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Tandem repeats of the 5′ non-transcribed spacer of *Tetrahymena* rDNA function as high copy number autonomous replicons in the macronucleus but do not prevent rRNA gene dosage regulation

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ABSTRACT

The rRNA genes in the somatic macronucleus of *Tetrahymena thermophila* are normally on 21 kb linear palindromic molecules (rDNA). We examined the effect on rRNA gene dosage of transforming *T.thermophila* macronuclei with plasmid constructs containing a pair of tandemly repeated rDNA replication origin regions unlinked to the rRNA gene. A significant proportion of the plasmid sequences were maintained as high copy circular molecules, eventually consisting solely of tandem arrays of origin regions. As reported previously for cells transformed by a construct in which the same tandem rDNA origins were linked to the rRNA gene [Yu,G.-L. and Blackburn,E.H. (1990) *Mol. Cell. Biol.*, 10, 2070–2080], origin sequences recombined to form linear molecules bearing several tandem repeats of the origin region, as well as rRNA genes. The total number of rDNA origin sequences eventually exceeded rRNA gene copies by ~20- to 40-fold and the number of circular replicons carrying only rDNA origin sequences exceeded rRNA gene copies by 2- to 3-fold. However, the rRNA gene dosage was unchanged. Hence, simply monitoring the total number of rDNA origin regions is not sufficient to regulate rRNA gene copy number.

INTRODUCTION

Generally, chromosome replication in eukaryotes is under cell cycle control. However, the high copy minichromosome that carries the ribosomal RNA genes (rDNA) in the somatic macronucleus of the ciliate *Tetrahymena thermophila* is a cellular replicon under additional replication control (1,2). This natural acentric minichromosome is a linear palindrome consisting of two ~10.5 kb halves, each containing an rRNA gene (Fig. 1A) and is found in the somatic nucleus (macronucleus) of *T.thermophila*. During macronuclear development, which occurs after the sexual phase of the ciliate life cycle, the rDNA is amplified to high copy number from a single copy rRNA gene in the germline micronucleus (reviewed in 3). It is maintained at ~10^4 copies/macronucleus during subsequent vegetative cell divisions.

While rDNA replication in vegetatively dividing *T.thermophila* cells is largely under cell cycle control (reviewed in 2,3), studies of alleles of the rDNA minichromosome differing in their origin control regions led to the proposal that rDNA replication is also regulated by a copy number control mechanism (1). Various allelic forms of the rDNA, some differing by only a single nucleotide position in a conserved repeated sequence element in the rDNA molecule, compete with each other *in vivo* for replication in the same macronucleus (1,4–6). Such competition is not predicted for chromosomes under strict cell cycle control, in which every chromosomal replicon is replicated once and only once per cell cycle (reviewed in 7). Therefore, physically or genetically marked allelic forms of the replicon can be used as a sensitive assay for differences in non-cell cycle controlled replication capability (1,6,8).

The replication origin of the rDNA minichromosome used during vegetative division has been localized by electron microscopy to within a ± 0.3 kb interval located in the 1.9 kb rDNA 5′ non-transcribed spacer (5′ NTS) (9). This interval includes two ~270 bp DNase I hypersensitive domains, one or both of which contain the functional origin of replication (10). Phylogenetically conserved repeated sequence elements, called Type I repeats, are located in these DNase I hypersensitive regions, as well as in the promoter (reviewed in 3). Molecular genetic studies have revealed *cis*-acting sequences, located both in the general area of the mapped origin and in the rRNA promoter, involved in control of replication of the normal 21 kb palindromic, linear rDNA (1,4). Mutations in the Type I repeats and promoter affect origin control (1,4,6,11 and reviewed in 3). Each of these mutations causes the rDNA minichromosome allele with the wild-type (strain C3) sequence to out-compete the rDNA allelic form with the mutated element during vegetative cell division, while maintaining the same overall rDNA copy number. Based on these molecular genetic findings, it was proposed that the total level of rDNA replicons in the macronucleus is determined by competition for limiting amounts of a *trans*-acting, positive replication factor that binds Type I repeats and that rRNA gene dosage is controlled by controlling the total copy number of its origin region (1).

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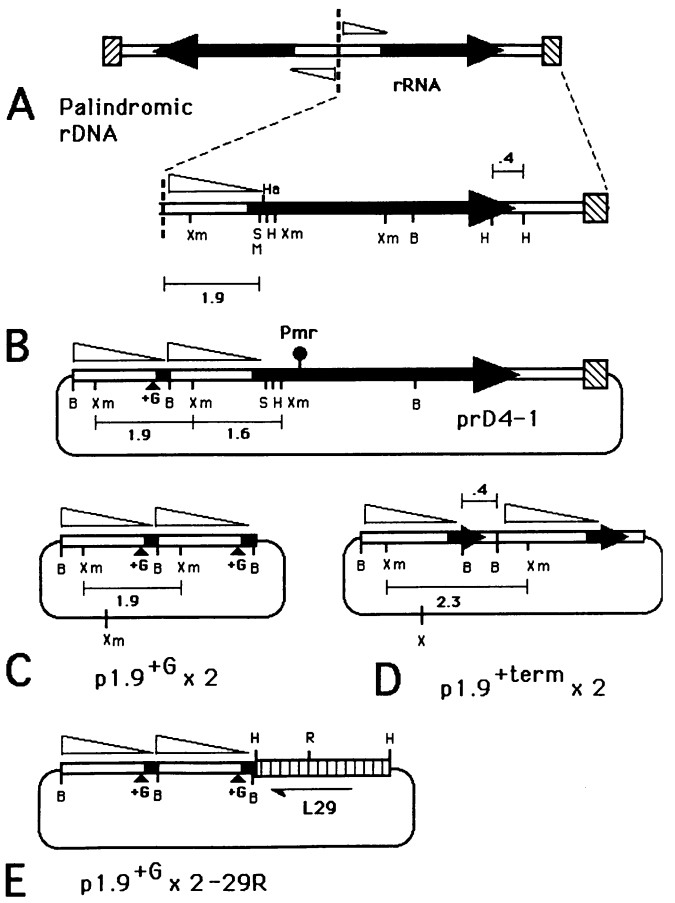

Figure 1. Maps of the linear rDNA and circular plasmids used in transformation of *T.thermophila* macronuclei. (**A**) The palindromic linear rDNA is shown schematically. The map below of half of the palindrome indicates relevant restriction enzyme sites and fragment sizes. Solid arrow, rRNA transcription unit; vertical dashed line, center of symmetry of rDNA; open bars, rDNA 5′ NTS and 3′ NTS; open triangle indicates orientation of the 1.9 kb 5′ NTS, which contains the replication origin; diagonally hatched box, telomeric sequence. The 0.4 kb *Hin*dIII fragment indicates the termination segment. B, *Bam*HI; H, *Hin*dIII; Ha, *Hae*III; M, *Mbo*I; S, *Sau*3AI; Xm, *Xmn*I. (**B**) Map of circular plasmid prD4-1 (14). Plasmid prD4-1 carries half the rDNA palindrome plus an extra tandem copy of the 5′ NTS plus a promoter subcloned from wild-type strain C3 rDNA (12) (present as a 1.9 kb *Bam*HI fragment) in the bacterial vector pBR322. Upward arrowhead, +G insertion mutation in the rRNA promoter; Pmr, base change causing paromomycin resistance. (**C**) Map of plasmid p1.9^{+G}×2. (**D**) Map of plasmid p1.9$^{wt+term}$×2. (**E**) Map of plasmid p1.9^{+G}×2-29R. Hatched bar, L29 gene with coding region indicated with arrow. R, *Eco*RI. Note that pUC or pBR322 vector sequences (curved lines) are not to scale.

