Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 8487–8491, November 1989
Genetics

Transformation of *Tetrahymena thermophila* with a mutated circular ribosomal DNA plasmid vector

(ciliate/microinjection/overexpression vector)

GUO-LIANG YU AND ELIZABETH H. BLACKBURN*

Department of Molecular Biology, University of California, Berkeley, CA 94720

Communicated by Joseph G. Gall, August 15, 1989 (received for review May 8, 1989)

ABSTRACT **A circular plasmid containing a complete *Tetrahymena thermophila* rRNA gene (rDNA), with a tandem repeat of a 1.9-kilobase-pair segment encompassing the replication origin and the rRNA promoter, and a polylinker in the 3′ nontranscribed spacer, was used to transform *T. thermophila* by microinjection. Most (20/21) stable transformants contained only recombinant linear palindromic rDNA molecules carrying rDNA sequences from both the donor plasmid and the recipient cell, as shown previously. However, in one transformant, the circular plasmid initially outreplicated the endogenous rDNA and was the major rDNA form for up to 65 generations. Stable circular replicons have not been reported previously in *Tetrahymena*. A single point mutation (+G) was identified in the repeated promoter of the plasmid maintained in this transformant. After recovery from the *Tetrahymena* transformant and recloning in *Escherichia coli*, the mutated circular plasmid again transformed *Tetrahymena* with stable maintenance of the circular rDNA plasmid. Transformants containing circular replicons were also obtained by using a similar plasmid from which the repeated promoter, but not the repeated replication origin, had been removed by BAL-31 deletion. We therefore propose that repeated rRNA promoters are deleterious *in vivo* in *Tetrahymena*, which normally lacks them. Transformants were obtained in 2–5 days compared with the 7–14 days required for transformation with unmutated rDNA plasmids by recombination. Similar results were obtained when a 550-base-pair segment containing the telomerase RNA gene of *T. thermophila* was inserted in the polylinker of the plasmid. We suggest that this plasmid is a useful vector system for transformation of *Tetrahymena*.**

The ribosomal RNA genes (rDNA) in the somatic macronucleus of the ciliated protozoan *Tetrahymena thermophila* are free, linear 21-kilobase-pair (kbp) palindromic molecules, unlike the rDNA in many eukaryotes, which consists of multiple rRNA gene copies arranged as tandem arrays within one or more chromosomal loci. In *T. thermophila*, the rDNA is differentially amplified to $\approx 10^4$ gene copies during the development of the macronucleus (1, 2). Different alleles of the rDNA of *T. thermophila* show differential replication properties. Previous work has shown that the rDNA from strain C3 has a replication advantage over a genetic variant rDNA from strain B (3, 4) and over the mutant C3 rDNAs C3-*rmm1* and C3-*rmm4* when they are present in the same macronucleus (3, 5–8). When the different rDNA alleles were compared, sequence differences were found in a repeated conserved element (type I repeat) in one of two nuclease hypersensitive domains in the replication origin region in the 5′ nontranscribed spacer (5′ NTS) (3, 8). It was therefore proposed that these elements are cis-acting positive regulatory elements for replication (3, 8).

These differential replication properties of the rDNA allelic forms have been exploited in transformation of *Tetrahymena* macronuclei by palindromic rDNA purified from macronuclei (4, 6, 9) and by circular rDNA plasmids containing *Tetrahymena* rDNA cloned in an *Escherichia coli* plasmid vector (5, 10). In these experiments, the donor rDNA used for transformation was the C3 allelic form, which has a replication advantage over the endogenous rDNA allele (B or *rmm1*) in the recipient cell.

We have shown previously that the usual outcome of transformation with a circular rDNA plasmid was homologous recombination between the injected rDNA plasmid and the endogenous macronuclear palindromic rDNA, generating palindromic rDNA species containing both donor rDNA and endogenous rDNA sequences. When a circular C3 rDNA plasmid construct containing an additional C3 origin region and promoter was used, the extra origin unexpectedly did not appear to have any effect on the outcome of transformation, because homologous recombination resulted in loss of the tandem repeat of the origin and promoter (5).

