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Accurate Processing and Amplification of Cloned Germ Line Copies of Ribosomal DNA Injected into Developing Nuclei of *Tetrahymena thermophila*

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The ciliate *Tetrahymena thermophila* contains a chromosomally integrated copy of the rRNA genes (rDNA) in its germinal (micronuclear) genome. These genes are excised from the chromosome through a process involving site-specific DNA breakage, become linear palindromic molecules with added telomeres, and are greatly amplified during development of the somatic nucleus (macronucleus). In this study, we cloned a 15-kilobase segment of the germ line DNA containing these genes and injected it into developing macronuclei of *T. thermophila*. Up to 11% of injected cells were transformed to the paromomycin-resistant phenotype specified by the injected DNA. Transformation efficiency was dependent on the developmental stages of the injected cells and the integrity of the injected DNA but not the DNA concentration or conformation. The injected DNA was apparently processed and amplified correctly to produce rDNA molecules with the expected linear palindromic structure which carried the appropriate physical markers. Thus, the 15-kilobase DNA contained all *cis*-acting sequences sufficient for the DNA-processing events leading to rDNA amplification in *T. thermophila*.

The genomes of some eucaryotes are known to undergo specific alterations during development. Processes such as gene amplification (reviewed in reference 23), DNA elimination and fragmentation (reviewed in references 25 and 26), and sequence rearrangements (reviewed in reference 6) are among the better-known examples. The molecular details of these processes are not fully understood. Two of these events, gene amplification and site-specific chromosome breakage, are known to occur in the ciliated protozoans of *Tetrahymena* spp. and drastically alter the organization of the rRNA genes (rDNA) (reviewed in reference 29). *Tetrahymena thermophila* contains two nuclei: a macronucleus responsible for most vegetative functions, and a micronucleus, which maintains the genetic continuity of the species (reviewed in references 7 and 22). The diploid micronucleus contains one copy of the rRNA genes on each copy of chromosome II (31). These genes are amplified when a micronuclear derivative is differentiated into a macronucleus during conjugation. In each mature macronucleus, there are roughly 45 copies of most other genomic sequences and approximately 18,000 copies of the rRNA genes (32). This macronuclear rDNA is organized into 21-kilobase (kb) linear palindromic molecules, each consisting of two inverted copies of these genes (12, 15, 18; Fig. 1). This molecule also contains telomeric sequences not present in the micronuclear rDNA (5, 17) Thus, developmental processing of rDNA in *T. thermophila* involves the formation of an extrachromosomal molecule, the formation of an inverted dimer, the addition of telomeric sequences, and preferential replication (Fig. 1; reviewed in reference 29).

The micronuclear rDNA is excised from the chromosome for amplification through a mechanism involving specific DNA breakage at sites near the two ends of the rDNA (28, 35). Site-specific breakage is also known to occur at 50 to 200 other sites in the *Tetrahymena* genome (2, 11, 33). This is a common phenomenon among many ciliates (reviewed in references 14 and 19) and may be similar to the chromosome fragmentation process associated with chromatin diminution in nematodes of the genus *Ascaris* (reviewed in reference 26). The developmental regulation of this process is not known. Studies of *Tetrahymena* DNA sequences have revealed a common 15-base-pair (bp) sequence (referred to as Cbs) near the two ends of the micronuclear rDNA (35) as well as five other breakage sites (34). Whether this sequence actually serves as the recognition signal for chromosome breakage remains to be determined.

In this study, we developed a system for testing directly the *cis*-acting sequences required for *Tetrahymena* rDNA processing and present evidence that a 15-kb DNA fragment including the rDNA is a sufficient substrate for all in vivo events leading to rDNA amplification, including site-specific breakage and palindrome formation. Taking advantage of the recently established method of *Tetrahymena* transformation (27), we introduced into developing macronuclei of conjugating cells copies of cloned micronuclear rDNA by microinjection and obtained transformed lines which expressed the phenotype specified by the injected rDNA. These foreign micronuclear rDNAs were processed and amplified accurately and became the predominant species of macronuclear rDNA in the transformed lines.

MATERIALS AND METHODS

Cells and growth conditions. *T. thermophila* strains from inbreeding line B were obtained originally from Eduardo Orias (strain SB255) and Peter Bruns (strains CU427 and CU428). Strains SL061 and SL062 were derived from a mating between CU427 and a strain of the C3 inbreeding line, C3-368, after mutagenesis as described earlier (27). Cells were maintained and grown in axenic media as described earlier (13).