The 5′ NTS has been further implicated in the control of rDNA replication and/or maintenance in experiments using circular rDNA constructs containing one 10.5 kb half palindrome to transform macronuclei. However, these constructs invariably recombined into the endogenous linear palindromic rDNA by homologous recombination (12). In contrast, when a tandem duplication of the 1.9 kb 5′ NTS (including the origin region and rRNA promoter) from strain C3 rDNA was placed next to the rest of the rDNA sequence in a circular construct, prD4-1 (Fig. 1B), intermolecular unequal homologous crossing over between tandem 1.9 kb repeats generated linear rDNA replicons containing progressively more tandem 1.9 kb repeats in the central region of the molecule (8,13). Eventually the cells accumulated linear rDNA forms carrying both rRNA coding sequences and >20–30 tandem 1.9 kb repeats/molecule (8,13). The recombination products with more 1.9 kb 5′ NTS repeats reproducibly out-competed those with fewer repeats, consistent with the importance of these 5′ NTS sequences in replication control.

The accumulation of high numbers of tandem 1.9 kb repeats on rDNA molecules carrying rRNA coding sequences was not predicted from the model described above, which proposed that rDNA chromosome copy number is determined by monitoring the total copy number of 5′ NTS sequences (1). Therefore, it was suggested that instead, replicons with more origins have an increased probability of replication, because they compete more effectively for replication factors (8). In this modified model, rRNA gene copy number is determined by the total number of replicons carrying origin control sequences.

Here we report a test of a prediction of this modified model: that additional copies of these sequences maintained on molecules separate from the rRNA genes should proportionately depress rRNA gene levels. However, we show that the dosage of rRNA genes in the macronucleus remains normal in the presence of a 2- to 3-fold excess of molecules containing only 1.9 kb repeats. Hence, rRNA gene levels are not controlled solely by monitoring the copy number of the 5′ NTS sequence.

In previous work the introduced rDNA replicons eventually attained and maintained high copy numbers, often completely replacing the endogenous rDNA allele (8). However, it was possible that their high copy number was a consequence of selection for the required high gene dosage of their physically linked rRNA coding sequences, since in eukaryotes normal cell growth generally requires a high rRNA gene dosage. The results presented here also show that a tandem array of the 1.9 kb 5′ NTS sequence, initially introduced as two tandem repeats, is sufficient to confer ARS (autonomously replicating sequence) function and maintenance at high copy numbers in *Tetrahymena* macronuclei, in the absence of any selection or rRNA transcription *in cis*. We show that these properties of the 1.9 kb region can be exploited for transformation of *Tetrahymena* with a selectable marker.

MATERIALS AND METHODS

Cell strains and culture

Tetrahymena thermophila SB2120 (C3-*rmml Pmr*$^{+}$) and strain SB210 (6,12) were generously provided by E. Orias (University of California at Santa Barbara). All the cells were grown at 30°C in 2% PPYS [2% proteose peptone (Difco), 0.2% yeast extract (Difco), 0.003% Sequestrine (Ciba-Geigy Corp., Summit, NJ)] with 100 U/ml penicillin, 250 pg/ml amphotericin B and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY) in flasks with aeration by gentle swirling on a gyratory shaker or without swirling in Petri dishes. Strains and transformant lines were maintained as stock cultures by passaging in 1% PPYS medium at 3 week intervals.

Transformation of *T.thermophila*

The microinjection procedure was essentially the same as that described previously (12). An estimated 10^2–10^3 molecules/macronucleus were injected, into the macronucleus of *T.thermophila* strain SB2120 so that mixtures of different constructs could be co-injected, allowing selection of a marker carried on an unlinked co-injected DNA molecule. To monitor transformation with a

selectable genetic marker, plasmid prD4-1 was used (14). All DNA samples for microinjection consisted of an ~1:1 molar ratio of plasmid prD4-1 and the specific plasmid of interest, the exception being p1.9^{+G}×2-29R, which carries its own cycloheximide resistance marker and was microinjected alone. *Escherichia coli* DH5α cultures harboring different plasmids were grown up separately and the two cultures were combined before plasmid DNA extraction. Plasmid preparations were purified by CsCl gradient centrifugation and after ethanol precipitation were dissolved in double-distilled H_2O to a concentration of 1–2 mg/ml. After the plasmids were injected, single cells or a group of five cells were transferred to 1 ml 2% PPYS in a 24-well plate. Serial transfers (1% of the culture) were made daily into 1 ml 2% PPYS in 24-well plates, both with (100 μg/ml paromomycin) and without selective medium up to the sixth transfer (~60 generations). When growth in paromomycin was observed, cells were grown up in 2% PPYS without paromomycin. Non-selective medium was used for the subsequent series of transfers. The drug selection procedure for p1.9^{+G}×2-29R injectants was the same as that for paromomycin selection, except that cycloheximide (15 μg/ml) was used instead of paromomycin.

DNA isolation from transformants

Total cellular DNA was isolated from 3 ml cultures after different numbers of cell transfers. An equal volume of prewarmed (55°C) NDS (500 mM EDTA, 10 mM Tris–HCl, pH 9.5, 2% SDS) was added to the cell pellet. Predigested pronase was added to 2 mg/ml, mixed and samples were incubated at 55°C for 16 h. DNA was purified by two phenol–chloroform extractions and two ethanol precipitations.

To quantitate levels of rRNA gene coding sequences and 1.9 kb origin sequences, DNA was extracted from 50 ml cultures of untransformed recipient SB2120 cells and transformant lines B and C that had been maintained for ~200 generations after transformation without paromomycin selection. The DNA was analyzed by Southern blotting, using as probe ^{32}P-labeled plasmid prD1, consisting of the entire rDNA (one half palindrome) plus pBR322 vector (12). Ratios of hybridization intensities of various bands were determined using a Molecular Dynamics PhosphorImager.

Plasmid construction

prD4-1. Plasmid prD4-1 consists of half of the palindromic rDNA and an extra 1.9 kb *Sau*3AI fragment inserted in the *Bam*HI linker at the 5′-end of the rDNA, creating *Bam*HI sites at both ends of the 1.9 kb fragment. A spontaneous +G mutation just upstream from the rRNA transcription initiation site, occurred in the extra 1.9 kb *Bam*HI sequence (Fig. 1; 14).

p1.9^{+G}×2. Two 1.9^{+G} segments derived from prD4-1 were cloned as tandem repeats of the *Bam*H1 fragment into the bacterial vector pUC119.

p1.9^{+term}×2. A wild-type 1.9 kb *Bam*H1 fragment was excised from prD2 (12) (in which the insertion of an extra G residue had not occurred; 14) and was ligated at its 3′-end to a 0.4 kb *Hin*dIII fragment [encompassing a transcriptional terminator sequence (term)]: *T.thermophila* rDNA bp 8365–8774 (15), creating a 2.3 kb fragment. The 2.3 kb segment was inserted as a pair of tandem direct repeats in pUC119.

Construction of a *Tetrahymena* ARS plasmid carrying a selectable cycloheximide resistance (L29) gene

The *T.thermophila* macronuclear genomic gene encoding ribosomal protein L29 (TL 29 gene) was cloned by exploiting its homology with the cloned L29 gene of *Saccharomyces cerevisiae*. Since genetically isolated cycloheximide-resistant mutants of yeast have a mutation of the Met 39 to Gln or Lys (see 16), we changed the Met39 of TL29 to a Lys. A 0.2 kb *Eco*RI–*Hin*cII fragment from the genomic TL 29 gene encompassing the Met39 codon was subcloned into pUC119. The plasmid was grown in *E.coli* RZ1032 (*dut*$^-$, *ung*$^-$, *F*′) and the cells were infected with phage VCS M13 (Stratagene). Single-stranded phage DNA was sequenced to verify the sequence of the insert. A complementary 22 base oligonucleotide with a Lys codon (CTT) in the center corresponding to the Met39 position of the TL29 gene was used for site-directed mutagenesis of the 0.2 kb *Eco*RI–*Hin*cII fragment which was recloned back into the genomic TL29 gene.