We report here our findings that led from the observation that in one transformant clone the entire rDNA plasmid with repeated origin and promoter regions was retained in its original circular form as a free replicon. After several cell generations, it largely replaced the palindromic rDNA form. We present evidence to show that removal or inactivation (mutation) of the repeated promoter, with retention of the repeated replication origin region, was necessary to obtain transformants retaining circular rDNA plasmids. Such transformants were obtained 3–4 times more rapidly than transformants requiring recombination with endogenous rDNA. The *T. thermophila* telomerase RNA gene (11), inserted into the polylinker in this circular rDNA plasmid, was maintained in the rDNA plasmid in transformants. Hence, this type of construct is a useful high copy number vector for transformation of *Tetrahymena* macronuclei.

MATERIALS AND METHODS

Culture and Transformation of *T. thermophila*. *T. thermophila* strain SB2120 (C3-*rmm1*, *Pmr*$^+$) was generously provided by E. Orias (University of California, Santa Barbara). Cells were grown at 30°C in 2% PPYS [2% proteose peptone (Difco)/0.2% yeast extract (Difco)/0.003% Sequestrine (CIBA–Geigy)] with penicillin (100 units/ml), amphotericin B (250 pg/ml), and streptomycin (100 μg/ml) (GIBCO) either in flasks, with aeration by gentle swirling on a gyratory shaker, or in cultures in Petri dishes. The paromomycin concentration in selective medium was 100 μg/ml in 2% PPYS. The microinjection procedure was essentially the same as described by Tondravi and Yao (4), adapted as described (5).

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Abbreviations: rDNA, ribosomal RNA-encoding DNA; NTS, nontranscribed spacer.

*To whom reprint requests should be addressed.

Plasmid Construction. Plasmid prD2 was constructed as described and consists of one-half of the palindromic rDNA and an extra 1.9-kbp *Sau*3AI fragment inserted at the *Bam*HI linker at the 5′ end of the rDNA, creating *Bam*HI sites at both ends of the fragment (5). Plasmid prD4 was constructed by partial *Ban* II digestion of prD2 and insertion of a 30-bp synthetic polylinker into the *Ban* II site in the 3′ NTS rDNA spacer (Fig. 1). The polylinker consisted of two complementary oligonucleotides with the sequences 5′-TCGAGGTACCCGGGCGGCCGCTGATCAGCC-3′ and 3′-TCGGAGCTCCATGGGCCCGCCGGCGACTAG-5′, which annealed to form a duplex with *Ban* II cohesive ends.

DNA Analysis. Total cellular DNA was isolated (12) from 50-ml cultures of *T. thermophila*. DNA preparations highly enriched in macronuclear rDNA were made from 2-liter cultures as described (13).

Densitometric measurements of DNA fragments in ethidium bromide-stained agarose gels were made with an LKB model 2202 Ultroscan laser densitometer equipped with a model 2220 recording integrator. Agarose gel electrophoresis, blotting of the DNA onto membranes, and hybridization to ^{32}P-labeled nick-translated probes were carried out by standard procedures (14).

Restriction fragments of purified rDNA were inserted into pUC118 and subcloned into M13mp19. DNA sequencing of the cloned fragments was done with a sequencing kit purchased from United States Biochemical.

RESULTS

Transformation of *T. thermophila* by a Circular rDNA Plasmid, prD4. We constructed a circular rDNA plasmid, prD4, which contains one complete rRNA gene, or half of the palindromic rDNA and, in addition, a tandemly repeated 1.9-kbp segment containing the 5′ NTS and rRNA promoter from C3 rDNA (Fig. 1). The promoter adjacent to the rRNA gene is designated the functional promoter, and the promoter in the repeated 1.9-kbp segment is designated the repeated promoter. The 17S rRNA gene in the plasmid had the selectable paromomycin-resistance marker *Pmr1* (13, 15) and a synthetic polylinker containing a *Not* I site was inserted into a *Ban* II site in the 3′ NTS. The additional 1.9-kbp *Sau*3AI fragment extended from the *Bam*HI linker at the original center of the palindromic rDNA to the *Sau*3AI site at position +20 in the rRNA transcription unit (5). Thus, prD4 contained

