MOLECULAR AND CELLULAR BIOLOGY, June 1995, p. 3372–3381 Vol. 15, No. 6
0270-7306/95/$04.00+0

Replication of an rRNA Gene Origin Plasmid in the *Tetrahymena thermophila* Macronucleus is Prevented by Transcription through the Origin from an RNA Polymerase I Promoter

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Received 2 December 1994/Returned for modification 24 January 1995/Accepted 23 March 1995

In the somatic macronucleus of the ciliate *Tetrahymena thermophila*, the palindromic rRNA gene (rDNA) minichromosome is replicated from an origin near the center of the molecule in the 5′ nontranscribed spacer. The replication of this rDNA minichromosome is under both cell cycle and copy number control. We addressed the effect on origin function of transcription through this origin region. A construct containing a pair of 1.9-kb tandem direct repeats of the rDNA origin region, containing the origin plus a mutated (+G), but not a wild type, rRNA promoter, is initially maintained in macronuclei as an episome. Later, linear and circular replicons with long arrays of tandem repeats accumulate (W.-J. Pan and E. H. Blackburn, Nucleic Acids Res, in press). We present direct evidence that the +G mutation inactivates this rRNA promoter. It lacks the footprint seen on the wild-type promoter and produces no detectable in vivo transcript. Independent evidence that the failure to maintain wild-type 1.9-kb repeats was caused by transcription through the origin came from placing a short DNA segment containing the rRNA gene transcriptional termination region immediately downstream of the wild-type rRNA promoter. Insertion of this terminator sequence in the correct, but not the inverted, orientation restored plasmid maintenance. Hence, origin function was restored by inactivating the rRNA promoter through the +G mutation or causing termination before transcripts from a wild-type promoter reached the origin region. We propose that transcription by RNA polymerase I through the rDNA origin inhibits replication by preventing replication factors from assembling at the origin.

Transcription at a DNA replication origin can have positive or negative effects on replication in both prokaryotic and eukaryotic systems. There are two previously published reports of a negative effect of transcription on origin function in eukaryotes. When transcription from a strong promoter proceeds through the ARS1 origin of *Saccharomyces cerevisiae*, function of that ARS is disrupted in vivo (26). Sequences thought to be transcriptional terminators protect the ARS from such functional inhibition. Similarly, it has been reported that replication of a class of autonomously replicating plasmids in human cells is inhibited by transcription through a region thought to be necessary for replication (14). Replication in this system was also restored by placing a transcription termination element downstream of the promoter. Here we address the effect of rRNA transcription through the origin region of the rRNA genes (rDNA) of the ciliated protozoan *Tetrahymena thermophila.*

T. thermophila is a single cell with two nuclei: a micronucleus and a transcriptionally active, polyploid macronucleus, containing acentric chromosomes, which is derived from the diploid, transcriptionally inactive germ line micronucleus. In vegetatively dividing cells the micronucleus divides mitotically; division of the macronucleus is amitotic, and as there is no mechanism to assure proper segregation of differing alleles, daughter cells may receive up to 8% different macronuclear DNA content (19). Following meiosis, *Tetrahymena* cells exchange haploid meiotic micronuclear products which fuse and then divide, a mitotic product of the new micronucleus differentiating into the macronucleus. Macronuclear formation entails site-specific chromosome breakages and telomere addition, DNA deletions, and gene amplification. All genes on the resulting long macronuclear chromosomes (average length, ~600 kb) are amplified to roughly 45 copies, except the rRNA genes, which are located on palindromic, 21-kb minichromosomes (rDNA) and amplified to 10^4 copies. The macronuclear rDNA minichromosome is derived from a single-copy micronuclear rRNA gene. This palindromic minichromosome contains two divergently transcribed copies of the rRNA genes. The 5′ nontranscribed spacer (NTS) of each transcription unit contains an origin of replication of the chromosome, mapped by electron microscopy for strain B rDNA (6) and, more recently, by a two-dimensional gel mapping method (2) for both strain B and C3 rDNA (17a). In both strains, since the rDNA replication origin is located near the center of the minichromosome, in the naturally occurring palindromic rDNA molecule the rRNA transcripts on both sides are directed outwards, away from the origin. Thus, within the natural rRNA transcription unit replication forks and transcription complexes proceed in the same direction (see Fig. 1A).

We have used the macronuclear rDNA chromosome of *T. thermophila* both as an experimental system for the exploration of the control of rDNA replication (24, 28) and for the design of rDNA origin-based vectors (24, 30). In the work reported here, circular constructs derived from the rDNA were introduced into *Tetrahymena* macronuclei and their replication properties were determined. Previous work reported transformation using circular plasmid constructs (prD constructs) containing one rRNA transcription unit and one copy (prD1) or two copies (prD2 and prD4) of the origin region of the chromosome (27, 30). In transformants obtained with each of the initial constructs, the constructs were not retained as autonomous replicons; instead, the rDNA sequences of the construct had invariably recombined into the endogenous linear rDNA

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(30). Thus, it was hypothesized that since the endogenous chromosome contains two origins of replication, incoming rDNA would also need to contain two functional origins in order to be maintained as an episome, because it must compete with the endogenous chromosome. However, when two origins of replication were present, as a pair of tandem direct repeats of a 5′ NTS segment containing the C3 rDNA origin plus the rRNA promoter (prD2 and prD4), only one prD4 transformant line showed evidence of episomal maintenance of the introduced DNA. When this episome (called prD4-1) was recovered by transformation of total transformant DNA into *Escherichia coli*, it was found to have a spontaneous mutation (+G) in the promoter of the upstream rDNA origin (27). rDNA replicons with more tandem 5′ NTS repeats were formed by unequal crossovers between the origin regions of prD4-1 and the endogenous rDNA and these outreplicated molecules with fewer repeats (28). It was proposed that in the original prD4 vector only the upstream origin is functional, because of transcriptional inactivation of the downstream origin by the rDNA promoter of the upstream origin region. Hence, in the mutated vector prD4-1 there would now be two functional origins (see Fig. 1A). In support of this hypothesis, deleting this upstream promoter of prD4 allowed its episomal maintenance (27).

Here we directly test the hypothesis that the +G mutation of prD4-1 inactivates the upstream promoter and that this prevents transcriptional inactivation of the downstream origin and allows maintenance of the vector as an episome. Recently we showed that a tandem direct repeat of a 1.9-kb rDNA origin region in which the rRNA promoter carries the +G mutation is sufficient to confer ARS function on circular replicons in the *Tetrahymena* macronucleus (24). It was shown that +G plasmids could be maintained on circular replicons separate from the functional rRNA genes. This provided an opportunity to examine the replication and maintenance properties of replicons containing just this rDNA origin region without compromising cell viability through interference with normal rRNA gene maintenance and expression. We show that the same plasmid construct, now containing only 1.9-kb repeats with wild-type promoters, is incapable of being maintained. We show that the +G mutation in the promoter abolishes transcription from this promoter as well as the DNase I footprint seen at the wild-type rRNA promoter. Inserting a functional rRNA transcriptional terminator fragment downstream of each wild-type promoter allowed maintenance of the tandem origins. We propose that without an intervening transcription terminator, transcription by RNA polymerase I through the rDNA origin inhibits replication by preventing replication factors from assembling at the origin.