DNA cloning. *Tetrahymena* micronuclei were isolated from strain SL062, and DNA was extracted by a method previously described (32). The micronuclear DNA was di-

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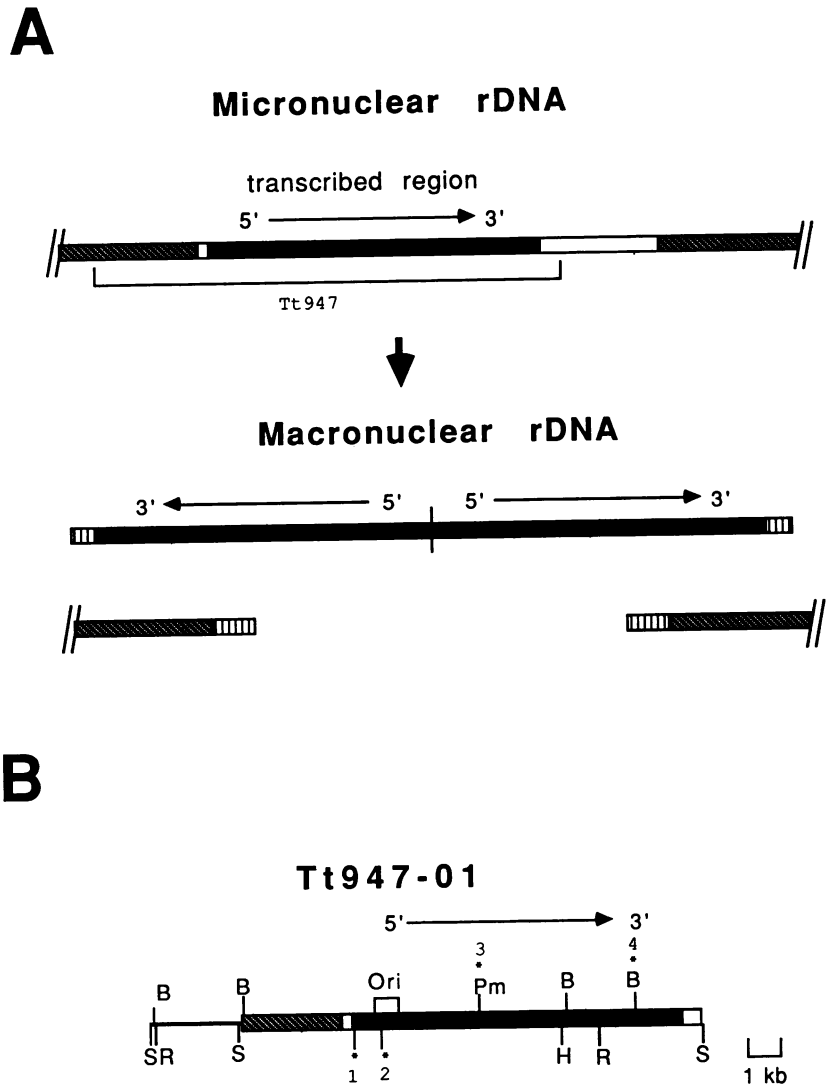

FIG. 1. Structure of *Tetrahymena* rDNA. (A) Structures of the micro- and macronuclear rDNAs of *T. thermophila*. Symbols: ▬, rDNAs; ↓, transcribed regions; ▭, immediate flanking sequences of the rDNA which are eliminated from the macronucleus; ▨, distal flanking sequences retained in the macronucleus; ▥, telomeric C_4A_2 repeats. The region of the micronuclear DNA cloned in Tt947 is indicated. (B) Linearized map of the circular micronuclear rDNA clone Tt947-01. Symbols for rDNA and flanking sequences are as described for panel A. The line to their left represents the plasmid vector DNA. Restriction sites for *Bam*HI (B), *Eco*RI (R), *Sph*I (H), *Sal*I (S), and *Sma*I (M) and approximate locations of the replication origin (Ori) and paromomycin resistance site (Pm) are indicated. Asterisks indicate known heteromorphic sites between *rdnA3* and *rdnA*$^+$ used in this study. The first (1) is a single-base-pair substitution at nucleotide position 71 detectable by oligonucleotide hybridization; the second (2) is a *Sph*I site which is present in *rdnA*$^+$ but not in *rdnA3*, which is the allele present in this clone; the third is the paromomycin resistance site, which is paromomycin sensitive in *rdnA*$^+$; and the fourth is the *Bam*HI site, which is missing from *rdnA*$^+$.

gested partially with *Mbo*I. Fragments of 10 to 20 kb in size were isolated with NaCl gradients and used to construct a recombinant DNA library by insertion into the *Bam*HI site of the bacteriophage lambda vector EMBL3. Approximately 10^5 clones were screened by hybridization in duplicate, using pTt506-4.4 and pTt220a as probes. pTt506-4.4 contains the 0.8-kb *Taq*I fragment flanking the 5′ end of the micronuclear rDNA (35). pTt220a contains a 4-kb *Eco*RI fragment including the 3′ end and the flanking region of the micronuclear rDNA. Three clones were found to hybridize with both probes and were further analyzed by restriction mapping. Two of these clones, Tt905 and Tt947, contained the two *Bam*HI sites expected for *rdnA3*, and the other contained the single *Bam*HI site expected for *rdnA*$^+$. Tt947 contained both 5′- and 3′-flanking sequences in addition to the rDNA. The insert from this clone was removed by digestion with *Sal*I and inserted into the *Sal*I site of the plasmid pIC19H, a derivative of pUC19, to generate Tt947-01.