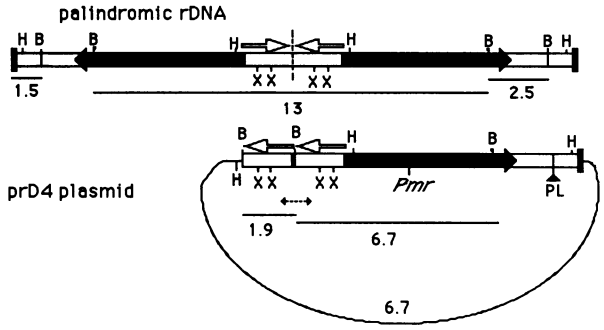

FIG. 1. Structures of palindromic rDNA and prD4 plasmid. Long solid arrows, rRNA transcription units; open arrows, repeated 5′ NTSs; vertical dashed line, center of the palindrome; solid vertical bars, telomeric sequences; horizontal dashed line with arrows, segment deleted by BAL-31 in prD4-d9; *Pmr*, paromomycin resistance marker. B, *Bam*HI; H, *Hin*dIII; X, *Xba* I sites. PL, synthetic polylinker. PL is inserted into the *Ban* II site in the 3′ NTS. Bs with a flag, *Bam*HI sites common to both plasmid and recipient palindromic rDNA. All *Bam*HI and *Xba* I sites are shown. Sizes of *Bam*HI fragments are shown. Only relevant *Hin*dIII sites are shown. Thin line in prD4 is the vector pBR322; its length is not proportional to actual size.

two complete tandem copies of both the replication origin region from wild-type C3 rDNA (3) and the highly conserved rDNA promoter sequence (16).

Transformation of the recipient strain SB2120, which contains C3-*rmm1*, paromomycin-sensitive (*Pmr*$^+$) rDNA in its macronucleus (see Fig. 1), was carried out by microinjection of prD4 plasmid DNA into the macronucleus. Injected cells were initially grown in rich nonselective medium (2% PPYS) for 2–3 days (15–20 cell generations). Subsequent transfers of the culture to fresh medium allowed the cells to grow so that the rDNA containing the donor replication origin could outreplicate the recipient rDNA, and then aliquots of the cultures were checked for transformation by transfer to medium containing 100 μg of paromomycin (PM) per ml. Of 48 total viable microinjected cells, 13 paromomycin-resistant transformants were isolated. An additional 8 independent transformants were obtained in a separate experiment from separate pools of injected cells. Twenty out of these 21 transformants contained heteropalindromic or homopalindromic rDNA, resulting from homologous recombination of the plasmid with one arm or both arms of the endogenous palindromic rDNA as expected from previous results (5). *Bam*HI digestion of all 20 of these transformants showed the expected restriction fragments for palindromic rDNA with one arm composed of donor sequences and one of recipient rDNA sequences. Fig. 2 (lane 3) shows results for a representative transformant. These transformants, and the frequency of transformation, were similar to those reported in our previous work with a similar circular rDNA plasmid (5). However, in one transformant, T5-1-1, the majority of the rDNA molecules had a different restriction map from that of palindromic linear molecules.

A Circular rDNA Plasmid Outreplicated the Endogenous Palindromic Linear rDNA. Approximately 50 generations after microinjection of the circular plasmid prD4 into strain SB2120 (C3-*rmm1*, *Pmr*$^+$), paromomycin-resistant cells appeared in the T5-1-1 line, as determined by replica transfers into selective PM medium. The culture was grown in paromomycin (100 μg/ml) for ≈15 generations, and then total cellular DNA was purified and analyzed by Southern blotting (Fig. 2, lane 4). At this stage (≈65 generations), there were predominantly two rDNA populations in the transformant clone: a minor population of the endogenous palindromic rDNA (lane 2) and a major population of rDNA molecules with the original prD4 restriction map (Fig. 1 and Fig. 2, lane 1). *Bam*HI (Fig. 2, lane 4), and *Hin*dIII (Fig. 3) digestions showed that the major rDNA species contained 6.7- and 1.9-kbp *Bam*HI fragments, and 4.4- and 3.0-kbp *Hin*dIII fragments, all diagnostic fragments of the circular plasmid (see Figs. 1 and 3*A*). When the same blots were probed with pBR322, only the 6.7-kbp *Bam*HI fragment and the 3.0- and 4.4-kbp *Hin*dIII fragments hybridized (data not shown), confirming that the major rDNA species had the circular restriction map of prD4. The plasmid prD4 contains a unique *Not* I site in the polylinker in the 3′ NTS. When the DNA from the 65-generation culture was digested with *Not* I and probed with pBR322, the pBR322-hybridizing plasmid band shifted down to the position of full-length linearized prD4 (≈15 kbp) (Fig. 3*B*). The circular restriction map of the molecules and their mobility change upon cleavage at the unique *Not* I site suggested that the major rDNA species replicating in T5-1-1 cells was the original circular form of prD4 (Fig. 3*C*). This was confirmed by transforming *E. coli* with total *Tetrahymena* DNA from the 65-generation stage of T5-1-1 cells. The plasmid DNAs isolated from all 6 of the single transformed *E. coli* colonies analyzed (from a total of 42 obtained) had the same size and restriction map as the original prD4.