RESULTS

Co-transformation with a circular origin region plasmid and a circular plasmid carrying a selectable rRNA gene

We determined whether two copies of a 1.9 kb rDNA segment, encompassing the replication origin plus rRNA promoter, were sufficient to allow episomal maintenance of a plasmid at high copy number in *Tetrahymena* macronuclei. The circular plasmid p1.9^{+G}×2 (Fig. 1C) was co-injected with prD4-1 into macronuclei. The cells (strain SB2120) transformed by microinjection contained a mutant rDNA allele, *rmm1*, which carries a point mutation in a Type I repeat (1), allowing prD4-1 sequences to out-replicate and eventually replace the endogenous *rmm1* rDNA (12–14). The dominant selectable paromomycin resistance marker, *Pmr1*, in the rRNA gene (17) of prD4 allows selection of prD4-1 transformants. In addition, the upstream 1.9 kb tandem repeat in prD4-1 includes a spontaneous mutation (+G) in the promoter sequence (Fig. 1B, solid triangle), abolishing the function of this upstream promoter (14,18). We refer to this 1.9 kb *Bam*HI fragment carrying the +G mutation as 1.9^{+G}.

Of the 228 injected single cells which gave rise to viable independent clonal lines, 13 were shown to be transformed by direct screening using Southern blot analysis with a bacterial vector probe, prior to any selection for paromomycin resistance. Figure 2, lanes 3–5, shows some of the results for representative transformant line A and two sublines of transformant B. In 11 lines, both the 6.7 and 3.2 kb *Bam*HI bacterial vector fragments, diagnostic of prD4-1 and p1.9^{+G}×2 respectively, were detected in amounts and ratios that varied from one transformant line to another. In the other two lines only the fragment from the p1.9^{+G}×2-vector was detectable. The presence of plasmid bands even before selection by exposure to paromomycin showed that both prD4-1 and p1.9^{+G}×2 accumulated to high copy numbers in the unselected cell population, in some transformant lines attaining an estimated average of >10^2 copies/macronucleus. All 13 transformant lines were replica plated to medium containing paromomycin and were shown to be paromomycin resistant; no other paromomycin-resistant lines were identified among the 228 injected lines. Paromomycin resistance is indicative of high copy numbers of the *Pmr1*

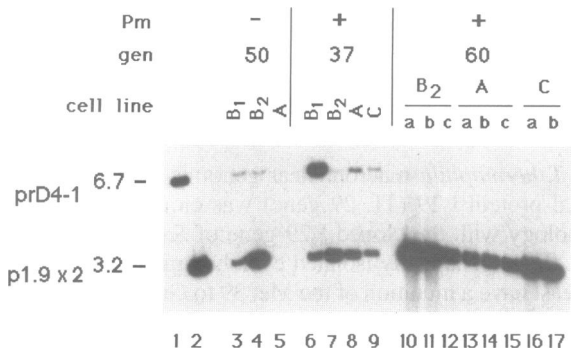

Figure 2. Co-transformation and maintenance of plasmids prD4-1 and p1.9^{+G}×2. Strain SB2120 *T.thermophila* cells were microinjected with a mixture of p1.9^{+G}×2 and prD4-1 in a 1:1 molar ratio. The injected cells were serially transferred into 2% PPYS medium without paromomycin. DNA was extracted at 50 generations from lines of injectants which had never been exposed to paromomycin, digested with *Bam*HI, fractionated in a 0.8% agarose gel, Southern blotted and hybridized to a ^{32}P-labeled pUC119 probe. At 30 generations an aliquot of each culture was also transferred to selective medium containing 100 mg/ml paromomycin, the remainder being transferred without selection. The paromomycin-resistant survivor cultures from this transfer were estimated to be at generation 37. Subsequent transfers were all in paromomycin-free medium and cell lines were periodically subcloned. Lanes 3–5, sublines of transformant B (B1, B2) and transformant line A at 50 generations (no paromomycin). The faint p1.9^{+G}×2 and prD4-1 bands in transformant A were clearly visible in other autoradiograms from the same DNA sample. At 37 and 60 generations (plus and minus paromomycin lanes respectively) DNA samples were extracted from transformant lines A and C and sublines B1 and B2 (lanes 6–17). Transformant lines and their generations are indicated. $B2_{a-c}$, A_{a-c} and $C_{a,b}$ were subclones of the B2, A and C cell lines respectively. Lanes 1 and 2, marker plasmids prD4-1 and p1.9^{+G}×2 mixed with recipient cell SB2120 DNA and digested with *Bam*HI before loading. See Figure 1 for *Bam*HI sites in both plasmids. *Bam*HI digestion generates a pUC-hybridizing 6.7 kb band characteristic of prD4-1 (8) and a 3.2 kb band diagnostic of the pUC vector sequence in p1.9^{+G}×2.

marker derived from prD4-1, which usually eventually integrates into high copy number linear rDNA by recombination (12,17).

Following paromomycin selection, the average copy numbers of both prD41 and p1.9^{+G}×2 bacterial vector sequences increased to ~10^3 or more per macronucleus, although only prD4-1 carries paromomycin resistance. Southern blot analyses for representative transformant lines after paromomycin selection are shown in Figure 2, lanes 6–9. This and additional Southern blot data also ruled out the possibility that p1.9^{+G}×2 had integrated by homologous recombination into prD4-1, since no restriction fragments of the sizes predicted from such integration were seen. We conclude that co-transformation by p1.9^{+G}×2 and prD41 occurred at a high frequency (13/13 transformants).

Molecular forms of the transforming DNA

The maintenance of p1.9^{+G}×2 and prD4-1 plasmid sequences was followed over the course of several hundred cell generations in several transformant lines with detailed analyses on DNAs at 30–40, ~60, ~100 and ~200 generations. Similar results were obtained with different transformant lines. However, the kinetics of appearance of different molecular forms of the transforming DNAs differed somewhat from line to line, as observed previously for cells transformed with prD41 alone (8).

Circular forms of p1.9×2 in transformants. By ~60 generations, the prD4-1-derived pBR322 sequence was barely detectable in the transformants (Fig. 2, lanes 10–17). This agreed with previous results using transformation with this molecule alone (8,14), in which by ~60 generations homologous recombination of prD4-1 with the linear endogenous rDNA had greatly reduced the level of prD4-1-derived pBR322 sequence (8). In contrast, the copy number of the 3.2 kb pUC vector fragment derived from p1.9^{+G}×2 had increased dramatically by ~60 generations (Fig. 2, lanes 10–17). Figure 3A shows Southern blot analyses of a representative transformant line (A) at ~60 generations and two of its sublines (A-1 and A-2), made at ~60 generations and analyzed at ~100 generations. With uncut transformant DNA, a pUC probe (Fig. 3A, lanes 1–6) and the 1.9 kb sequence (data not shown) both hybridized to two slowly migrating DNA species in transformant A (Fig. 3A, open triangles). The most prominent of these species migrated more slowly than limit mobility linear DNA (thick bar in Fig. 3A) or any of the bands present in the original uncut input p1.9^{+G}×2 and prD4-1 plasmids (Fig. 3A, lanes 1 and 2). An rRNA coding sequence probe (black bar in Fig. 1A) showed only a trace of hybridization to a low mobility species, which did not differ between untransformed and transformed cell DNAs (Fig. 3A, lanes 9 and 10, upper arrow). To confirm that the majority of the pUC-hybridizing low mobility DNA species contained only p1.9^{+G}×2 sequences, transformant DNA was digested with *Hpa*I or *Bgl*II. Each enzyme cuts within the rRNA coding sequence (Fig. 4B), but not in p1.9^{+G}×2. Such digestion did not reduce the amount of the slowly migrating forms detected with the pUC probe. This is shown in Figure 3B, lanes 5–7, for *Hpa*I-digested DNA (compare Fig. 3A, uncut DNA lanes 4–6).

