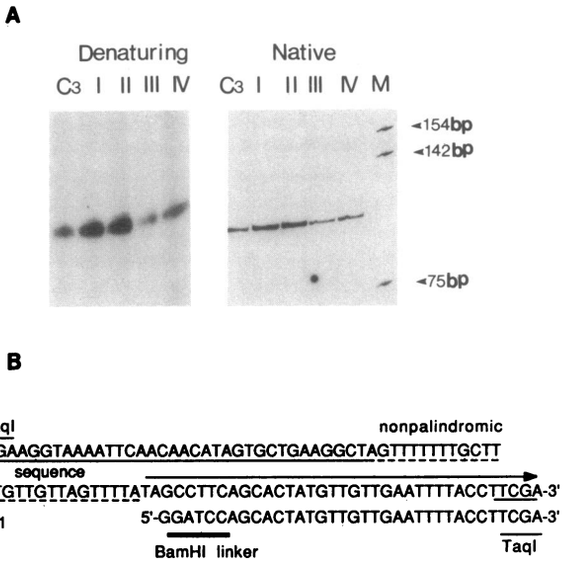

FIG. 5. The central sequence of the rDNA in transformants is indistinguishable from that of recipient rDNA. (*A*) The 5′-end-labeled central *Taq* I fragment from each of the same DNA preparations shown in Fig. 4 analyzed by electrophoresis in denaturing (*Left*) and nondenaturing (*Right*) 5% polyacrylamide gels. Double-stranded DNA size markers (M) are shown for the nondenaturing gel. (*B*) Sequence of the central *Taq* I fragment of natural palindromic rDNA (top two lines), and of the corresponding region at the 5′ end of plasmids prD1 and 2 (bottom line) are aligned. Conserved inverted repeats (12) (arrows) are shown flanking the central nonpalindromic sequence (broken underline) (7).

central *Taq* I fragment in the transformants would be different from that of the recipient rDNA. However, the central *Taq* I fragment from each transformant, when 5′ end-radiolabeled and analyzed by native and denaturing polyacrylamide gel electrophoresis (Fig. 5*A*), was identical in migration properties to the central 98 bp *Taq* I fragment of the recipient rDNA. In addition, all other restriction analysis of the palindromic rDNA of each transformant indicated that it is completely colinear with the recipient rDNA, and hence identical to it in size and form (Fig. 2 and data not shown). Together, our results provide strong evidence that the injected plasmids recombined with endogenous rDNA by homologous recombination.

DISCUSSION

We have shown here that *Tetrahymena* can be efficiently transformed by microinjection of circular plasmids. The transformed cells all contained palindromic rDNA molecules carrying rDNA sequences from the microinjected circular plasmids. These molecules eventually completely replaced the original recipient rDNA. Although in some transformants homopalindromes were formed, the results shown in Fig. 5 argue strongly against processing of the injected plasmids to form palindromic molecules. Instead, our results are most consistent with homologous recombination between plasmid rDNA sequences and the endogenous palindromic rDNA of the recipient cells.

Although the numbers of experiments were small, transformation frequencies by plasmid microinjection are estimated to be comparable to those in a previous report (1), when rDNA was microinjected as palindromic molecules. In an additional experiment, 70 single cells were isolated after rDNA plasmid microinjection. Of the 48 surviving cells, 13 were transformed by the same type of homologous recombination as in experiments II to IV (data not shown). We have no information on whether the presence of two C3 origins in prD2 had any effect on its efficiency of transformation compared with prD1. In one of the three plasmid transformation experiments, a small amount of the prD1 plasmid was maintained stably through many vegetative cell divisions with its original restriction map. We have not determined whether this was circular prD1, prD1 linearized at many different points around the plasmid, or prD1 integrated by homologous recombination into the rDNA palindrome.

The results presented here provide strong evidence for the replication advantage conferred on palindromic rDNA by cis-acting sequences in the origin region of wild-type C3 rDNA. Since all the rDNA coding and transcribed-spacer regions that have been sequenced are identical between wild-type C3 and B rDNAs (3), the more polymorphic 5′ or 3′ spacer regions are the likely candidates for variable sequences. Because the donor rDNAs had C3 rDNA sequences only in the 5′ and not the 3′ spacer, these 5′ sequences must be sufficient for the replication advantage over B and *rmm1* rDNAs. This conclusion is reinforced by the finding that, in experiment IV, after prolonged vegetative growth the proportion of homopalindromes with two C3-type 5′ spacers increased relative to the heteropalindromes with one C3- and one B-type 5′ spacer.

Various pathways leading to the observed recombinant palindromes can be envisaged. One example of a simple pathway that would generate a heteropalindrome would begin with a single crossover between homologous rDNA sequences in the plasmid and endogenous rDNA, to integrate the plasmid into the linear rDNA. This could be followed by gene conversion out to the rDNA telomere, or by an intramolecular reciprocal recombination event, resulting in replacement of recipient rDNA sequences with plasmid rDNA sequences, and excision of the reciprocal circular product. Alternatively, the telomeric repeats in the integrated plasmid could be processed to form a telomere, with loss of the distal recipient rDNA sequences.

Because the plasmids were injected in circular form, we have no evidence to show whether the gene replacement process was initiated by a double-stranded break, as has been proposed in yeast (reviewed in ref. 13). One crossover must have taken place 5′ to the rDNA origin region, replacing the *rmm1* or B sequences with the donor C3 sequence in this region, to account for the replication properties of the recombinant rDNAs. Specifically, we infer that this crossover occurred in the ≈1-kb region 3′ to the nonpalindromic sequence at the center of the palindrome and 5′ to domain 2, which differs between C3, B, and *rmm1* rDNAs (9). In experiment IV this crossover occurred 5′ to the *Sph* I site located on the 5′ side of domain 2. In each transformant, gene replacement by donor sequences extended through the *Pmr* marker to the 3′ end of the rDNA, since the *Bam*HI, *Taq* I, and *Sau*3AI sites, located 1.2, 1.0, and 0.4 kb, respectively, from the telomeric (CCCCAA)·(TTGGGG) repeats, were all of the donor type. The same results have been found in five additional transformant cell lines (data not shown). This was unexpected, because a second crossover anywhere in the 7-kb region 3′ to the *Pmr* marker would generate paromomycin resistance. These results suggest that either resolution of a Holiday intermediate after gene conversion or a second reciprocal crossover occurred at or near the telomere. Thus the telomeric repeats may be a hot spot for either of those events or for processing as described above. In experiments III and IV, formation of a heteropalindrome could have been followed by mitotic recombination of the recombinant arm of the palindrome with the other arm and gene conversion, forming a homopalindrome.

Introduction of rDNA plasmid prD2 into *Tetrahymena* by electroporation has also resulted in formation of recombinant palindromes (G.-L.Y., J. Karttunen, and E.H.B., unpublished work). Thus, integration of introduced DNA sequences by homologous recombination, resulting in gene replacement, may prove generally useful in *Tetrahymena* for targeted insertion of genes.

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