Retransformation of *T. thermophila* with Mutant Plasmid. The unusual outcome of transformation in the T5-1-1 line

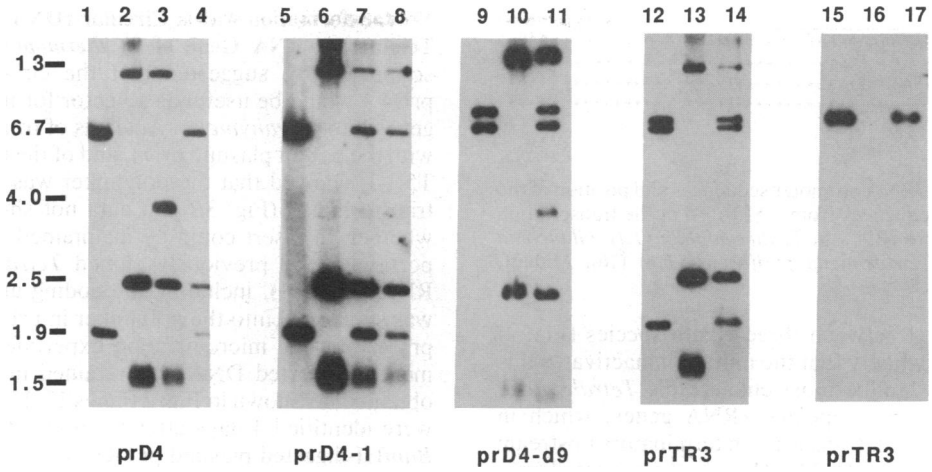

FIG. 2. *Bam*HI digestion of DNA from transformants. Lanes: 1 and 5, prD4 plasmid; 2, 6, 10, and 13, untransformed recipient SB2120; 3, a typical (20/21) normal transformant; 4, the transformant T5-1-1; 7 and 8, prD4-1 transformants; 9, prD4-d9 plasmid; 11, a prD4-d9 transformant; 12, prTR3 plasmid; 14, a prTR3 transformant; 1–14, probed with nick-translated prD4 plasmid; 15–17, duplicates of lanes 12–14, probed with a nick-translated 550-bp fragment containing the telomerase RNA gene. The 13-, 2.5-, and 1.5-kbp *Bam*HI fragments are expected from recipient's palindromic rDNA. The prD plasmids lack a *Bam*HI site 1.5 kbp from the telomeric sequence; hence, recombinant palindromic rDNA contains a 4.0-kbp *Bam*HI fragment (lanes 3 and 11). prD4 and prD4-1 plasmids contain two 6.7-kbp fragments and a 1.9-kbp fragment. prD4-d9 plasmid contains a 6.7- and a 7.9-kbp *Bam*HI fragment.