***Tetrahymena* transformation.** Injection of *T. thermophila* was carried out by a method described previously (27). A Zeiss IM35 microscope equipped with phase-contrast optics was used for most of the study. Vegetative cells were grown to late log phase (approximately 2×10^5 cells per ml) before injection. Mating cells were prepared by mixing prestarved CU427 and CU428 and fed with an equal volume of enriched peptone medium (13) 1 to 2 h before injection. In several experiments, matings were initiated by terminating the shaking of premixed cells of different mating types in 10 mM Tris

solution (pH 7.4). Pairing occurs approximately 1 h sooner in this type of procedure (8). For better comparison, the time at which shaking terminated was treated as 1 h after mating began. Only one of the two macronuclear anlagen in each conjugating cell was injected. In most cases, the mating efficiencies were between 80 and 90%. Cells that failed to mate were easily recognized under the microscope and were not injected. Both injected and noninjected (unmated) cells were cloned into approximately 20 μl of the enriched peptone medium and incubated for 3 days, at which time most clones had grown to saturation. Most mating pairs had separated by the time of injection or cloning. However, a small number stayed together and were cloned into single wells. Normally about 20% of the clones failed to grow, probably because of handling or inability of some pairs to produce viable progeny. The viable clones were replicated to NEFF medium (0.25% protease peptone, 0.25% yeast extract, 0.5% dextrose, 0.333 mM $MgSO_4$, 0.167 mM $CaCl_2$, 0.0333 mM $FeCl_3$) containing 100 μg of paromomycin per ml. Sensitive cells were killed in 1 to 2 days, and the resistant clones grew to saturation in 2 to 3 days. The rate of transformation was calculated on the basis of the number of resistant clones as a percentage of injected cells. Since not all injected cells gave viable clones and some injected pairs were grown as single clones, these values should be slightly lower than the percentages of viable injected cells that became drug resistant.

DNA analysis. The transformed cells were transferred to 10 to 50 ml of enriched peptone medium containing 100 μg of paromomycin per ml and grown to near saturation. DNA was isolated from these cells by a method described earlier (3). Restriction enzyme digestion, agarose gel electrophoresis, Southern blotting, and DNA hybridization were carried out by standard methods (3). Oligonucleotides were synthesized in an Applied Biosystems automated DNA synthesizer at this institution.

RESULTS

Cloning of micronuclear rDNA. Two genetic components of *T. thermophila* rDNA are relevant to this study: a site in the 17S rRNA-coding region which confers resistance to paromomycin in some mutants (9, 24), and the replication origin, which is probably responsible for the interallelic competition in replication (20, 27). The allele *rdnA3* contains the paromomycin-resistance marker and the C3 replication origin, which is dominant over the B origin of the paromomycin-sensitive allele *rdnA*$^+$ (20, 27). Injection of only a few copies of the *rdnA3* macronuclear rDNA into an *rdnA*$^+$ homozygote results in high rates of transformation and complete replacement of the host rDNA in the transformed lines (27). *rdnA3* and *rdnA*$^+$ also differ in other parts of their sequences, which can be distinguished by restriction enzyme digestion or oligonucleotide hybridization (see below).

To clone the micronuclear copy of *rdnA3*, we prepared a micronuclear DNA library from an *rdnA3 rdnA*$^+$ heterozygote (SL062). The library was screened by hybridization with two separate probes, one containing the 5′-flanking sequence and the other containing the 3′ portion and flanking sequence of a micronuclear *rdnA*$^+$ clone previously isolated (28, 35). Three clones that hybridized with both probes were isolated. Two were found to contain the *Bam*HI site which distinguishes *rdnA3* from *rdnA*$^+$ (Fig. 1); one of these two, Tt947, appeared to contain an intact copy of the rDNA plus 3.4 kb of the 5′-flanking and 0.5 kb of the 3′-flanking sequences. Figure 1 shows a restriction map of this clone. Its structure agrees well with the known structure of *Tetrahymena* micronuclear rDNA (28, 35). The *Tetrahymena* DNA insert in Tt947 was recloned into plasmid pIC19H to generate clone Tt947-01, which was used for the rest of the study.

TABLE 1. Transformation of *T. thermophila* with micronuclear rDNA

Developmental stage, or time (h) in mating	No. of cells transformed/ no. injected	% Transformation
Vegetative[a]	0/198	<0.5
Vegetative	0/268	<0.4
Vegetative[a]	0/104	<1.0
Vegetative[a]	0/153	<0.7
3.3	0/166	<0.6
4	0/80	<1.3
9.3[b]	5/177	2.8
10.5	17/204	11.3
11	8/83	9.6
11.4[b]	3/154	1.9
11.5	3/128	2.3
12	2/212	0.9
13	1/176	0.6
14	0/143	<0.7
16	2/114	1.8
21	0/260	<0.4

[a] Uncut DNA was used for injection.
[b] Cells were not refed before injection.