MATERIALS AND METHODS

Cell strains and culture. *T. thermophila* SB2120 (C3-*rmml Pmr*$^+$) was generously provided by E. Orias (University of California, Santa Barbara) (30). All the cells were grown at 30°C in 2% PPYS (2% Proteose Peptone [Difco Laboratories, Detroit, Mich.], 0.2% yeast extract [Difco], 0.003% Sequestrine [CIBA-GEIGY Corp., Summit, N.J.]) with 100 U of penicillin per ml, 250 pg of amphotericin B per ml, and 100 μg of streptomycin (GIBCO Laboratories, Grand Island, N.Y.) per ml in flasks with aeration by gentle swirling on a gyratory shaker or in cultures in petri dishes.

Plasmid construction. (i) prD4-1 (15.2 kb). Construction of plasmid prD4-1 was described previously (27). The parent plasmid prD2 consists of one-half of the palindromic rDNA and an extra 1.9-kb *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 (27, 30). A spontaneous single site mutation, the insertion of a G residue in a run of G residues 16 to 21 bp upstream from the transcription initiation site, occurred in the extra 1.9-kb *Bam*HI sequence, allowing tandem repeats of the 1.9-kb segment to be maintained in rDNA replicons in transformant macronuclei (27, 29) (Fig. 1A). The extra 1.9-kb *Bam*HI segments, with and without the G insertion, are referred to as 1.9^{+G} and 1.9, respectively. A mutation in the 17S rRNA coding region, the *Pmr1* mutation (4), confers paromomycin resistance and served as a selectable marker for identification of prD4-1 transformants.

(ii) $p1.9^{+G} \times 2$ (6.8 kb). Construction of the $p1.9^{+G} \times 2$ plasmid is described elsewhere; the plasmid consists of two tandem direct repeats of the 1.9^{+G} *Bam*HI fragment derived from prD4-1 cloned into the *Bam*HI site in the polylinker of plasmid pUC119 (24).

(iii) $p1.9^{wt} \times 2$ (6.8 kb). This plasmid has the same structure as $p1.9^{+G} \times 2$ except that the two 1.9-kb repeats were derived from plasmid prD2 and contain the wild-type C3 rRNA promoter lacking the +G mutation (30).

(iv) $p1.9^{wt+term} \times 2$ (7.6 kb). The 1.9-kb wt (1.9^{wt}) *Bam*HI fragment from $p1.9^{wt} \times 2$ was ligated, via a custom-made linker, to a 0.4-kb *Hin*dIII fragment derived from the *T. thermophila* rDNA sequence (bp 8365 to 8774 [10]), destroying the *Hin*dIII sites and creating a 2.3-kb *Hin*dIII fragment. On the basis of mapping of the rRNA transcription unit (10), this 0.4-kb *Hin*dIII fragment (referred to as term) encompasses the site of the 3′ end of the rRNA transcript. It also includes all the evolutionarily conserved sequence elements, downstream from the transcription termination site, thought to be involved in termination function (7). The 0.4-kb term fragment was ligated in its correct orientation relative to the rRNA promoter. $p1.9^{wt+term} \times 2$ was constructed by inserting two 2.3-kb (1.9 + term) fragments as tandem repeats into pUC119.

(v) $p1.9^{wt+inv\text{-}t} \times 2$ (7.6 kb). $p1.9^{wt+inv\text{-}t} \times 2$ has the same structure as $p1.9^{wt+term} \times 2$, except that the 0.4-kb term fragment was ligated in inverted orientation to the 1.9^{wt} fragment.

Preparation of plasmid DNA for *Tetrahymena* transformation. *E. coli* DH5α cultures harboring the two plasmids to be coinjected were grown in parallel but separately; the two cultures were combined before DNA extraction. For microinjection into macronuclei, plasmid DNA preparations were purified in a CsCl gradient and dissolved in double-distilled H_2O to a concentration of 1 to 2 mg/ml.

Transformation of *T. thermophila* macronuclei. The microinjection procedure used was as described previously (30). Microinjections were performed with a Zeiss model CZG micromanipulator attached to a Zeiss IM35 inverted microscope. DNA was injected into macronuclei with an Eppendorf model 5242 microinjector. An estimated 2×10^3 to 4×10^3 plasmid molecules were injected per cell. Single cells or a group of five cells were transferred to 1 ml of 2% PPYS in microtiter well plates. Serial transfers of 1% of the culture were made daily into cultures in microtiter well plates. Each day a sample of the culture was also transferred to selective medium (2% PPYS containing 100 μg of paromomycin per ml) for up to six transfers (an estimated 40 to 50 cell generations). When growth in paromomycin was observed, an aliquot of the cell culture was grown up in 2% PPYS without paromomycin. Nonselective medium was used for the subsequent series of transfers.

All test plasmids were constructed without a selectable marker. Therefore, each plasmid to be tested was injected as a mixture with the rDNA plasmid prD4-1 (Fig. 1A). prD4-1 contains a single rRNA gene carrying the paromomycin resistance marker *Pmr1* in the 17S rDNA coding sequence, plus a tandem repeat of the 5′ NTS and +G promoter. As shown previously, the C3 rDNA of SB2120 has a point mutation (the *rmm1* mutation) which allows it to be outcompeted by rDNA replicons carrying the wild-type C3 5′ NTS (18).

Transformation by electroporation with prD4-1 alone was performed as described elsewhere (12b), using essentially the method of Gaertig and Gorovsky (12).

Isolation of cellular DNA from transformants. Total cellular DNA was isolated from 3 ml of culture, spun down in a single microcentrifuge tube by pelleting the first 1.5 ml of culture at full speed for 15 s and then adding the second 1.5 ml of culture and respinning. The cell pellet was resuspended in the residual 100 μl of medium, and to this was added an equal volume of prewarmed (55°C) NDS (500 mM EDTA, 10 mM Tris hydrochloride [pH 9.5], 2% sodium dodecyl sulfate). Predigested pronase (50 μl; 200 μg/ml) was added and mixed well. Samples were incubated at 55°C for 5 to 16 h. DNA was purified by phenol-chloroform extraction two times and ethanol precipitation two times.