Non-linear DNA forms such as circles or catenanes characteristically migrate slowly behind the limit mobility position for linear DNA. The presence of circular forms was confirmed by transformation of *E.coli* with DNA prepared from *T.thermophila* transformant cells at various intervals up to ~150 generations after microinjection and recovery of p1.9^{+G}×2 plasmid sequences from *E.coli* transformants. The numbers of *E.coli* transformants were roughly reflective of the total levels of bacterial vector sequences present in each of the *T.thermophila* DNA samples, as shown by Southern blot analyses similar to that shown in Figure 2. In addition, representative *E.coli* transformants were analyzed by restriction digestion. In each case the ratio of p1.9^{+G}×2- and prD4-1-derived plasmids reflected the relative ratios of these plasmids in the corresponding *T.thermophila* DNA. Both monomeric and dimeric forms of p1.9^{+G}×2 were recovered in the *E.coli* transformants.

Taken together, these results showed that, up to at least ~100 generations, p1.9^{+G}×2 was maintained in circular forms that were often larger than the original input plasmid.

Integration of p1.9 kb sequences into linear rDNA and other sequences. Southern blot analysis also showed that p1.9^{+G}×2 integrated into linear rDNA. When the rRNA coding sequence probe was hybridized to uncut transformant DNA, essentially all hybridization was to both 21 kb and longer rDNA forms (Fig. 3A, lanes 10–12). These were interpreted to be linear from the restriction digestion analyses described below and because they migrated at or faster than the limit mobility position (black bar to left of Fig. 3A). These longer linear DNAs also hybridized with the pUC probe (Fig. 3A, lanes 4–6). To determine their structure, transformant DNA was digested with various restriction

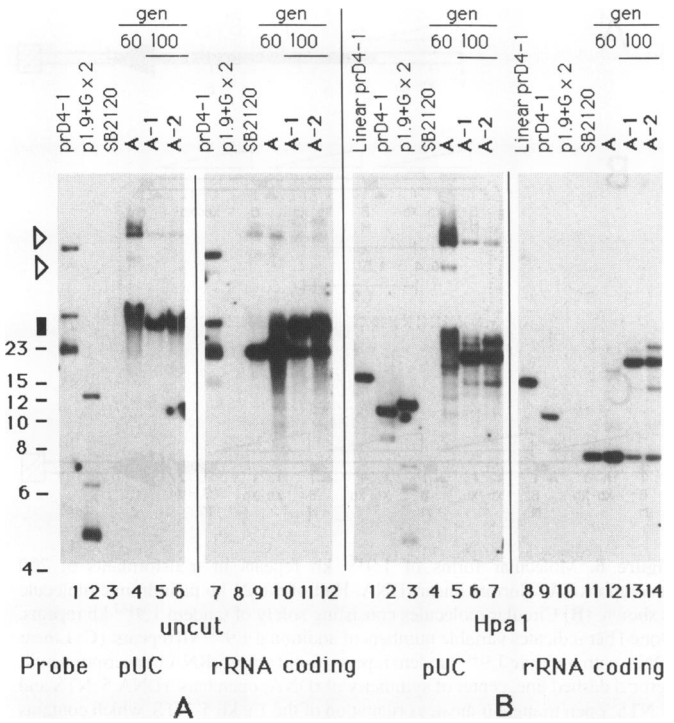

Figure 3. Molecular forms of pUC bacterial sequence retained in p1.9^{+G}×2 transformants up to 100 generations. DNA was extracted from transformant A at 60 generations (A, lanes 4 and 10; B, lanes 5 and 12) and from two subclones of transformant A, isolated as single cell lines (A-1 and A-2) at 100 generations (A, lanes 5, 6, 11 and 12; B, lanes 6, 7, 13, 14). Transformant A is the same DNA sample shown in the *Bam*HI-digested lane 13 of Figure 2. Southern blotted DNAs were probed with either a pUC119 probe (A, lanes 1–6; B, lanes 1–7) or the rRNA coding sequence probe shown in Figure 1B (A, lanes 7–12; B, lanes 8–14). Bar, limit mobility position for linear DNAs; arrowheads, major circular forms of p1.9^{+G}×2 sequences in transformants. Marker fragment sizes are indicated in kb. (**A**) Uncut DNA. Lanes 3 and 9, untransformed strain SB2120 control DNA; lanes 1, 2, 7 and 8, prD4-1 and p1.9^{+G}×2 plasmid marker lanes respectively (plasmid DNA mixed with untransformed SB2120 DNA). (**B**) *Hpa*I-digested DNA. Lanes 1 and 8, linear prD4-1 marker lanes; lanes 4 and 11, untransformed strain SB2120 control DNA; lanes 2, 3, 9 and 10, prD4-1 and p1.9^{+G}×2 plasmid marker lanes (for marker lanes, plasmid DNA was mixed with untransformed SB2120 DNA).

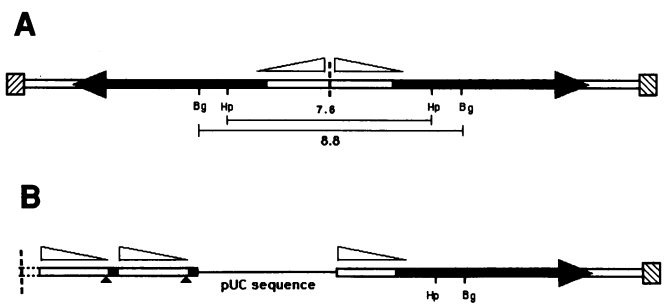

Figure 4. Homologous recombination to produce linear rDNA molecules containing p1.9×2 sequences. (**A**) Endogenous 21 kb palindromic rDNA. Hp, *Hpa*I site. (**B**) Linear rDNA form resulting from homologous recombination of one or more pUC1.9×2 copies in 21 kb linear rDNA.

enzymes, including *Hpa*I and *Bgl*II, which cut the 21 kb palindromic rDNA to give a 7.6 (*Hpa*I) or 8.8 kb (*Bgl*II) band which spans the central region of the palindrome and also hybridizes with the rRNA coding sequence probe (Fig. 4). For each digest this band was still present in transformant DNA at ~60 and ~100 generations [Fig. 3B, lanes 12–14 (*Hpa*I) and data not shown], showing that some of the rDNA was still present in the normal 21 kb form. However, in the A-1 and A-2 sublines at 100 generations the rRNA coding sequence probe (as well as the pUC and 1.9 kb probes) also hybridized to a major and a few minor longer *Hpa*I fragments (Fig. 3B, lanes 6, 7, 13 and 14). This and additional Southern blot data from *Bgl*II and other digests (data not shown) established that the 1.9 kb segment and its physically linked pUC sequence had integrated by homologous recombination into the endogenous linear rDNA, producing a predominant linear form of the rDNA with an extra 13–15 kb inserted in the central region of the palindrome, as shown in Figure 4B.

We also detected, at much lower abundance, linear DNA molecules that hybridized to pUC sequences but which did not appear to be associated with rRNA coding sequences by two criteria: they did not hybridize with the rRNA coding sequence and their heterogeneous *Bgl*II and *Hpa*I fragments containing pUC sequences were of different sizes from those predicted by homologous recombination into rDNA [Fig. 3B, lanes 5–7 (faint *Hpa*I bands between 10 and 15 kb) and data not shown]. These were not analyzed further.