Injection of the micronuclear rDNA. Previous studies have shown that injection of native macronuclear rDNA of the allele *rdnA3* into macronuclei of a *rdnA*$^+$ homozygote results in transformation at rates up to 66% (16, 27). We therefore tested the ability of the cloned micronuclear rDNA to transform vegetatively growing *T. thermophila*. Tt947-01 DNA was microinjected into the macronucleus of an *rdnA*$^+$ homozygote strain (SB255) during vegetative growth, using previously established methods (27). The injected cells were cloned, grown for 3 days, and tested for drug resistance. No transformants were obtained in four different attempts using either supercoiled DNA or DNA digested with *Sal*I, which cuts at the junctions between the *Tetrahymena* DNA insert and the vector DNA (Table 1). This result is in direct contrast to those obtained previously by using native macronuclear rDNA for injection. The cloned micronuclear rDNA contained essentially all of the sequence information present in the native macronuclear rDNA but did not contain telomeric DNA, was not palindromic, and was methylated differently. In addition, it contained flanking sequences that are not normally present in the macronuclear rDNA. Any of these differences, except perhaps the difference in methylation (36), could have contributed to the failure of transformation by the cloned micronuclear rDNA.

We next tested the transformation by using conjugating cells. Two strains homozygous for *rdnA*$^+$ (CU427 and CU428) were induced to mate synchronously and harvested at various time points for injection with *Sal*I-digested Tt947-01 DNA. Injection was directed at the developing macronuclei (or meiotic nuclei in the early time points). The injected cells were cloned and tested for paromomycin resistance (described in Materials and Methods). Unlike injections of vegetative cells, injections of mating cells led to significant transformation (Table 1). The transformed cells were resistant to more than 500 μg of paromomycin per ml, essentially identical to the mutant strain containing *rdnA3* (SL062) (data not shown). The transformation frequencies ranged from 1 to 11% when injections were performed between 8 and 18 h

TABLE 2. Effect of DNA concentration and conformation on transformation

DNA concn (μg/ml)	*Sal*I cut	No. transformed/ no. injected	% Transformation
500[a]	+	30/627	4.8
25	+	3/132	2.3
10	+	25/372	6.7
80	−	13/151	8.6
80	−	4/131	3.1
8	−	7/189	3.7
1.6	−	3/186	1.6

[a] Combined results from four experiments shown in Table 1, in which refed cells were injected between 10 and 12 h after mating began.

after mating was initiated. The highest rates (9.6 and 11.3%) were obtained among cells injected 10 to 12 h after mating began. This is approximately the time when genome reorganization occurs in this species (12 to 14 h in starvation buffer after mating begins; 3; unpublished observations). Injection outside of this 2-h period gave lower rates of transformation. Injection at a much later time point (21 h) did not lead to transformation. Therefore, the rates of transformation were related to the developmental stages of the injected cells, being highest among cells injected around the time of genome reorganization, which suggests a relationship between transformation and genome reorganization. These results agree with the interpretation that the injected micronuclear rDNA is processed, amplified, and expressed properly to give the transformed phenotype. The lower rates of transformation obtained from cells injected outside of the 2-h period may be significant and suggest that the machinery for amplification is operative, although less efficiently, even after the normal genome reorganization processes are completed. However, the slight degree of asynchrony in mating among the injected cells complicates this interpretation.

Transformation frequency is independent of the concentration or conformation of injected DNA. In the experiments described above, approximately 500 μg of DNA per ml was used for injection, which would give roughly 2,500 copies of molecules in a nucleus if the injected volume is 10% of the nuclear volume. This is far greater than the four to eight copies of micronuclear rDNA normally present in these developing nuclei (1, 3). To determine whether the injected rDNA was in excess, various dilutions of either uncut or *Sal*I-digested Tt947-01 DNA were used for injection (Table 2). Within 300-fold dilutions, no significant difference in transformation was detected. In addition, both linear and supercoiled DNA molecules transformed the cells at similar rates (Table 2). These results imply that molecules of either conformation are good substrates for the processing events leading to transformation unless interconversion of these two forms occurs readily after injection.

Transformation requires intact rDNA. To further explore the nature of the transformation process, Tt947-01 DNA was digested with *Eco*RI or *Bam*HI and used for injection. These enzymes cut once in the transcribed region of the rDNA and once (*Eco*RI) or three times (*Bam*HI) in the rest of the plasmid DNA. In either case a large fragment containing most of the rDNA sequence was produced, which by itself was not sufficient for generating normal rDNA because of the lack of the 3′ end sequences but carried the necessary genetic markers for converting the host rDNA into the paromomycin-resistant and replication-dominant molecule by a process such as recombination or gene conversion. In three different attempts, no transformation was detected by using *Eco*RI- or *Bam*HI-digested DNAs for injection, whereas DNA digested with *Sal*I or *Sma*I, which do not cut within the rDNA, was capable of transformation (Table 3). Therefore, efficient transformation depended on the injection of intact rDNA.

TABLE 3. Transformation with fragmented DNA

Cutting enzyme	No. transformed/ no. injected	% Transformation
*Sal*I[a]	30/627	4.8
*Sma*I	5/184	2.7
*Eco*RI	0/208	<0.5
*Eco*RI	0/222	<0.5
*Bam*HI	0/145	<0.7

[a] Combined results from four experiments shown in Table 1, in which refed cells were injected between 10 and 12 h after mating began.