RNA analysis by primer extension. Total RNA was extracted from 25 ml of the transformant cell culture during log-phase growth by RNA zol B (Biotecx Laboratories) following the manufacturer's instructions. Oligonucleotide primers were 5′ end labeled with ^{32}P-ATP by bacteriophage T4 polynucleotide kinase (New England BioLabs). Between 30 and 40 μg of cellular RNA was annealed to 1 pmol of ^{32}P 5′-end-labeled primer with 5×10^6 to 10×10^6 cpm in 7.5 μl of primer extension solution (120 mM Tris-HCl [pH 8.5], 67 mM KCl, 10 mM $MgCl_2$, 12 mM dithiothreitol, 33 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] 7% glycerol, all four ribonucleoside triphosphates [0.25 mM each], 5 U of avian myeloblastosis virus reverse transcriptase (Boehringer-Mannheim). Following incubation at 42°C in a water bath for 40 min, reactions were stopped by the addition of EDTA. DNA was ethanol precipitated and analyzed on an 8% polyacrylamide DNA sequencing gel. For an RNA dependence control, two RNA samples were subjected to RNase digestion before serving as substrates for primer extension. RNase A was added to these samples, to a final concentration of 25 μg/ml, and the samples were incubated at 37°C for 2 h. After two phenol-chloroform extractions, the digests were precipitated with ethanol before the primer extension reaction.

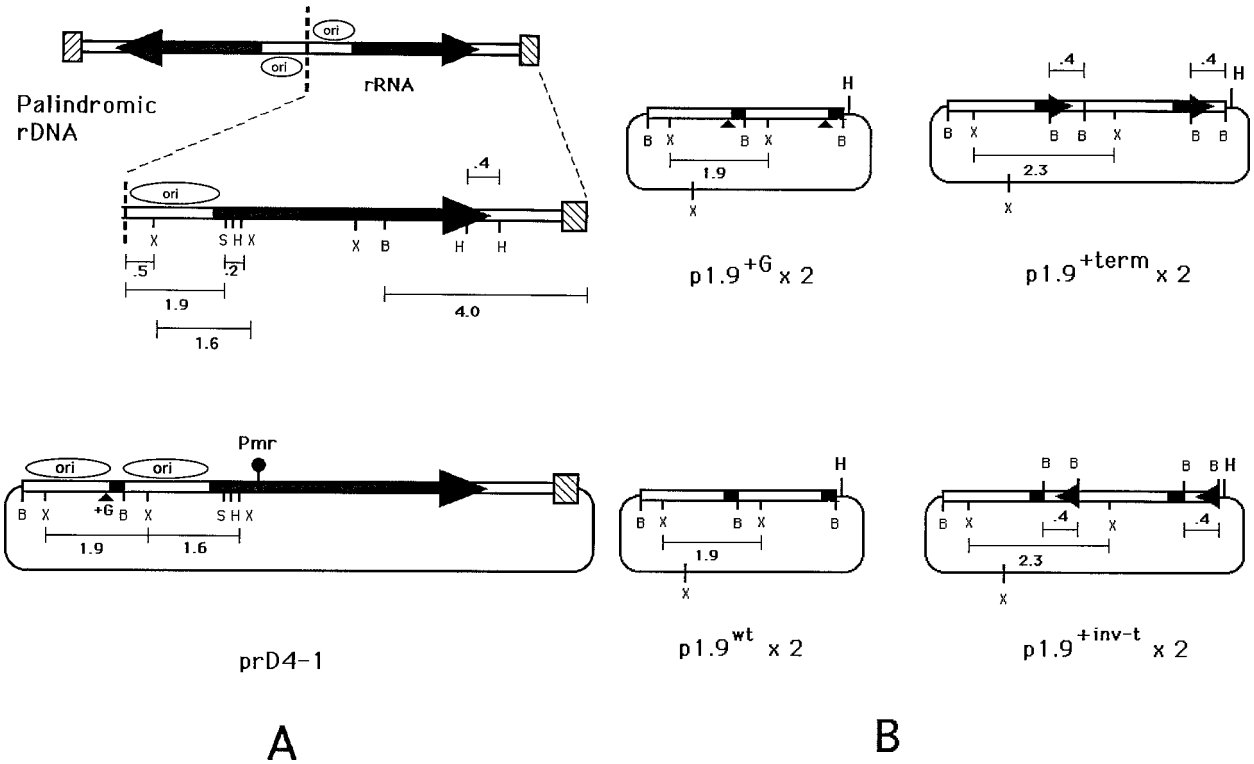

FIG. 1. Structure of palindromic rDNA and its derivative plasmids. (A) (Top) The naturally occurring macronuclear 21-kb palindromic rDNA. Vertical dashed line, center of palindromic rDNA; long solid arrow, rRNA transcription unit (arrowhead denotes transcription termination site); open bars, 5′ NTS and 3′ NTS; open triangle, 1.9 or 1.9^{+G} region and its orientation; striped box, telomere. (Bottom) Plasmid prD4-1. Solid triangle, +G insertion mutation; Pmr, paromomycin resistance marker; thin line, 2.7-kb pBR322 vector (between *Bam*HI and *Pvu*I sites). B, *Bam*HI site; H, *Hin*dIII site; S, *Sau*3A I site; X, *Xmn*I site. Only relevant restriction sites are shown. Fragment lengths are roughly to scale for the rDNA regions. Bacterial vector regions (curved lines) are not to scale. (B) Plasmids $p1.9^{+G} \times 2$, $p1.9^{wt+term} \times 2$, $p1.9^{wt} \times 2$, and $p1.9^{wt+inv-t} \times 2$. The inverted arrows for $p1.9^{wt+inv-t} \times 2$ indicate direction of the 0.4-kb termination segment. Thin line, 3.2-kb pUC119 vector. All other symbols are as in Fig. 1A.

DNase I footprinting analyses. (i) Growth of cells and isolation of nuclei. *T. thermophila* SF137, a strain hemizygous for wild-type C3 rDNA, was created by a cross between SB1934 and CU374 (17) and was used as the wild-type control. prD4-1 transformant cells were CU428 crossed with B2086, transformed with the vector prD4-1, and passaged for about 330 generations. $p1.9^{wt+term} \times 2$ transformant cells were grown up from a stock tube. The number of mutant repeats was checked by Southern analysis and was identical to the number present when the primer extension analysis was done. One liter or 200 ml of cells was grown at 30°C in 2% PPYS containing penicillin, streptomycin, and fungizone to a density of approximately 3×10^5 cells per ml. Nuclei were isolated by lysis in Nonidet P-40 as described previously (5), except that nuclei were resuspended in 700 μl of buffer A (60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 15 mM Tris-HCl [pH 7.4], 2 mM $CaCl_2$, 15 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride).