could have been the result of a change either in the transforming DNA plasmid or in the recipient cell. To distinguish between these two possibilities, we retransformed SB2120 cells by using circular prD4 plasmid that had been recovered from the T5-1-1 transformant *Tetrahymena* cells by transformation into *E. coli* as described above. This recovered plasmid, named prD4-1, was then microinjected into recipient SB2120 *Tetrahymena* cells. Two transformant cell clones were obtained from 12 single injected cells. Paromomycin-resistant cells first appeared ≈20 generations after microinjection, when an aliquot of each microinjected cell culture was tested by transfer into PM medium. After ≈15 more generations of growth, cellular DNA was prepared. Fig. 2 (lane 5) shows the *Bam*HI-digested prD4 plasmid DNA used for the original transformation. The cellular DNA of the untransformed recipient cell line is shown as a control in lane 6 of Fig. 2. The major rDNA species in both transformants contained the 6.7- and 1.9-kbp *Bam*HI fragments predicted from the circular prD4 map, in addition to fragments from the recipient palindromic rDNA (lanes 7 and 8). We estimated the relative amounts of circular plasmid and recipient palindromic rDNA in the macronucleus of each of these transformants by densitometric scanning of a negative of a photograph of an ethidium bromide-stained gel containing total cellular DNA digested with *Bam*HI. In both transformants, 60–80% of the rDNA present was in the circular plasmid form (data not shown). An additional three transformants obtained from different experiments also had similar proportions of circular rDNA. Thus, transformation with prD4-1 reproduced, in five separate new transformants, the unusual outcome of transformation first seen in transformant T5-1-1. These results indicated that an alteration of the prD4 plasmid DNA, rather than of the host cell, was responsible for its ability to be retained as a circular replicon.

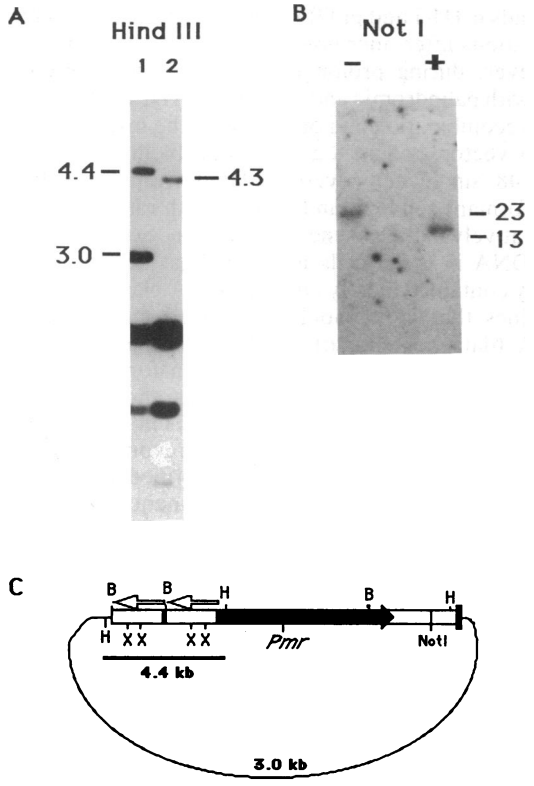

FIG. 3. The major rDNA form at 65 generations was a circular plasmid. (*A*) *Hin*dIII digestion of recipient and 65-generation T5-1-1 DNA. Lanes: 1, T5-1-1 at 65 generations; 2, recipient SB2120 DNA. The blot was probed with nick-translated prD4 plasmid. The 3.0- and 4.4-kbp fragments are diagnostic of the circular prD4 plasmid. The 4.3-kbp fragment in lane 2 is the central fragment of the palindromic 21-kbp rDNA. Bands below 3.0 kbp are all rRNA coding fragments. (*B*) T5-1-1 65-generation DNA with (+) or without (–) digestion by *Not* I, probed with nick-translated pBR322. The size markers are shown. (*C*) The structure of the rDNA in T5-1-1 at 65 generations. The symbols are the same as in Fig. 1. The diagnostic 3.0- and 4.4-kbp *Hin*dIII fragments are indicated.