Macronuclear rDNA of the transformed cells. To determine whether the injected rDNA was properly processed, structures of the macronuclear rDNA of the transformed cells were determined (Fig. 2). DNA from 12 transformed lines grown for 22 to 25 doublings in the presence of paromomycin was digested with *Bam*HI, subjected to agarose gel electro-

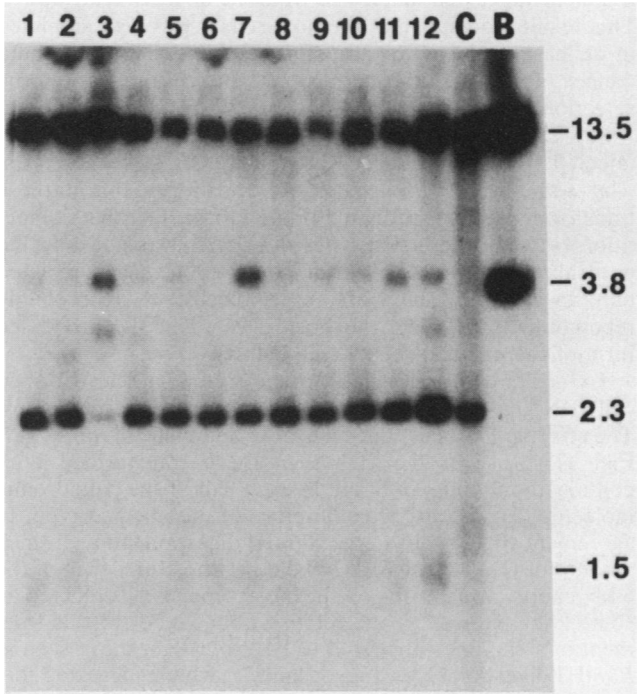

FIG. 2. *Bam*HI digestions of DNAs of transformed lines. Whole-cell DNAs (2 to 5 μg) isolated from 12 independent paromomycin-resistant transformed lines were digested with *Bam*HI, separated in a 1.0% agarose gel, blotted onto a nitrocellulose filter, and hybridized with the rDNA clone Tt947, which was labeled with ^{32}P by nick translation. Lanes 1 through 12 contain transformed-cell DNA; lanes C and B contain control DNAs from strains SL062 and CU427, respectively. SL062 contains the macronuclear form of the injected rDNA (*rdnA3*), and CU427 contains the host rDNA (*rdnA*$^+$). Numbers on the right indicate estimated sizes (in kilobases) of the detected DNA fragments. The 3.8-kb fragment in CU427 was cleaved to form the 2.3- and 1.5-kb fragments found in SL062. Most of the rDNAs in the transformed cells were similar to those found in SL062, which suggests that the injected micronuclear rDNA was accurately processed and amplified in these cells.

phoresis, and hybridized with a probe containing the entire rDNA sequence (Tt947). The major form of rDNA found in all transformants was identical to the rDNA in SL062 (13.5-, 2.3-, and 1.5-kb bands). The top 13.5-kb band contained the center fragment of the palindromic macronuclear rDNA. The bottom 1.5-kb band contained the terminal fragments. The broadness of this band was characteristic of the heterogeneous-sized telomeric DNA of *Tetrahymena* cells (5, 28). This fragment was slightly smaller in the transformants than in SL062, probably because of telomere length variation (27). The presence of the 1.5- and 2.3-kb fragments indicated the presence of the extra *Bam*HI site specific to *rdnA3*. Thus, the transformed cells contained typical macronuclear rDNA carrying the paromomycin resistance marker and the *Bam*HI site marker of the injected molecule. This evidence strongly supports the argument that the injected micronuclear rDNA is processed through site-specific DNA breakage, telomere formation, palindrome formation, and selective amplification to give rise to the mature macronuclear rDNA responsible for the drug-resistant phenotype. Although the rDNA contents of these transformed lines have not been accurately measured, they appear similar to that of normal cells.

In some transformed lines, a 3.8-kb *Bam*HI fragment was also detected (e.g., Fig. 2, lanes 3 and 7). The size of this fragment suggested that it was from the host rDNA (*rdnA*$^+$). This result indicates that the host rDNA was also amplified in at least some of the transformed lines. The low abundances of rDNA in these lines probably resulted from drug selection and rDNA allelic competition during growth, which can lead to replacement of the host *rdnA*$^+$ by the injected *rdnA3*, as observed previously (16, 27). The reason why some *rdnA*$^+$ molecules were still present in these transformed lines is probably related to the fact that competition between these two types of rDNAs was not detected until about 20 doublings after conjugation was completed (20). In addition, some of these molecules were probably recombinants between the host rDNA and the processed form of the injected rDNA (see below).