(ii) Treatment of nuclei and naked DNA with DNase I. Aliquots (100 μl) of nuclei in buffer A, containing approximately 10^6 macronuclei or about 11 μg of macronuclear DNA, were preincubated at 25°C for 2 min. DNase I at 0, 1.25, 2.5, 5, 10 or 20 U/ml in 100 μl of buffer A containing 20 mM $MgCl_2$ was then added, so the final $MgCl_2$ concentration was 10 mM and the final DNase I concentration was half that in 20 mM $MgCl_2$. Samples were mixed for 10 s and incubated for 1 min total at 25°C in the presence of DNase I. Reactions were stopped by the addition of EDTA to 30 mM and by the addition of 200 μl of 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1% sodium dodecyl sulfate (SDS), and 0.5 mg/ml proteinase K. Samples were incubated at 37°C overnight, extracted with phenol-chloroform, and precipitated with 2.5 M ammonium acetate and ethanol. DNA was resuspended in 20 μl of TE (10 mM Tris-HCl [pH 7.4], 1 mM EDTA) with RNase at 10 μg/ml to a final concentration of approximately 500 ng/μl. Naked DNA was prepared from nuclei in the same way except that there was no preincubation at 25°C or incubation in the presence of nucleases. (Preincubation for 2 min at 25°C in the presence of buffer A containing 10 mM $MgCl_2$ had no effect on the subsequent pattern of cleavages seen with naked DNA and so was not done routinely). Naked DNA was resuspended in 20 μl of buffer A and 10-μl aliquots were added to 90 μl of buffer A and treated with DNase I as described above for nuclei except that the DNase I concentrations were one-tenth those used for chromatin. DNA was then prepared from these samples in exactly the same way as described above for chromatin and was resuspended in TE and RNase at approximately 500 ng/μl.

(iii) Primer extension reactions. Approximately 500 ng of DNA was mixed with 1 pmol of end-labeled oligonucleotide containing approximately 10^6 cpm in a 5.5-μl volume. DNA was denatured in the presence of oligonucleotide at 90°C for 2 min. A second denaturation was carried out after the addition of 3 μl of 5× *Taq* reaction buffer (250 mM Tris-HCl [pH 9.0], 10 mM $MgCl_2$). The oligonucleotide was annealed to the denatured DNA for 15 min at 37°C. A *Taq* reaction mix (12.5 μl) containing 160 μM deoxynucleoside triphosphates, reaction buffer, and 0.5 U of *Taq* polymerase was added, and primer extension took place at 60°C for 10 min. Sequencing reactions were performed on approximately 40 ng of prD4-1 plasmid DNA. For these samples the primer extension mix included either ddATP or ddTTP. Primer extension was terminated by the addition of 170 μl of DNA preparation solution (10 mM Tris-HCl [pH 8.0], 2.5 mM EDTA, 0.5% SDS, 2.5 μg of tRNA per ml), and the reaction mixtures were incubated at 80°C for 30 min. Samples were extracted with phenol-chloroform and precipitated with the addition of 10 μl of 5 M NaCl, and ethanol. Precipitated DNA was resuspended in 6 μl of sequencing dyes. Each sample (3 μl) was run on a 6% acrylamide–8 M urea sequencing gel for 4 to 6 h at 42 W. These were exposed to X-ray film at −80°C with an intensifying screen for 3 days to 2 weeks.

(iv) Primer sequences. Primer 11, GCTCTAAATTAAATTAGACTTAGTG, begins at −222 from the start site of transcription and extends 5′ to 3′ towards the coding region. Primer 36, CACGAAGTCTCAAAAGTTG, is unique to the C3 rDNA and to domain 2. It is contained within the 42-bp B strain deletion (10) and extends 5′ to 3′ towards the center of the rDNA.

RESULTS

Failure of tandem 1.9^{wt} repeats to be maintained in *Tetrahymena* transformants. A series of test plasmids was constructed, and their ability to be maintained at high copy number was tested by microinjection into the somatic macronucleus of vegetatively growing strain SB2120 *T. thermophila* cells. Each test

TABLE 1. Maintenance of plasmids at high copy numbers in transformants

Test plasmid injected with prD4-1	No. of viable injected cells[a]	No. of Pm-R transformants[b]	High copy no.[c]		Probability that result is due to random fluctuation[d]
			prD4-1	Test plasmid	
p1.9^{+G} × 2	228	13	13	13	
p1.9^{wt} × 2	346	7	6	0	0.027
p1.9$^{wt+term}$ × 2	368	10	9	8	1.000
p1.9$^{wt+inv-t}$ × 2	334	10	4	0	0.006

[a] After microinjection, single cells were isolated and grown in individual wells in the absence of selection. Some injected cells do not survive the injection and transfer.
[b] Wells that grew up from single cells were replica transferred to media containing paromomycin. Cells that grew up in these wells were paromomycin-resistant prD4-1 transformants (Pm-R).
[c] The copy number of rDNA-derived vectors in *T. thermophila* has been determined by comparing known amounts of rDNA with a defined copy number to the plasmid amount. The copy number of the rDNA vectors is roughly 10^3.
[d] Fisher exact test calculation (13) uses "No. of Pm-R transformants" column for calculation of probability that the number of observed transformants that failed to maintain the non-prD4-1 plasmid at high copy number is due to random fluctuation.

plasmid DNA was microinjected as a ~1:1 molar mixture with the plasmid prD4-1 (Fig. 1A). Plasmid prD4-1 was coinjected to provide the dominant selectable marker *Pmr1*, which confers paromomycin resistance and has been shown previously to allow identification and selection of transformants in coinjection experiments with p1.9^{+G} × 2 (24, 27). Coinjection with prD4-1 also provides an internal control for transformation.

The plasmid p1.9^{wt} × 2, containing two tandem direct repeats of the C3 strain rDNA 5′ NTS with its wild-type rRNA promoter, was constructed and is shown schematically in Fig. 1B, along with the map of plasmid p1.9^{+G} × 2. Plasmid p1.9^{+G} × 2 differs from p1.9^{wt} × 2 only in the +G mutation in the run of six G's at −16 to −21 from the transcription start site, present in both copies of the rRNA promoter (27). We have shown elsewhere, using microinjection, that the frequency of cotransformation of *T. thermophila* macronuclei by p1.9^{+G} × 2 with prD4-1 is high (>90%) (24). Accordingly, in the present study of p1.9 × 2 derivatives, transformants were identified by selection for paromomycin resistance carried by prD4-1. Furthermore, even in the absence of selection for the paromomycin resistance marker carried on prD4-1, the p1.9^{+G} × 2 and prD4-1 sequences attain high copy numbers in transformant lines (24). Therefore, after selection, total cellular DNA was extracted following different numbers of cell transfers up to ~60 generations and analyzed by restriction digestion and Southern blotting to a pUC119 sequence probe. This analysis was used to determine whether the injected test plasmid could replicate and be maintained at high copy numbers in the transformant.