A Point Mutation in the Repeated Promoter of prD4-1. We reasoned that the retention of circular rDNA plasmid at high copy number in transformant T5-1-1 may have been caused by an alteration in the tandemly repeated 1.9-kbp segment in prD4. By sequence analysis, we found a single site mutation, the insertion of a G residue in a run of 6 G residues 16–21 bp upstream from the transcription initiation site, in the repeated promoter in the circular rDNA recovered from transformant T5-1-1 (Fig. 4). No other mutations were found in the entire 1.9-kbp repeated *Sau*3AI fragment in three separate clones analyzed (data not shown). As discussed later, the position of this mutation in a sequence of the rRNA promoter that is

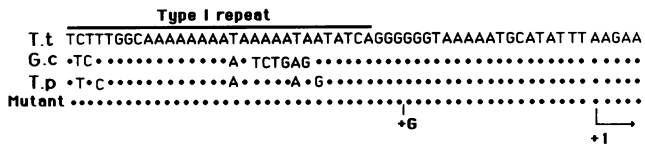

FIG. 4. Conserved rRNA promoter sequences and position of the mutation in prD4-1. Sequences from −51 to +5 at the transcription initiation site of the pre-rRNA in *T. thermophila* (*T.t*), *Glaucoma chattoni* (*G.c*), and *Tetrahymena pyriformis* (*T.p*) (16). Mutant, prD4-1 sequence.

absolutely conserved between three ciliate species (Fig. 4; ref. 16) makes it very likely that the mutation inactivates this repeated promoter. Unlike most eukaryotes, *Tetrahymena* does not have tandemly repeated rRNA genes, which in many eukaryotes contain transcription terminators upstream of the rRNA promoters (21–23). Hence, the repeated promoter in the prD4 construct represents an abnormal situation for *Tetrahymena*.

In contrast to the repeated promoter, sequence analysis showed that the functional promoter adjacent to the rRNA gene in prD4-1 was identical to wild type. The sequence of both copies of the promoter in the original prD4 plasmid preparation used for microinjection was also shown to be identical to wild type. These results support the hypothesis that a spontaneous +G mutation in a string of 6 G residues at −16 to −21 of the repeated promoter resulted in the ability of plasmid prD4-1 to be maintained in a circular form in transformants.

Transformants by a Circular rDNA Plasmid with a Deleted Repeated Promoter. To test directly whether inactivation of the repeated promoter by the +G insertion was the cause of the ability of prD4-1 to be maintained as a free replicon, we carried out a BAL-31 deletion of the repeated promoter. This deletion was done by first linearizing the prD4 plasmid by partial *Bam*HI digestion. A mixture of the three possible full-length molecules, linearized at one of the three *Bam*HI sites in prD4, was isolated from other partial digestion products by agarose gel electrophoresis. BAL-31 digestion was used to remove ≈300 bp of sequence from each side of the *Bam*HI sites. The deleted rDNA plasmids were recircularized and cloned in *E. coli*, and their structures were determined by *Bam*HI, *Hin*dIII, *Alu* I, *Dra* I, and *Hin*fI restriction analysis (data not shown). Plasmids with BAL-31 deletions extending from the correct *Bam*HI site—that is, between the *Bam*HI fragment containing the 1.9-kbp origin repeat and the 6.7-kbp *Bam*HI fragment carrying the rRNA coding sequence (see Fig. 1)—were identified and used for transformation experiments.

The recipient strain SB2120 was transformed by one such deleted plasmid, prD4-d9, in which a total of ≈600 bp, including the repeated promoter, had been removed as shown in Fig. 1. In one experiment, of 11 viable microinjected cells, one transformant line was identified by its resistance to paromomycin 5 days after microinjection. The results obtained from Southern analysis are shown in Fig. 2 (lanes 9–11). The *Bam*HI-digested prD4-d9 plasmid DNA is shown in lane 9. Lanes 10 and 11 show the *Bam*HI-digested cellular DNAs of the recipient and this transformant, respectively. Identical fragments of prD4-d9 are seen in lanes 9 and 11. An additional transformant by prD4-d9 was obtained in a separate experiment. Southern analysis showed that the rDNA in this transformant also contained the same restriction fragments as those of the donor plasmid. We conclude that, as with prD4-1, plasmid prD4-d9 can transform by self-replication and expression of the circular rDNA plasmid without the necessity for recombination with endogenous palindromic rDNA.