To further determine their genetic origins, the transformed cell rDNAs were analyzed at two other polymorphic sites. The enzyme *Sph*I cuts once in *rdnA3* and twice in *rdnA*$^+$ (17; Fig. 1). Most of the rDNA in these transformed lines contained only one *Sph*I site in each half of the palindromic molecule (Fig. 3) and was therefore of the injected type. In agreement with results of the *Bam*HI digestion study, a small amount of rDNA containing two *Sph*I sites (presence of the 6-kb band), apparently of the host type, was detected in some lines. However, the relative abundances of this fragment in some lines appeared to be lower than those seen in *Bam*HI digestion (e.g., Fig. 3, lane 7), which suggested that some molecules probably contained the host-type *Bam*HI site and the injected-type *Sph*I site. This type of molecule could have been produced through recombination between the host rDNA and the processed version of the injected rDNA at the interval between the *Bam*HI and the *Sph*I heteromorphic sites. The other polymorphic site examined was at nucleotide position 71 (from the 5′ end of the rDNA sequence), which is a C in the host rDNA and an A in the injected rDNA (Fig. 1; unpublished observation). An oligonucleotide (20-mer) containing the *rdnA3* version of this sequence was synthesized and used as a probe to hybridize with *Sph*I-digested DNA from the transformed lines. Under stringent conditions, the transformed cell DNA, but not the host DNA, hybridized well with the oligonucleotide probe

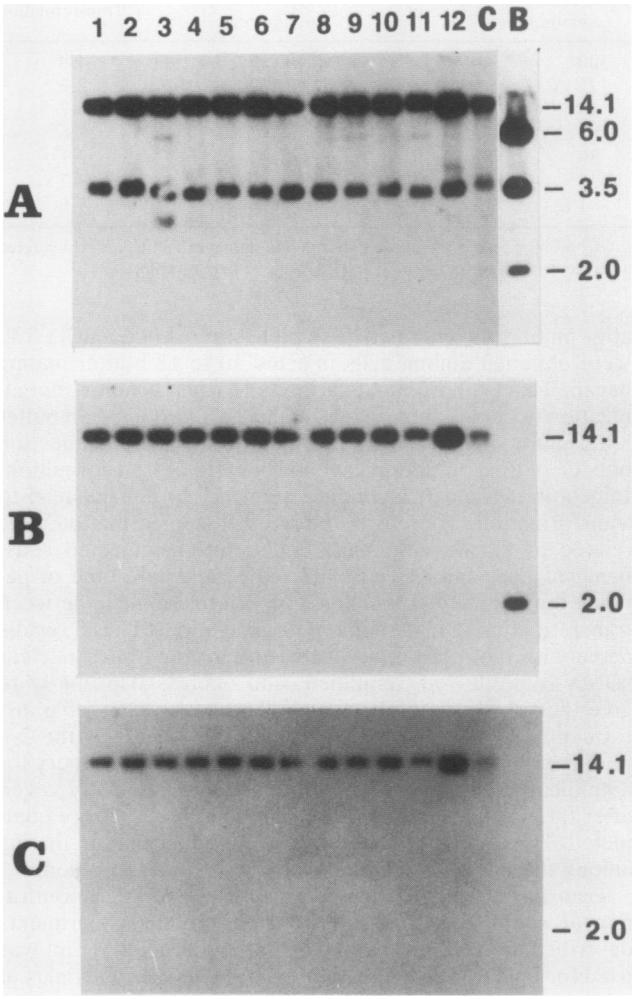

FIG. 3. *Sph*I digestion of transformed-cell rDNA. The transformed-cell and control DNAs shown in Fig. 2 were analyzed by the method described in the legend to Fig. 2 except that *Sph*I was used for the digestion. (A) DNA hybridized with the rDNA clone Tt947. The 14.1-kb fragment in SL062 DNA (lane C) was cleaved to form the 2.0-kb fragment and two identical 6.0-kb fragments seen in CU427 DNA (lane B). Again, most of the transformed cell rDNAs (lane 1 through 12) were similar to the rDNA of SL062 (lane C), although small amounts of the host-type sequence were also detected in some of the lines (e.g., lanes 3, 9, and 11). (B and C) The same DNAs hybridized with the oligonucleotide probe (5′-CTTC GAAAATAACTTAAAAT) which matches the sequence between nucleotide position 61 and 80 of the injected rDNA (*rdnA3*). The host rDNA contain a C instead of the A in position 71 (35; M.-C. Yao and C.-H. Yao, unpublished data). (B) Hybridization in which the posthybridization wash was carried out with 1× SSC (SSC is 0.15 M NaCl plus 0.015 sodium citrate) at 37°C, which did not remove the mismatched hybrid with the host-type DNA (the 2.0-kb band in lane B). (C) The same hybridized DNA washed in 0.5× SSC at the same temperature, which eliminated the mismatched hybrid in lane B but not the perfectly matched hybrid in lane C or the other lanes. Therefore, this injected-type sequence was present in the DNAs of all transformed lines. The result in panel A was obtained by using the same filter-bound DNA that was used to obtained the results shown in panels B and C (after removal of all radioactive materials).