When p1.9^{wt} × 2 was coinjected into macronuclei in an equimolar mixture with prD4-1, no p1.9^{wt} × 2 was retained in the macronucleus (Table 1). This finding contrasted with the result of the same experiment using coinjection of p1.9^{+G} × 2 with prD4-1. As reported elsewhere (24) and included in Table 1, in an experiment in which 228 cells injected with a mixture of p1.9^{+G} × 2 and prD4-1 gave rise to viable cell lines, 13 independent transformant lines were obtained. These were initially identified by screening the 228 clonal lines by Southern hybridization to the pUC119 probe, prior to selection for the *Pmr1* marker. In these transformants both plasmids were present at high copy numbers (24) (Table 1). As shown in Fig. 2, lanes 6 to 8, for three such representative transformants, the pUC119 probe detects a characteristic 6.7-kb *Bam*HI fragment from prD4-1 and a 3.2-kb band from p1.9^{+G} × 2. The 1.9^{+G} repeat unit plus its flanking bacterial vector sequence at high copy numbers in the transformants became detectable between the first and third transfers. Once detected in a transformant line, these sequences became more abundant over the course of the next transfers (reference 24 and data not shown).

Coinjection of 346 cells with an equimolar mixture of prD4-1 and p1.9^{wt} × 2 resulted in seven paromomycin-resistant (Pm-R) transformants (Table 1). However, although the 6.7-kb *Bam*HI fragment containing bacterial vector sequence from prD4-1 plasmid attained high copy numbers in six of the seven Pm-R lines, as seen by Southern blotting analysis, none of the seven transformants showed any evidence for maintenance of p1.9^{wt} × 2. Two representatives of the six prD4-1-hybridizing transformants are shown in Fig. 2, lanes 10 and 11; only the 6.7-kb, prD4-1-derived bacterial vector-hybridizing fragment was detectable. Figure 2, lane 9, shows the single remaining transformant in which no bacterial vector fragment from either plasmid was detectable by Southern blotting. To confer paromomycin resistance, the *Pmr1* marker must be maintained at high copy numbers (4), but we have shown previously that this can also be achieved by homologous recombination with the endogenous rDNA, resulting in loss of the bacterial vector sequence (27, 28, 30). Such recombination accounts for the paromomycin resistance of this transformant line (data not shown).

The lack of transformants that maintained p1.9^{wt} × 2 as well as prD4-1 (0 of 6 transformants) was compared with the frequency of maintenance of p1.9^{+G} × 2 (13 of 13 transformants) and was statistically significant according to the Fisher exact test (13) (Table 1).

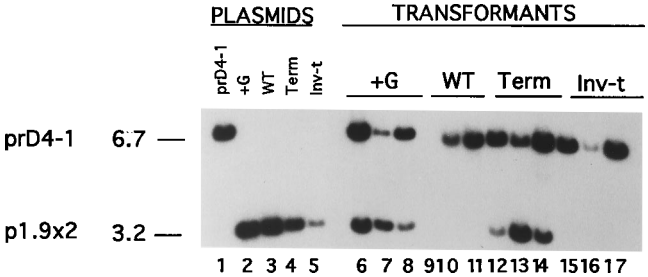

FIG. 2. Replication and amplification of injected plasmids. Plasmids p1.9^{+G} × 2, p1.9^{wt} × 2, p1.9$^{wt+term}$ × 2, and p1.9$^{wt+inv-t}$ × 2 were mixed separately with prD4-1 and injected into *T. thermophila* SB2120. Transformants were selected by paromomycin. Total DNA was fractionated on a 0.8% agarose gel after *Bam*HI digestion, Southern blotted, and hybridized to ^{32}P-labeled pUC119 probe. The 6.7-kb hybridization band is characteristic of prD4-1, and the 3.2-kb band is from the test plasmid (Fig. 1). Lanes 1 to 5, plasmids prD4-1, p1.9^{+G} × 2, p1.9^{wt} × 2, p1.9$^{wt+term}$ × 2, and p1.9$^{wt+inv-t}$ × 2 mixed with total DNA of recipient SB2120 cells before being loaded onto an agarose gel. Lanes 6 through 17, DNA from transformants transformed by mixtures of prD4-1 and p1.9^{+G} × 2 (lanes 6 to 8), p1.9^{wt} × 2 (lanes 9 to 11), p1.9$^{wt+term}$ × 2 (lanes 12 to 14), or p1.9$^{wt+inv-t}$ × 2 (lanes 15 to 17). Three transformant DNA samples were picked at random from each group of transformants.

Placing the rRNA transcriptional termination region between the rRNA promoter and the rDNA origin allows tandem origin-plus-promoter repeats to be maintained at high copy number. Since the 1.9-kb 5′ NTS origin-plus-promoter in p1.9^{wt} × 2 has the wild-type strain C3 rDNA sequence, both the origin and promoter are functional. Furthermore, two copies of the same C3 1.9-kb sequence (in palindromic orientation) confer on an rDNA replicon the ability to outreplicate the endogenous rDNA of the recipient C3-rmm1 (rDNA maintenance mutant) strain cell (18, 30). In spite of this replication advantage conferred by the wild-type C3 5′ NTS, transformants failed to maintain p1.9^{wt} × 2 yet were able to maintain p1.9^{+G} × 2 at high copy numbers. This suggested that transcription from the active rRNA promoters in the circular p1.9^{wt} × 2 construct may block its replication. To test this directly, a transcription terminator was placed downstream of each wild-type promoter sequence. The plasmid p1.9$^{wt+term}$ × 2 was constructed, in which a 0.4-kb *Hin*dIII fragment encompassing the rDNA transcriptional terminator sequence, here called term, was ligated to the 3′ end of the 1.9^{wt} *Bam*HI fragment. In the resulting 2.3-kb segment (1.9$^{wt+term}$) the wild-type promoter is followed by the rRNA transcriptional terminator in its correct orientation relative to the rRNA promoter. Two 2.3-kb 1.9$^{wt+term}$ fragments were inserted as a pair of tandem repeats into pUC119, creating plasmid p1.9$^{wt+term}$ × 2 (Fig. 1B). Out of 368 cells injected with a 1:1 mixture of p1.9$^{wt+term}$ × 2 and prD4-1, 10 transformants were identified by selection for paromomycin resistance. Plasmid prD4-1 sequences were maintained at high copy numbers in 9 of 10 transformants, while p1.9$^{wt+term}$ × 2 sequences were maintained at high copy numbers in 8 of 10 transformants (Table 1, and representative results in Fig. 2, lanes 12 to 14).