Transformation with a Circular rDNA Vector Carrying the Telomerase RNA Gene of *T. thermophila*. The results described above suggested that the circular rDNA plasmid prD4-1 would be useful as a vector for introduction of other genes into *Tetrahymena*. Analysis of transformants obtained with the parent plasmid prD4, and of the prD4-1 transformant T5-1-1, showed that the polylinker was maintained in these transformants (Fig. 3*B* and data not shown). To determine whether an insert could be maintained in this polylinker, a portion of the previously cloned *Tetrahymena* telomerase RNA gene (11), including the coding and flanking regions, was subcloned into the polylinker in prD4-1 to form plasmid prTR3. In one microinjection experiment, this 550-bp segment of inserted DNA was retained in both transformants obtained, as shown in Fig. 2 (lanes 12–17). The transformants were identified 4 days after microinjection. Lane 12 shows *Bam*HI-digested plasmid prTR3, lane 13 shows the *Bam*HI-digested recipient cellular DNA as a control, and lane 14 shows the *Bam*HI-digested cellular DNA of one transformant, all hybridized with nick-translated prD4 plasmid. Lanes 15–17 show the same DNA samples as in lanes 12–14 hybridized with a purified telomerase RNA gene probe. This experiment was repeated three times with similar results. We conclude that the telomerase RNA gene can be maintained in the rDNA vector in the transformants. Analysis of the expression of the telomerase RNA in these transformants will be reported elsewhere.

Stability of the Circular rDNA Plasmids. The circular rDNA plasmids prD4-1 and prTR3 were stably maintained for 50–80 generations after microinjection, as shown in Figs. 2 and 3. However, during prolonged mass culture, overgrowth by cells with palindromic and other linear rDNA forms, resulting from recombination and often loss of the inserted gene in the rDNA vector, becomes a serious problem. To examine this loss, 48 single cells were isolated from a single prD4-1 transformant cell line, and their growth rates were observed qualitatively in the absence of selection. Southern analysis of the rDNA in these cells indicated that cell lines that grew slowly contained mostly circular rDNA plasmid, whereas the cell lines that grew rapidly contained mostly palindromic rDNA (data not shown). Under selective or nonselective conditions, prD4-d9 and prTR3 transformants also grew noticeably more slowly than controls and were eventually outgrown in the cell population by cells in which the circular plasmids had undergone homologous recombination with the endogenous rDNA. As will be described elsewhere, such recombination events also included unequal crossovers, resulting in linear recombinant rDNA molecules containing multiple origins that had a replication advantage over the circular plasmids.

To solve this problem of loss of the insert in the rDNA vector, subculturing of the initial transformants was carried out. In one experiment with a prTR3 transformant, 16 single cell lines were isolated 35 generations after microinjection and grown without paromomycin selection for an additional 15 generations, and their DNA was then analyzed. In 8 of the 16 lines, the majority of the rDNA was recombinant linear rDNA molecules containing the telomerase RNA gene. In an independent experiment, in 5 of 7 transformed single cell lines isolated 35 generations after microinjection of prTR3, the telomerase RNA gene was still maintained in the circular rDNA vector after an additional 15 generations. At this point, a total of 23 single cells were then isolated from 3 of these 5 cell lines. After growth for 15 generations, all 23 cell lines still contained the telomerase RNA gene insert, with comparable amounts in the circular rDNA vector and linear recombinant rDNA molecules (data not shown). These results indicated that, by subculturing, the transformed gene could be maintained for at least 65 generations.

DISCUSSION

Cellular Selection for the Repeated Promoter Mutation in prD4-1. Of 21 transformants obtained from microinjection of prD4 plasmid into the C3-*rmm1* strain SB2120, transformant T5-1-1 was unique in containing the rDNA plasmid in circular form. Although the number of transformants is not large enough for accurate statistics, T5-1-1 appears to be an unusual case. The occurrence of a point mutation in prD4 explains the initial low frequency (1/21) of this type of transformant. Since the usual outcome of plasmid transformation is homologous recombination with endogenous rDNA to produce recombinant palindromic rDNA, our results indicate that in transformant T5-1-1 the mutation took place either before or soon after the plasmid was injected into the *Tetrahymena* macronucleus. The fact that the mutation took place spontaneously and the mutant molecule largely replaced the endogenous palindromic rDNA provides clear evidence that the mutant rDNA had a replication advantage over the recipient's rDNA and was not grossly deleterious to the cell. The total number of origin control regions in plasmid prD4-1 is the same as in the palindromic form of wild-type C3 rDNA, which was previously shown to have a replication advantage over *rmm1* rDNA. A fuller study of the replication properties of various rDNA forms in prD4-1 transformants will be reported elsewhere.