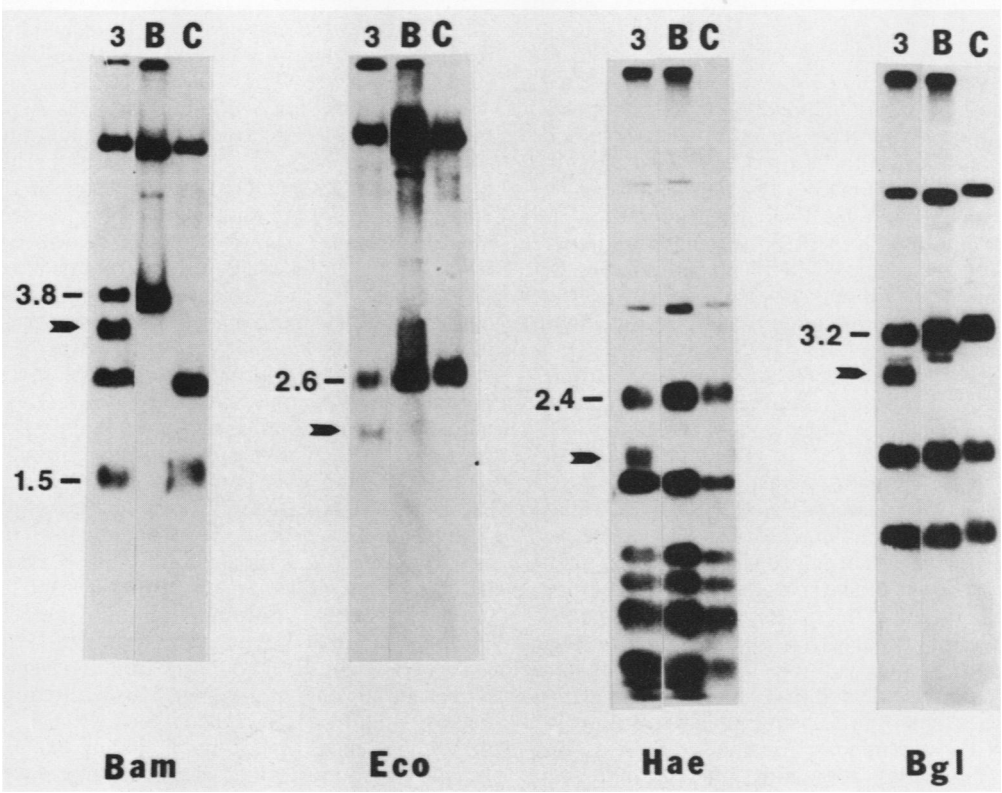

FIG. 4. Restriction mapping of the aberrant rDNA in a transformed line. The transformed-line DNA shown in lanes 3 of Fig. 2 and 3, which contained an extra fragment not seen in control DNAs, was further analyzed by digestions with other restriction enzymes. Lane 3 in each digestion contains DNA from this transformed line. Lanes B and C contain the control DNAs as described in Fig. 2. Bam, Eco, Hae, and Bgl indicate the enzymes used for the digestions (*Bam*HI, *Eco*RI, *Hae*III, and *Bgl*II, respectively). Numbers to the left of each panel are the estimated sizes (in kilobases) of the terminal fragments of normal rDNA. Arrows point to the unusual fragments. In each case, the fragment was approximately 0.7 kb smaller than the terminal fragment, which suggests a deletion within this region of the rDNA. On the basis of other digestion studies (not shown), the deleted region appears to include the heteromorphic *Bam*HI site, which explains why the aberrant fragment was larger than the 1.5-kb fragment in the *Bam*HI-digested sample.

(Fig. 3), which again demonstrated the presence of *rdnA3*-specific sequence in the transformed-cell rDNA.

Abnormality in rDNA processing. In most transformed lines, the macronuclear rDNA appeared to have a normal structure. However, on rare occasions (4 of 36 lines analyzed), small proportions of abnormal rDNAs were also detected. These abnormal rDNAs appeared to contain small deletions near the termini of the molecule. Figure 2 (lane 3) shows an example of this rDNA, which contains a 3.1-kb terminal *Bam*HI fragment. Restriction mapping studies localized the deletion to within the terminal 2.3 kb of the molecule, including the extra *Bam*HI site of *rdnA3* (Fig. 4). The exact sizes of these deletions are difficult to measure because of telomere heterogeneity, but they are around 0.7 kb. Similar deletions were observed at somewhat elevated frequencies when in vitro-mutated versions of Tt947-01 were used for transformation (R. Sweeney and M.-C. Yao, EMBO J., in press; R. Godiska and M.-C. Yao, unpublished observations). The deletion was not terminal, since a synthetic oligonucleotide (18-mer) containing the sequence 68 bp internal from the telomere addition site at the normal 3′ end of rDNA (17) was found to hybridize with this DNA (data not shown). Therefore, these deletions could not have been produced from errors in breakage at the 3′ end of the rDNA, which could produce terminal deletions. The cause for this internal deletion is unknown. Deletions of this kind have not been found among native rDNA of this species or in transformed lines injected with native macronuclear rDNA. They are therefore related to processing of the injected micronuclear rDNA. It should be pointed out that internal deletions occur as regulated events at more than 5,000 specific sites in this genome during macronuclear development (4, 10, 30; reviewed in reference 14). It would be interesting to know whether these two deletion events are related.