An array of tandem 1.9^{+G} repeats alone is an autonomously replicating sequence (ARS)

Although the copy numbers of pUC bacterial vector sequence in p1.9^{+G}×2 transformants remained high for up to ~150 generations, these dropped progressively during subsequent cell transfers. In contrast, the copy numbers of the 1.9^{+G} segment became very high. Representative results are shown for two transformant lines, B and C, analyzed at ~200 generations (Fig. 5). With undigested DNA (Fig. 5A) there was significant hybridization with the labeled 1.9 kb probe to DNA forms that did not enter the gel (solid arrowhead) or that entered the gel but migrated more slowly than the limit mobility position for linear DNA (open arrowhead). As discussed above, such slow migration is characteristic of circular DNA. Neither rRNA coding sequence nor pUC vector probes hybridized detectably to these regions (data not shown) and untransformed cell DNA showed no hybridization of the 1.9 kb probe to these regions (lane S). Thus the 1.9 kb hybridization to these regions of the gel was specific to transformed cells and to this probe. The slowly migrating DNA was not cut by restriction enzymes which cut in the rest of the rDNA and in the pUC119 vector sequence but not in the 1.9 kb repeats (Fig. 5B, *Sph*I, *Hae*III, *Hin*dIII and *Pst*I lanes; see rDNA restriction sites in Fig. 6A). Conversely, digestion with restriction enzymes which do cut within the 1.9 kb repeat sequence in the constructs converted all the 1.9 kb sequence to the expected sizes for monomeric repeat units (Fig. 5B, *Xba*I, *Mbo*I and *Bam*HI lanes; see Fig. 6 for restriction maps).

The 1.9 kb probe (Fig. 5A) and rRNA coding sequence probe (data not shown) also hybridized to the limit mobility position for linear DNA (Fig. 5A, vertical bar). The 1.9 kb probe also hybridized to a series of faster migrating bands which were resolvable into a ladder with steps at ~1.9 kb intervals after *Hae*III or *Hin*dIII digestion (Fig. 5B). At this stage (~200 generations) all the 21 kb palindromic rDNA had been replaced by these longer linear forms (Fig. 5A). Long autoradiographic exposures showed only very faint pUC hybridization in this linear molecule region (data not shown). These results indicated that linear rDNAs with

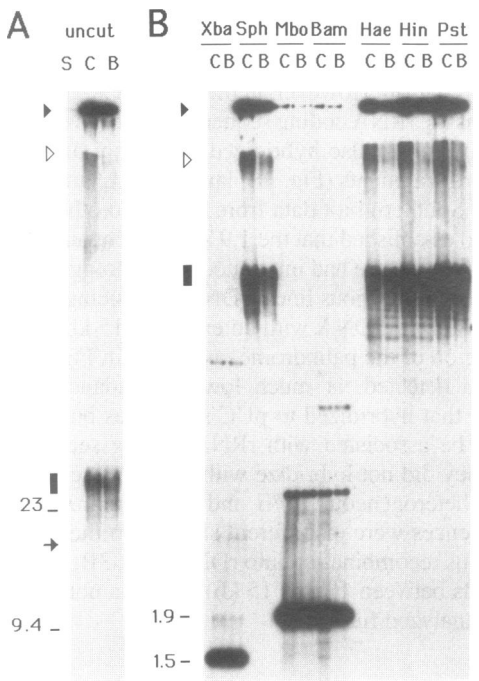

Figure 5. Non-linear molecules containing 1.9 kb repeats in transformants at 200 generations. Total DNA samples were extracted from the control untransformed recipient strain SB2120 and from transformant cell lines B and C that had been maintained for 200 generations without paromomycin selection. After treatment with various restriction enzymes the DNA was fractionated by 0.8% agarose gel electrophoresis, Southern blotted and hybridized to a 1.9 kb probe. (**A**) Undigested DNA. S, untransformed SB2120 DNA; C and B, transformants C and B at 200 generations. Solid bar, position of limit mobility for linear DNA; solid and open arrowheads, circular DNA forms; arrow, position at which any 21 kb linear DNA present would have run. (**B**) DNA from transformants C and B at 200 generations digested with *Xba*I, *Sph*I, *Mbo*I, *Bam*HI, *Hae*III, *Hin*dIII or *Pst*I as indicated. The expected sizes for monomeric units of tandem 1.9 kb repeats: 1.5 + 0.4 kb for *Xba*I digestion and 1.9 kb for *Bam*HI or *Mbo*I digestion (see Fig. 6). The 0.4 kb *Xba*I fragment from the 1.9 kb repeat is not included in the figure.

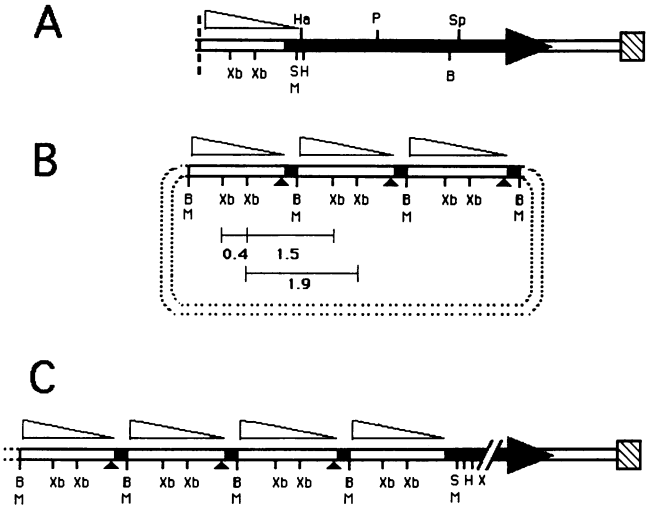

Figure 6. Molecular forms of 1.9^{+G} kb repeats in transformants at 200 generations. (**A**) Normal linear rDNA. Half of the 21 kb palindromic molecule is shown. (**B**) Circular molecules consisting solely of tandem 1.9^{+G} kb repeats. Dotted bar indicates variable numbers of additional 1.9^{+G} kb repeats. (**C**) Linear rDNA with multiple 1.9^{+G} tandem repeats. Solid arrow, rRNA transcription unit; vertical dashed line, center of symmetry of rDNA; open bars, rDNA 5′ NTS and 3′ NTS; open triangle indicates orientation of the 1.9 kb 5′ NTS, which contains the replication origin; diagonally hatched box, telomeric sequence. B, *Bam*HI; H, *Hin*dIII; Ha, *Hae*III; M, *Mbo*I; P, *Pst*I; S, *Sau*3AI; Sp, *Sph*I; X, *Xmn*I; Xb, *Xba*I. Note that unlike *T.thermophila* B strain rDNA, the C3 strain-derived rDNA lacks an *Sph*I site at position 1014 (15).

many tandem 1.9^{+G} repeats eventually accumulated during the course of vegetative cell divisions (Fig. 6C).

Hence, by ~200 generations, 1.9 kb 5′ NTS repeats were found in two predominant forms: large circular molecules consisting solely of multiple tandem 1.9 kb repeats which had lost pUC vector sequences and tandem 1.9 kb repeats integrated by homologous recombination into the central region of linear rDNA, which also largely lacked pUC sequences.