These results were consistent with the interpretation that the term fragment terminated transcripts from the wild-type promoter in p1.9$^{wt+term}$ × 2 before they reached sequences in the origin, preventing transcriptional inactivation of the origin. Furthermore, inverting the orientation of the 0.4-kb term fragment, in plasmid p1.9$^{wt+inv-t}$ × 2 (Fig. 1B), prevented plasmid maintenance. Of 10 paromomycin-resistant transformants obtained by coinjecting a mixture of p1.9$^{wt+inv-t}$ × 2 and prD4-1, four had high copy numbers of prD4-1, but none showed any evidence of p1.9$^{wt+inv-t}$ × 2 maintenance (Table 1 and Fig. 2, lanes 15 to 17). As described above, the paromomycin resistance of the remaining six transformants was likely due to recombination of the *Pmr1* marker into the endogenous rDNA. The finding that tandem 1.9^{wt} rDNA origin repeats were maintained only when a transcription terminator was interposed, in the correct orientation, between the wild-type promoter and the origin supported the hypothesis that RNA polymerase I transcription through the origin region inhibits origin function.

An additional G in the −16 to −21 G tract in the rRNA promoter inactivates promoter function. Two types of analysis showed that the mutant +G promoter was nonfunctional.

(i) Transcript analysis. To test directly whether the +G mutation destroyed promoter function, RNA primer extension was used to compare levels of transcripts from tandem 1.9^{+G} versus 1.9^{wt} promoters. Specific primers were used to distinguish between transcripts initiated from these promoters and the normal rRNA transcripts made from the functional rRNA gene promoters.

A subline of one of the eight transformants that maintained high copy numbers of p1.9$^{wt+term}$ × 2, subline 42b, was grown to a total of ~100 generations by successive transfers in nonselective medium. A portion of a 50-ml culture of 42b cells in logarithmic growth phase was used for DNA extraction, and another aliquot was used for extraction of RNA for primer extension. First, the arrangement and copy number of the wt+term repeats in 42b were determined by Southern blotting analysis of the 42b DNA. The 1.9$^{wt+term}$ sequence derived from 1.9$^{wt+term}$ × 2 was present in high copy number as tandem arrays arranged as in Fig. 3A, as shown by digestion with *Xmn*I, *Hin*dIII (Fig. 3C), *Bam*HI, and *Nhe*I (data not shown). In the *Xmn*I digests the strong 2.3-kb band hybridized to a 1.9-kb 5′ NTS fragment probe (Fig. 3C, lane 2) as well as to a 0.4-kb term probe (data not shown). The control DNAs analyzed in this experiment were untransformed cell DNA (Fig. 3B and C, lanes 1) and DNA from a cell line (RG) transformed by prD4-1 alone (Fig. 3B and C, lanes 3). As expected from previous results (28), long *Hin*dIII fragments extending to limit mobility were found in the DNAs of both the p1.9$^{wt+term}$ × 2 and prD4-1 transformants (Fig. 3A and Fig. 3C, lanes 5 and 6). These long fragments result from unequal crossovers between tandem 1.9-kb 5′ NTS repeats (28), and they replace the 4.3-kb band spanning the center of palindromic rDNA seen in untransformed cells (compare Fig. 1A and Fig. 3A; Fig. 3C).

The 1.6-kb *Xmn*I fragment common to transformants and

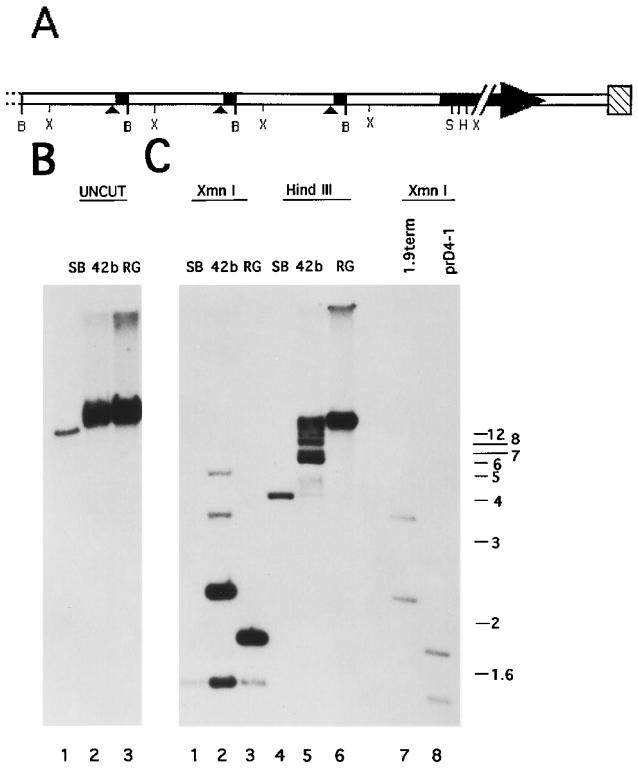

FIG. 3. Amplification of 1.9^{+term} and 1.9^{+G} sequences in transformants. (A) Depiction of one half of a molecule that consists of the endogenous rDNA into which mutant origin-promoter regions from the incoming vector have recombined. Symbols are as in Fig. 1A. Note that since *Hin*dIII cuts only in the transcription unit the central *Hin*dIII fragment increases in length in increments of 1.9 or 2.3 kb (for 1.9+term [0.4-kb] regions) as more and more exogenous origin regions recombine into the endogenous chromosome. (B) DNA samples were extracted from cell line RG (transformed by prD4-1), cell line 42b (transformed by a mixture of prD4-1 and p$^{1.9+term}$ × 2), and untransformed recipient cell line SB2120. Samples were separated on a 1.2% agarose gel, Southern blotted, and hybridized to a ^{32}P-labeled 1.9-kb 5′ NTS probe. Lane 1, SB2120; lane 2, 42b; lane 3, RG. (C) Southern blotting analysis of DNAs (Fig. 3B) digested with *Xmn*I (lanes 1 to 3, 7, and 8) or *Hin*dIII (lanes 4 to 6) and hybridized to a ^{32}P-labeled 1.9-kb 5′ NTS probe. Lanes 1, SB2120; lanes 2, 42b; lanes 3, RG; lane 4, SB2120; lane 5, 42b; lane 6, RG; lane 7, p1.9$^{wt+term}$ × 2 plasmid DNA; lane 8, prD4-1 plasmid DNA. Size markers (in kilobases) are shown at right.