DISCUSSION

We have shown that injections of cloned copies of the micronuclear rDNA of *T. thermophila* into developing macronuclei produce transformed cells that contain normal macronuclear rDNA with the genetic markers specific to the injected molecules. These results indicate that the injected molecules are processed normally and adequately amplified. Therefore, the 15-kb DNA used for injection must contain all *cis*-acting sequences required for *Tetrahymena* rDNA amplification. Formation of the macronuclear rDNA involves at least four events: site-specific breakage, palindrome formation, telomere addition, and preferential replication. The injected molecules contain, in addition to the rDNA, 3.4 kb of the 5′-flanking and 0.5 kb of the 3′-flanking sequences, including some distinct sequence elements that may have a role in these events. The most prominent element is the 15-bp sequence Cbs, of which there are two complete and one partial copy in the 5′-flanking region and one complete

copy in the 3'-flanking region. This sequence has been shown to be tightly associated with chromosome breakage sites in *Tetrahymena* cells and could be a recognition signal for this event. Another feature is the 42-bp inverted repeats at the 5' end of the rDNA. The structure, location, and evolutionary conservation of these repeats suggest a possible role in palindrome formation (35). The third feature is the hexanucleotide 5'-CCCCAA-3', one copy of which is present at the 3' end of the rDNA. Its location and similarity to telomeric DNA suggests a possible role in determining the point of telomere addition, although its presence is obviously not required for telomere addition at other breakage sites (34, 35). The signal for preferential replication is not known but is likely localized within the rDNA, since chromosome breakage probably precedes preferential replication. Whether these sequences, or other sequences within the injected DNA, play a specific role in rDNA processing or amplification can now be directly investigated.

Recombination between injected and host rDNA has recently been shown to occur in *Tetrahymena* vegetative cells (21, 36; Sweeney and Yao, in preparation; M.-C. Yao, unpublished observations). This type of recombination may have also occurred in some of the transformed lines obtained here. It is therefore important to ask whether the transformation observed in this study is the result of recombination between the injected molecule and the amplified host macronuclear rDNA rather than of processing and amplification of the injected rDNA. Recombinations might produce, for example, hybrid molecules containing the paromomycin resistance marker and the replication origin of the injected molecule but the palindromic structure and telomeric sequences from the host macronuclear rDNA. We do not think that this is the case for the following reasons. First, the process is developmental stage specific, being most effective when injection is carried out at the time when rDNA amplification normally occurs. It does not occur in vegetative cells, in which recombination between rDNAs is also known to occur. Second, DNA fragments containing the paromomycin resistance marker and the replication origin but without the 3' end of rDNA are ineffective in transformation. This result is not expected if recombination is the mechanism. Third, most of the rDNA from the transformed cells contains all four heteromorphic markers specific to the injected molecule. Three of them are physical markers not known to affect growth rates. Together they occupy a region approximately 9 kb in length, or 85% of the rDNA. Although some host markers are present in some transformed lines, it is important to stress that all three injected-type physical markers are present in all lines and, in most cases, as the predominant species. The chance that this large piece of DNA is cotransferred with the paromomycin resistance marker to the host rDNA by recombination or conversion in every transformed line is remote. Moreover, one of these sites is only 71 bp from the center of the palindromic sequence. If recombination is the mechanism, at least one crossover will have to occur within this 71-bp interval in every transformed line to produce the result observed, which is most unlikely. Taken together, these results provide strong evidence that the macronuclear rDNAs of the transformed cells are not produced through recombination between the injected molecule and the host macronuclear rDNA. The second and third points of evidence also argue against recombination between the injected molecules and the host micronuclear rDNA, which might still be present at the time of injection. We therefore conclude that the transformation is the result of accurate processing and amplification of the injected micronuclear rDNA and does not involve recombination with the host rDNA as an obligatory step.

We are impressed by the high rates of transformation achieved in this study. The highest rates are comparable to those obtained by injection of mature macronuclear rDNA at similar developmental stages reported earlier (27). Thus, processing of the injected molecules probably occurs efficiently and is not the limiting step in transformation. This is in agreement with the observation that injections of only a few molecules are sufficient for high rates of transformation. It is interesting that injection of larger amounts of DNA neither improves nor diminishes the transformation rates. At the highest point, as many as 5,000 potential breakage sites are injected, which is more than all of the breakage sites normally present in the genome at these stages of development, and this is apparently not lethal to the cell. It is possible that the enzymes involved in chromosome processing are present in excess and not easily reduced in amount by the injected DNA.

In most transformed lines obtained in this study, the host-type rDNA is present in low abundance or is not detected. This ability to replace host rDNA with a cloned copy offers the opportunity for studying rDNA function through the method of gene replacement. Gene replacement has been a powerful tool for studying gene function and regulation in other organisms. However, this approach has been difficult for rDNA mostly because of the redundancy of this gene in most organisms, including *Escherichia coli* and yeasts. Our study provides a unique solution to this problem. By injecting reconstructed rDNA clones into *Tetrahymena* cells, one should be able to produce cells containing only altered rDNA and thus be able to study the biological consequences of these mutations. This idea has now been successfully tested (R. Sweeney and M.-C. Yao, in press).

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