Normal levels of rRNA coding sequences are maintained in the presence of excess rDNA origin regions

We tested directly whether the molecules carrying rDNA origins alone depressed the copy number of molecules carrying the rRNA genes plus origin regions. DNA samples were extracted from equal numbers of cells from duplicate logarithmic phase cultures at ~200 generations and, after *Hin*dIII/*Xba*I double digestion, analyzed by Southern hybridization. The results are shown in Figure 7A for two transformant lines, B and C, and the control untransformed cells. The transformant C DNA is the same DNA sample as that analyzed in Figure 5. *Hin*dIII/*Xba*I digestion of the rDNA generates several fragments common to both the transformants and untransformed controls (bands without asterisks, Fig. 7A). In all lines the hybridization intensities of these bands relative to total DNA were similar, indicating that the rRNA gene copy number was the same in transformants and untransformed control cells. The 0.4 and 1.5 kb *Xba*I fragments (asterisks in Fig. 7A) together comprise the tandem 1.9 kb origin repeats derived from plasmid $p1.9^{+G}\times2$ (see Fig. 6B and C). The intensities of the 0.4 kb band in transformants C (lanes 1–2) and B (lanes 3–4) were respectively ~40 and ~20 times that in the untransformed cell controls (lanes 5–8). As the normal rDNA abundance is 10^4 copies/cell, the total copy number of the 1.9 kb rDNA origin segments was ~$2–4 \times 10^5$ copies/cell in these transformants at ~200 generations. Hence, the 1.9 kb origin region escaped copy number control in the transformants, while the copy number of the rRNA coding sequence and its linked 3′ NTS remained wild-type.

As was shown in Figure 5, the excess 1.9 kb sequences were present both in long linear molecules carrying rRNA genes and physically unlinked, apparently circular molecules consisting entirely of tandem 1.9 kb repeats. The sizes of these slowly migrating bands in Figure 5 that entered the gel were estimated by comparing their migration with relaxed circular DNAs of known lengths (data not shown). These experiments indicated a maximum size of ~20 kb (~10 1.9 kb repeats) for the circular forms. Partial restriction digestions and reversed field gel electrophoresis showed that the long linear forms typically contained 20–30 1.9 kb repeats (see also 8). Quantitating their hybridization intensities and correcting for their length differences allowed the ratio of the circular 1.9 kb only replicons to molecules carrying rRNA genes to be conservatively estimated as at least 1:1. In some transformant sublines the ratio was over 3:1 (data not shown).

By ~200 generations all the transformant cell lines and their sublines showed abnormal morphologies: all cells were either

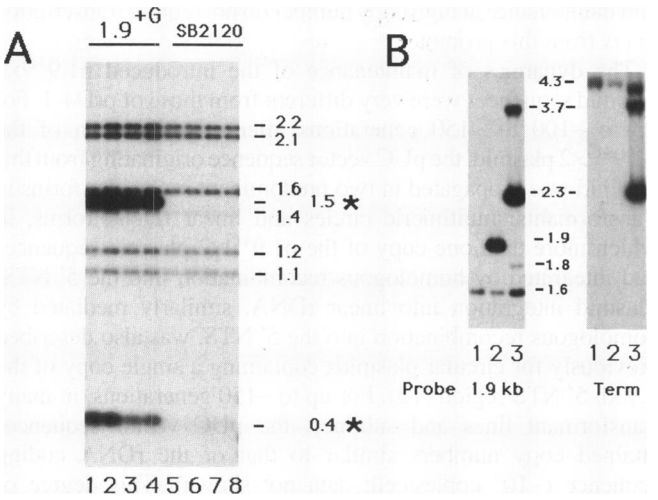

Figure 7. Copy numbers of mutant and wild-type tandem 1.9 kb sequences in transformants. (**A**) DNA samples were extracted from the same numbers of log phase cells transformed by a mixture of p1.9^{+G}×2 plus prD4-1 and untransformed control SB2120 cells. DNA was double digested with *Hin*dIII and *Xba*I, fractionated by 1.4% agarose gel electrophoresis, Southern blotted and hybridized to a ^{32}P-labeled probe containing the entire sequence of prD4-1. Lanes 1–4, duplicate DNA samples extracted independently from transformants C and B respectively; lanes 5–8, quadruplicate samples extracted independently from untransformed recipient strain SB2120. The 2.2, 2.1, 1.6, 1.2 and 1.1 kb bands are *Hin*dIII fragments derived from the rRNA coding sequence, its junction with the 5′ NTS and the 3′ NTS adjacent to the telomeric sequence (8). The faint 1.4 kb band is the 5′ NTS *Xba*I fragment spanning the palindromic rDNA center (Fig. 6B) The 0.4 and 1.5 kb bands (asterisks) are fragments derived from the 1.9 kb sequence repeats (see Fig. 6B). (**B**) Total DNA was extracted at 100 generations from cell lines transformed by a mixture of p1.9$^{wt+term}$×2 and prD4-1 (lanes 3), by prD4-1 only (lanes 2) and from untransformed recipient strain SB2120 (lanes 1). *Xmn*I-digested DNA fractionated by 1.4% agarose gel electrophoresis, Southern blotted and hybridized to a ^{32}P-labeled 1.9 kb probe (left panel). The filter was stripped of probe then hybridized to a second probe, the 0.4 kb *Hin*dIII rDNA term fragment (see Fig. 1A) These were shown to be in circular form and integrated into linear rDNA (data not shown), as described above for the 1.9^{+G} repeats in p1.9^{+G}×2 transformants. The strong 2.3 kb band, seen only in the p1.9$^{wt+term}$×2 transformant (lanes 3) is derived from tandem repeats of the 1.9 + 0.4 kb term segment (see Fig. 1D). The 3.7 and fainter 1.8 kb bands hybridizing to the 1.9 kb probe in (B) lane 3 (and to a pUC119 probe, data not shown) are the right and left junctions of the 1.9$^{wt+term}$ region and the pUC119 vector in p1.9$^{wt+term}$×2 (see Fig. 1D). The 3.7 kb band hybridizing to the term probe in (B) lane 3 is the right junction fragment. The 4.3 kb band shared by all three samples in the term-probed panel is the terminal *Xmn*I fragment of the linear rDNA (see Fig. 1A). In the p1.9$^{wt+term}$×2 transformant there was no evidence of 1.9^{+G} repeats derived from prD4-1 (if there were, a 1.9 kb band would occur in lane 3). However, earlier (at 65 generations) this transformant and other transformant lines showed the expected 1.9 kb band (data not shown).

rounded, irregular or variable in size. Furthermore, the mean cell doubling time in culture in the absence of paromomycin selection was prolonged to 4 h, from the 2.5 h seen for the untransformed control cells. After passaging transformant lines A, B and C for 32 months, the 1.9 kb repeat had accumulated until they comprised ~5–10% by weight of the total cellular DNA.

Origin fragments containing a wild-type rRNA promoter also escape from copy number control

The 1.9^{+G} origin–promoter segment used in the experiments described above contained an extra G residue in a highly conserved sequence in the rRNA promoter (14). This mutation destroys promoter function (14,18). Therefore, to test whether the +G mutation had allowed the tandem 1.9^{+G} repeats to escape copy number control, the construct p1.9$^{wt+term}$×2 was made. In this construct the two 1.9^{+G} repeats were replaced by two tandem repeats of a 2.3 kb unit consisting of a C3 strain 1.9 kb segment containing the wild-type rRNA promoter plus a 0.4 kb *Hin*dIII fragment encompassing the rDNA transcriptional termination region (term; see Fig. 1A). This created an ~180 base rRNA minigene transcription unit (Fig. 7A; 18). The rRNA terminator was included in p1.9$^{wt+term}$×2 to prevent transcription through the origin region interfering with normal origin function (18–20).