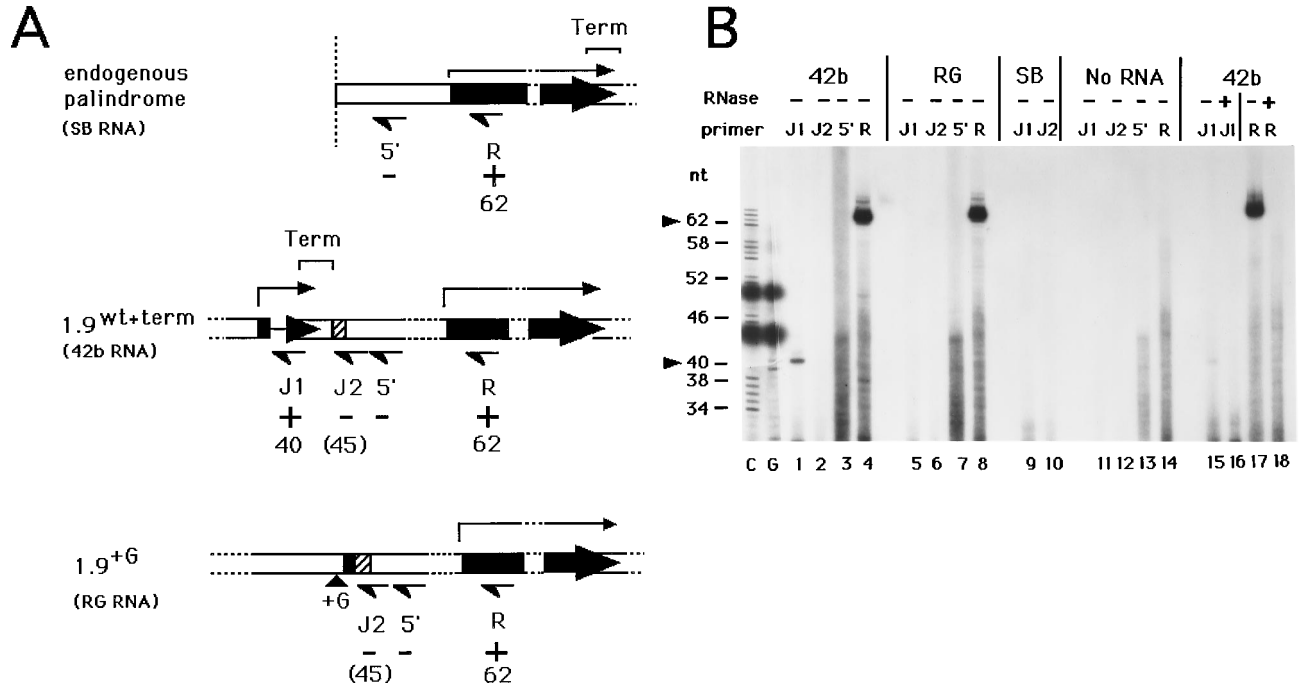

FIG. 4. Primer extension analysis of RG and 42b RNA transcripts. (A) Diagrams of four oligonucleotide primers and tandem repeats of plasmid sequences integrated in transformant's rDNA. Endogenous palindrome, one half of native palindromic rDNA; $1.9^{wt+term}$, tandem repeats of $1.9^{wt+term}$ in the rDNA of $p1.9^{wt+term} \times 2$ transformants; 1.9^{+G}, tandem repeats of 1.9^{+G} in the rDNA of prD4-1 transformants. Corresponding RNA transcripts (Fig. 4B) are indicated in parentheses. Vertical dashed line, center of rDNA. Vertical line, border of tandem repeats of $1.9^{wt+term}$ or 1.9^{+G}. Open bar, rDNA 5′ NTS and 3′ NTS. Solid bar, rDNA transcriptional unit. Solid arrowhead, terminator sequence. Single thin line, 10-bp junction between 1.9-kb sequence and terminator sequence generated during plasmid construction. Solid triangle, +G insertion mutation. Half-arrow, four oligonucleotide primers and their 5′-3′ direction. J1, a 16-mer (5′-CAAGCTGACTCTAGAG-3′). J2, a 16-mer (5′-CAACAACATAGTGCTG-3′). 5′, a 21-mer (5′-ACATCTAATTCTTATCTACTC-3′). R, a 25-mer (5′-TCTTACTGAAGCTCAAATCGAGCTG-3′). Plus and minus signs indicate the results expected if the +G mutation inactivates the promoter and the term segment results in transcription termination. Numbers indicate the corresponding expected lengths of transcripts. (45) is the length expected if the +G promoter were active. Fragment lengths shown are not proportional to their actual sizes. (B) Primer extension of 42b and RG RNA substrates. RNA substrates and primers are marked on the figure. The absence or presence of added RNase is indicated. The two left lanes (C, G) are size markers. Nucleotides (nt) are indicated to the left. Arrows point to the 62- and 40-nt expected sizes for extension products made with primers R and J1, respectively. 42b RNA was extracted from a cell line transformed by a mixture of prD4-1 and $p1.9^{wt+term} \times 2$. The $p1.9^{wt+term} \times 2$-derived 1.9+term sequence was abundantly amplified as tandem repeats in the transformants (line 42b). RG RNA was extracted from cell lines transformed by prD4-1 only. The prD4-1-derived 1.9^{+G} was abundantly amplified as tandem repeats in cell line RG. RNA was also extracted from untransformed recipient strain SB2120. All oligonucleotide primers were described in Fig. 4A. RNA primer extension was carried out under conditions described in Materials and Methods.

untransformed controls (Fig. 3C, lanes 1 to 3) consists of the junction of the 5′ NTS and the rRNA transcription unit of the endogenous rDNA of the recipient cell (Fig. 3A). The gene dosage of rRNA genes is regulated at $\sim 10^4$ per macronucleus even in the presence of high copy numbers of 1.9-kb sequences (24). Therefore, the ratio of intensities of the 2.3- and 1.6-kb bands of an *Xmn*I digest can be used to estimate the copy number of $1.9^{wt+term}$. In lane 2 densitometry indicated that the copy number ratio of $1.9^{wt+term}$ repeats to rDNA coding sequences was about 6.5:1, or an estimated 6.5×10^4 $1.9^{wt+term}$ repeats per cell. The absence of a 1.9-kb hybridizing band in Fig. 3C, lane 2, also showed that at this stage in transformant 42b there were no detectable 1.9^{+G} repeats derived from prD4-1. In the prD4-1-alone transformant RG, the intensity of the 1.9-kb *Xmn*I band relative to the 1.6-kb band (Fig. 3C, lane 3) indicated that 1.9^{+G} repeats derived from prD4-1 were present at over 10^5 copies per cell. Together with the *Hin*dIII results, these data indicated that large numbers of $1.9^{wt+term}$ or 1.9^{+G} repeats had accumulated in the central regions of the rDNA in 42b and RG, respectively, in cells that had grown for greater than 100 generations after transformation.