The rDNA sequence from −26 to +20 is absolutely conserved between the ciliates *Tetrahymena* and *Glaucoma*, although surrounding sequences differ (ref. 16; Fig. 4). The repeated 1.9-kbp *Bam*HI fragment in the prD4 plasmid contains all of this conserved sequence as well as all of the upstream spacer. The mutation in the repeated promoter in prD4-1 is predicted to decrease transcription initiation from this promoter because an additional G residue to the string of 6 G residues at −16 to −21 alters both the spacing between +1 and the type I repeats and changes the conserved promoter sequence. Direct contact of RNA polymerase I with the rRNA transcription initiation factor is inferred to be essential for transcription initiation in several systems (17–20). In *Acanthamoeba* and mouse rRNA genes, a 1-bp alteration of the spacing between the transcription initiation factor binding site in the rRNA promoter and the +1 position significantly reduces the level of transcription (17, 20).

The extra promoter in prD4 and also in a similar construct, prD2 (5), appears to be invariably eliminated in transformants: it is removed by homologous recombination in most transformants and was inactivated by a mutation in the one case of T5-1-1. We showed that deliberate removal of the extra promoter by BAL-31 deletion also resulted in retention and self-replication of an rDNA plasmid with a repeated origin of replication. From these observations, we propose that this extra promoter is deleterious in *Tetrahymena*. It may compete with the functional promoter adjacent to the rRNA gene for transcription factors or, by initiating transcription upstream of the true rRNA promoter, transcribe through replication control elements and prevent binding of replication factors. Since *Tetrahymena* does not contain tandemly repeated rRNA genes, it may lack the transcription terminator found just upstream of the rRNA promoter in many other eukaryotes. Such a transcription terminator may prevent promoter occlusion by the passage of transcribing polymerase (21–24), since, in a dimeric *Acanthamoeba* rRNA promoter, RNA polymerase I has been shown to transcribe from the upstream promoter and read through the downstream promoter (24).

A General High Copy Number Vector for Transformation of *Tetrahymena* Macronuclei. This paper reports a plasmid molecule capable of replicating as a circular replicon and of being maintained and expressed for many generations in *Tetrahymena* macronuclei. In *Paramecium*, circular plasmids microinjected into macronuclei were randomly linearized and acquired telomeres (25, 26). The circular plasmids in *Tetrahymena* are not stable indefinitely because of the high frequency of homologous recombination. We surmise that a single rRNA gene per circular replicon instead of the two rRNA genes per palindromic rDNA molecule results in a cell growth disadvantage. Therefore, subculturing of the transformants appears necessary to prevent overgrowth of large cultures by recombinants with two rRNA copies per rDNA replicon. Retransformation of prD4-1 resulted in the appearance of paromomycin-resistant transformants 2–5 days after microinjection, compared to the usual 7–14 days needed to precede drug selection when the rDNA plasmid has to undergo a recombination event with endogenous rDNA (5). In a recent study in which a single rRNA gene on a circular plasmid without a repeated origin was microinjected into vegetative cells, no transformants were seen (10). The reason for this discrepancy is likely to be that in that study only a small number of generations elapsed after microinjection before transformants were scored. We showed previously that transformants are observed only after 50 generations following microinjection of a similar circular rDNA plasmid. The plasmid prD4-1 contains a polylinker in the 3′ NTS, and we have shown than an unrelated *Tetrahymena* gene can be inserted into this polylinker and can be maintained (Fig. 3) and expressed in transformants (G.-L.Y., L. Attardi, and E.H.B., unpublished results). We therefore suggest that prD4-1 will be a useful general high copy number vector for transformation of *Tetrahymena* macronuclei.

We thank E. Robinson and C. Wyman for critical reading of the manuscript. This work was supported by National Institutes of Health Grant GM32565 to E.H.B.

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