SB2120 cells were co-transformed with p1.9$^{wt+term}$×2 and prD4-1 as described (18) and transformant DNA was analyzed at ~85 generations by digestion with *Xmn*I, Southern blotting and successive hybridization to 1.9 and 0.4 kb term fragment probes (Fig. 7B, lanes 3). The hybridization patterns were compared with those of cells transformed with prD4-1 alone (Fig. 7B, lanes 2) and untransformed control cells (Fig. 7B, lanes 1). The strong 2.3 kb band characteristic of p1.9$^{wt+term}$×2 in transformant DNA (Fig. 7B, lanes 3) was shown by additional Southern blot analyses to be derived from tandem repeats of the 1.9 + 0.4 kb term segment, either in circular forms or integrated into linear rDNA (data not shown). The 1.6 kb fragment common to transformed and untransformed cells (Fig. 7A) spans the junction of the rDNA 5′ NTS with the 5′-end of the rRNA coding sequence (see Fig. 1B). The ratio of the origin segment to the rRNA coding sequence in the transformant line shown was 6:1, as calculated from PhosphorImager measurement of the hybridization intensities of the 2.3 and 1.6 kb bands. Thus, like the 1.9^{+G} sequence, tandemly repeated wild-type 1.9 kb sequence containing a functional rRNA promoter can also escape copy number control.

A cloning vector for *T.thermophila* based on the maintenance properties of the 1.9^{+G} sequence

The finding that tandemly repeated 1.9^{+G} kb segments alone can confer both ARS function and high copy number on a replicon suggested the utility of this region as a vector for transformation of *Tetrahymena* macronuclei. A dominant selectable drug resistance marker was constructed using the macronuclear gene encoding ribosomal protein L29, cloned from *T.thermophila* DNA and mutated by site-directed mutagenesis to encode an altered L29 protein (L29^{MK39K}). Cloning of the same L29 gene from *T.thermophila* and a similar strategy for converting it into a cycloheximide resistance marker have been reported independently (16) and, using a different vector, expression of this similarly mutated L29 gene has previously been shown to confer cycloheximide resistance in *Tetrahymena* (16). The L29^{MK39K} gene was inserted into p1.9^{+G}×2 to construct plasmid p1.9^{+G}×2-29R (Fig. 1E). This construct was microinjected alone into macronuclei of vegetatively dividing *T.thermophila* strain SB210. Four cycloheximide-resistant transformants were obtained from 118 viable microinjected cells. They remained cycloheximide resistant for as long as they were tested (several hundred cell generations). All four transformant lines exhibited resistance to similar levels of cycloheximide (45 mg/ml compared with 1.5 mg/ml cycloheximide for the untransformed control strain SB210) and successive transfers into cycloheximide did not increase their resistance levels.

Molecular analyses similar to those described above showed that the introduced L29^{M39K} gene was maintained in three

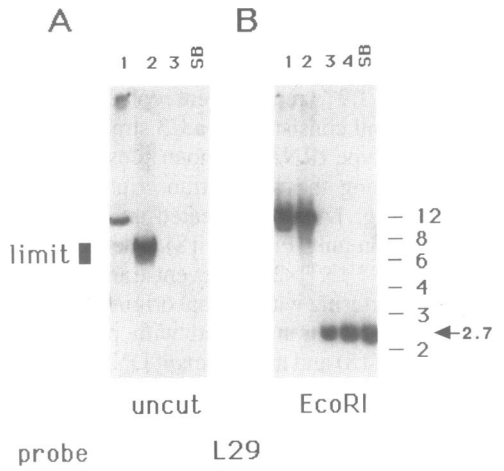

Figure 8. Southern blot analysis of DNA from cell lines transformed by and probed with 1.9^{+G}×2-29R (see Fig. 1E). (**A**) Undigested DNA. Lanes 1–3, transformants 1–3 respectively; SB, untransformed SB2120 control. (**B**) DNA digested with *Eco*RI. Lanes 1–4, transformants 1–4 respectively; SB, untransformed SB2120 control. Note that in (B) lanes 3, 4 and SB are loaded with 10 times more DNA than lanes 1 and 2.

predominant forms. In transformants 1 and 2 the $L29^{M39K}$ gene was present as tandem arrays in high copy number, retaining the restriction map of the original transforming $p1.9^{+G}$×2-29R construct (Fig. 8 and data not shown). The amplified form was predominantly circular in transformant 1 and predominantly linear in transformant 2 (Fig. 8A, uncut DNA lanes). In both transformants the $L29^{M39K}$ gene copy number was up to 30 times higher than the normal copy number of the endogenous L29 gene. In a separate experiment, four additional independent transformants were obtained, all with the same amplified circular forms as transformant 1. Restriction analyses showed that in transformant 2 the plasmid sequences, including the $L29^{M39K}$ gene, had integrated into the rDNA (data not shown). However, transformants 3 and 4 contained no additional hybridization of the L29 probe above that in the untransformed SB210 control (Fig. 8B) and no detectable 1.9 kb repeats (data not shown). The high cycloheximide resistance exhibited by transformants 3 and 4 suggested that, as described previously for this gene (16), the M39K mutation had been introduced into the endogenous macronuclear L29 gene copies by homologous recombination and/or gene conversion.

DISCUSSION

We have shown that a tandem array of the 1.9 kb 5′ NTS region of the rDNA, physically unlinked to other *Tetrahymena* or bacterial sequences, is sufficient for long-term maintenance at high copy number in the somatic macronucleus of *T.thermophila*. We separated the rDNA origin and its known control sequences from the rRNA gene carrying a selectable drug resistance marker and found that origin only molecules were able to co-transform and be maintained, initially together with a vector containing a selectable marker and later on their own. Thus this ARS activity does not require selection for the replicon carrying the 1.9 kb repeats. These findings were made with both mutated (+G) 1.9 kb repeats and the wild-type 1.9 kb repeats. As the +G 1.9 kb repeat lacks a functional rRNA promoter sequence, such ARS activity and maintenance at high copy number do not require transcription *in cis* from this promoter.

The dynamics of maintenance of the introduced $p1.9^{+G}$×2 plasmid sequences were very different from those of prD4-1. For up to ~100 to ~150 generations after microinjection of the $p1.9^{+G}$×2 plasmid, the pUC vector sequence originating from this plasmid was propagated in two predominant molecular forms in transformants: multimeric circles and linear rDNA forms, in which more than one copy of the $p1.9^{+G}$×2 plasmid sequences had integrated by homologous recombination into the 5′ NTS. Plasmid integration into linear rDNA, similarly mediated by homologous recombination into the 5′ NTS, was also described previously for circular plasmids containing a single copy of the 1.9 kb 5′ NTS region (12). For up to ~150 generations, in many transformant lines and sublines, the pUC vector sequences attained copy numbers similar to that of the rDNA coding sequence (~10^4 copies/cell; data not shown). This degree of retention of pUC vector sequences from $p1.9^{+G}$×2 contrasts with the rapid loss of the bacterial vector sequences from prD4-1, seen both in these experiments and in previous work with cells transformed by prD4-1 alone (8).

From ~150 generations onwards the pUC sequences derived from $p1.9^{+G}$×2 were progressively lost from transformant cells and by ~200 generations the tandem arrays of 1.9 kb sequences lacked intervening pUC vector sequences. The slowly migrating 1.9 kb sequences were arranged in tandem arrays lacking any pUC vector or other rDNA sequences (Fig. 6B). Linear molecules with arrays of 1.9^{+G} kb rDNA repeats lacking pUC bacterial vector sequences flanked by rRNA genes on linear rDNA had been found previously in cells transformed with prD4-1 alone (8). However, a form consisting of 1.9 kb arrays on circular DNA molecules unlinked to rRNA genes has not been reported previously in *Tetrahymena* macronuclei.

In previous studies when tandem 1.9 kb repeats joined to the rRNA coding sequence were introduced into the macronucleus, the total number of tandem 1.9 kb repeats increased, but the total copy number of rDNA replicons containing the 1.9 kb repeats was not obviously altered (8). In this case the tandem 1.9 kb repeats remained physically joined to the rRNA genes. While these experiments supported the conclusion that rDNA molecules with more origins have an increased probability of replication, because they compete more effectively for *trans*-acting factors (8), they suggested the possibility that the mechanism for setting the rRNA gene dosage involves monitoring the total number of rDNA replicon molecules. However, we show here that even when a significant proportion of the 20-fold excess of total 1.9 kb repeats are in replicons that are physically unlinked to the rRNA genes, the normal copy number of rRNA genes is maintained in macronuclei. We conclude that the level of rRNA gene copies is not set solely by monitoring the copy number of 1.9 kb-containing replicons. Other regions of the rDNA (the rRNA coding region or 3′ NTS) must be required to regulate copy number, acting through a still unknown mechanism.

Replication origins differ considerably in extent and complexity in different eukaryotic systems. *Cis*-acting ARS regions, in the yeast *S.cerevisiae*, include the replication origin itself, as well as the *cis*-acting sequences that augment replication (21,22). These chromosomal replication origins of *S.cerevisiae* resemble those of certain mammalian viruses (21). However, *cis*-acting control regions for chromosomal origins in metazoans appear to be more complex, extending over regions of a few kilobases or more

(24,25 and reviewed in 21). Here we have shown that the 1.9 kb origin region of *T.thermophila* rDNA contains sufficient *cis*-acting sequences for ARS function. Sequence comparisons with other known origin regions suggest that the 1.9 kb region contains multiple potential origin control sequences besides the Type I, II and III repeats and the promoter (26), but these have not been dissected functionally. The recovery of arrays of multiple tandem 1.9 kb repeats may be analogous to a situation which has been demonstrated for human sequences and the ARS consensus sequence of *S.cerevisiae*, which do not by themselves confer ARS activity in a single copy, but can do so when present in a tandem array (27,28). Alternatively, multimeric repeats of the 1.9 kb region may accumulate because increased DNA length increases stability or confers some other replication or maintenance advantage, as was suggested recently for a trypanosome replicon (29).

The accumulation of large amounts of DNA consisting solely of 1.9 kb repeats was somewhat deleterious to cell growth, yet these sequences were maintained at high levels for over 2 years in stock cultures. This situation constitutes an interesting case of 'selfish DNA' (reviewed in 30). We propose that the eventual abundance of these 1.9 kb-containing forms (up to 10% by weight of the total cell DNA) reflects the balance in the cell population between their replication advantage and the consequent growth disadvantage imposed by high copy numbers of these sequences. The high level of replicons attained is particularly striking, because these replicons were not under externally imposed selection, such as drug selection. In contrast amplified ADA genes in a mammalian system (31) which came to represent up to 20% by weight of the cellular DNA, were only maintained at such high levels under continuous selective pressure for high level expression of these genes. The unselected persistence of excess 1.9 kb DNA sequences may therefore provide a useful model system for studying aspects of genome evolution (30).

This work also shows that the $L29^{M39K}$ gene, encoding a mutated ribosomal protein which confers cycloheximide resistance, when introduced on the self replicating plasmid vector with tandem 1.9 kb repeats, can transform macronuclei and be maintained as a high copy number replicon. Transformation of *T.thermophila* macronuclei with other vectors usually occurs by homologous recombination with the endogenous locus, resulting in the inability to recover the complementing sequence (12,16,32). We propose that by introducing a *Tetrahymena* library cloned into the $p1.9^{+G}\times2$-29R plasmid tested here, the associated $p1.9^{+G}\times2$ vector sequences can be used as a tag or shuttle vector to recover the complementing sequence of interest from transformants. The vector $p1.9^{+G}\times2$-29R has recently been successfully tested for macronuclear transformation by electroporation of cells undergoing new macronuclear development (J. Gaertig and M. Gorovsky, personal communication).

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REFERENCES

1 Larson,D.D., Blackburn,E.H., Yaeger,P.C. and Orias,E. (1986) *Cell*, **47**, 229–240.
2 Larson,D.D., Umthun,A.R. and Shaiu,W.-L. (1991) *J. Protozool.*, **38**, 258–263.
3 Kapler,G.M. (1993) *Curr. Biol.*, **3**, 730–735.
4 Yaeger,P.C., Orias,E., Shaiu,W.L., Larson,D.D. and Blackburn,E.H. (1989) *Mol. Cell. Biol.*, **9**, 452–460.
5 Yao,M.-C. and Yao,C.-H. (1989) *Mol. Cell. Biol.*, **9**, 1092–1099.
6 Kapler,G.M., Orias,E. and Blackburn,E.H. (1994) *Genetics*, **137**, 455–466.
7 Coverley,D. and Laskey,R.A. (1994) *Annu. Rev. Biochem.*, **63**, 745–776.
8 Yu,G.-L. and Blackburn,E.H. (1990) *Mol. Cell. Biol.*, **10**, 2070–2080.
9 Cech,T.R. and Brehm,S.L. (1981) *Nucleic Acids Res.*, **9**, 3531–3543.
10 Kapler,G.M. and Blackburn,E.H. (unpublished).
11 Gallagher,R. and Blackburn,E.H. (unpublished).
12 Yu,G.L., Hasson,M. and Blackburn,E.H. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5151–5155.
13 Romero,D.P. and Blackburn,E.H. (1995) *J. Eukaryotic Microbiol.*, in press.
14 Yu,G.L. and Blackburn,E.H. (1989) *Proc. Nat. Acad. Sci USA*, **86**, 8487–8491.
15 Engberg,J. and Nielsen,H. (1990) *Nucleic Acids Res.*, **18**, 6915–6919.
16 Yao,M.C. and Yao,C.H. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 9493–9497.
17 Bruns,P.J., Katzen,A.L., Martin,L. and Blackburn,E.H. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 2844–2846.
18 Pan,W.J., Gallagher,R. and Blackburn, E.H. (1995) *Mol. Cell. Biol.*, submitted.
19 Snyder,M., Sapolsky,R.J. and Davis,R.W. (1988) *Mol. Cell. Biol.*, **8**, 2184–2194.
20 Haase,S.B., Heinzel,S.S. and CalosM.P. (1994) *Mol. Cell.Biol.*, 2516–2524.
21 Depamphilis,M.L. (1993) *Annu. Rev. Biochem.*, **62**, 29–63.
22 Marahrens,Y. and Stillman,B. (1992) *Science*, **255**, 817–823.
23 Bell,S.P. and Stillman,B. (1992) *Nature*, **357**, 128–134.
24 Orr-Weaver,T.L., Johnston,C.G. and Spradling,A.C. (1989) *EMBO J.*, **8**, 4153–4162.
25 Delidakis,C. and Kafatos,F.C. (1989) *EMBO J.*, **8**, 891–901.
26 Dobbs,D.D., Shaiu,W.-L. and Benbow,R.L. (1994) *Nucleic Acids Res.*, **22**, 2479–2489.
27 Krysan,P.J., Smith,J.G. and Calos,M.P. (1993) *Mol. Cell. Biol.*, **13**, 2688–2696.
28 Zweifel,S.G. and Fangman,W.L. (1990) *Yeast*, **6**, 179–186.
29 Metzenberg,S. and Agabian,N. (1994) *Proc. Natl. Acad. Sci. USA*, **91**, 5962–5966.
30 Charlesworth,B., Sniegowski,P. and Stephen,W. (1994) *Nature*, **371**, 215–220.
31 Virta-Pearlman,V.J., Gunaratne,P.H. and Chinault,A.C. (1993) *Mol. Cell. Biol.*, **13**, 5931–5942.
32 Gaertig,J. and Gorovsky,M.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 9196–9200.