Total cellular RNA samples were extracted from an aliquot of the same 50-ml cultures of 42b and RG transformants used for the DNA analysis shown in Fig. 3. Primer extension analysis by avian myeloblastosis virus reverse transcriptase was used to determine whether transcripts were made from the $1.9^{wt+term}$, 1.9^{+G}, and functional rRNA promoters. The positions of the four DNA oligonucleotide primers used, and the results of RNA primer extension from each primer expected if the +G mutation inactivates the promoter and if the term segment terminates transcription, are shown schematically in Fig. 4A. The 16-nucleotide primer J1 matched the upstream 6 bases of 0.4-kb term, plus 10 bases of the junction between 1.9 and term that was created during the construction of $p1.9^{wt+term} \times 2$. The 3′ end of J1 was 24 bp downstream of the transcription initiation position predicted for this wild-type copy of the rRNA promoter. Hence, transcription from this promoter is expected to generate a 40-base primer extension product. Oligonucleotide J2 matched the 16 most upstream bases (37 to 52) (10) of the rDNA 5′ NTS present in the 1.9-kb repeat (30). The 3′ end of J2 was located 29 bases downstream of the transcription initiation site position, so that if the 1.9^{+G} promoter in prD4-1 functioned the primer extension product would be 45 bases in length. Oligonucleotide primer 5′ matched a different stretch of the rDNA 5′ NTS (sequence 131 to 111) (10), located downstream of the transcription termination region in $p1.9^{wt+term}$. Transcripts initiated from the $1.9^{wt+term}$ promoter were expected to terminate before reaching this region, and hence 5′ provided a negative control. Primer R, a 25-mer positive control, matched a stretch of the

functional rRNA transcription unit. The 3′ end of primer R was located 37 bases downstream of the wild-type rRNA transcription initiation site, and the normal rRNA transcript from the functional rRNA gene promoter is expected to generate a 62-base primer extension product.

The results shown in Fig. 4B were reproducible and in complete agreement with the predictions schematized in Fig. 4A. Primer 5′ was not extended by RNA from either 42b or RG (Fig. 4B, compare lanes 3 and 7 with their primer-only control in lane 13). In contrast, in both transformants primer R gave a strong predicted primer extension product of 62 bases (Fig. 4B, lanes 4 and 8) which was not generated with primer alone (lane 14), with an RNA preparation that had been treated with RNase A prior to primer extension (lane 18), or with the total cell DNA preparations (data not shown). As predicted, extension of primer J1 resulted in the 40-base primer extension product with RNA extracted from 42b (lane 1) but not with RNA from RG (lane 5) or untransformed recipient SB2120 cells (lane 9). This 40-base primer extension product was abolished by prior treatment of the 42b RNA preparation with RNase A (lane 16). Finally, as predicted, no 45-nucleotide primer extension product was produced from primer J2 by using RNA from either transformant (lanes 2 and 6) or untransformed cells (lane 10). These results showed that the 1.9^{wt} promoter in the $1.9^{wt+term}$ repeat was active, while the 1.9^{+G} promoter produced no detectable transcript.

(ii) Footprinting analysis of wild-type and +G mutant promoters. As the primer extension analysis measured steady-state transcript levels, it could not be excluded that the differences between levels of transcripts from the wild-type and +G rRNA promoters were attributable to different transcript stabilities. Independent evidence that the +G promoters were inactive came from DNase I footprinting analysis of these promoters. Nuclei were prepared from prD4-1 transformants and from control wild-type untransformed C3 cells and subjected to DNase I footprinting. Control naked DNA digestions were performed in parallel on DNA extracted from the same nuclear preparations but deproteinized before DNase I digestion, as described in Materials and Methods. The positions of DNase I cleavages were determined by extension of one of two ^{32}P 5′ end-labeled DNA primers with *Taq* polymerase, followed by analysis of the primer extension products on DNA sequencing gels. Figure 5A, wild-type lanes, shows that a clear DNase I footprint extends from nucleotide 1775 to the transcription start site on the wild-type functional rRNA gene promoter in C3 cells which contain normal 21-kb palindromic rDNA. With the same $p1.9^{wt+term} \times 2$ transformants analyzed in Fig. 3C, a similar promoter footprint was seen (Fig. 5B). In these transformants the ratio of upstream $1.9^{wt+term}$ promoters to rRNA gene promoters was 6.5:1, as described above. Thus, the strength of the footprint in Fig. 5B shows that the majority of these wild-type tandemly repeated upstream promoter repeats, in the context of the 2.3-kb fragment, are also footprinted. In contrast, the corresponding sequence of the +G promoter probed in nuclei from prD4-1 transformant cells showed no difference in DNase I cleavage pattern from the naked DNA pattern (Fig. 5A, transformant lanes). This suggests that the majority of the +G rRNA promoters are either naked or have randomly positioned nucleosomes over them. Note that the primer does not distinguish between the natural and the tandemly repeated promoters so that the footprint is a composite of both. In the case of the +G transformants we estimated from the length of the *Hin*dIII fragment in these cells that the ratio of mutant to wild-type promoters was at least 10:1 (data not shown). Hence, the footprint is derived primarily from the +G mutant promoters. In control footprinting primer extension analyses on the same DNase I-treated samples, a different region of the 5′ NTS was probed (Fig. 6). In the 1.9^{+G} repeats of prD4-1 transformants, a DNase I footprint can be found over a precisely positioned nucleosome located ~800 bp upstream of the promoter in the 5′NTS (12a). This footprint was identical to the DNase I footprint found over this region in the natural 5′ NTS in untransformed cells (Fig. 6). Hence, the lack of a footprint over the promoter regions in the 1.9^{+G} repeats is unlikely to be due to a general disruption of the chromatin structure of the mutant repeats in this unnatural tandem repeat form.

Together, the lack of a detectable footprint and the results of the RNA primer extension analyses provide strong evidence that the +G rRNA promoter is inactive.

DISCUSSION

We have shown here that *T. thermophila* transformants can maintain tandem direct repeats of segments containing the rDNA origin and an rRNA promoter copy only if this promoter is inactive or if the rRNA transcription terminator is inserted between the wild-type rRNA promoter and the downstream origin.

In previous work, in the rDNA plasmid construct prD4, the promoter in the tandem repeat upstream of the functional rRNA gene promoter was wild type and prD4 was not maintained. It was hypothesized that a spontaneous mutation (insertion of a G residue) in this upstream copy of the promoter (Fig. 1A) inactivated this upstream rRNA promoter and thereby allowed the mutated derivative, prD4-1, to be maintained, in contrast to its parent construct prD4 (27). Two possible explanations were advanced for why such upstream promoter inactivation allowed prD4-1 maintenance. The first was that transcription from the upstream promoter through the rDNA replication origin region directly interferes with origin function and that the +G mutation in the upstream promoter of prD4-1 prevents this transcription. However, rRNA gene function is required for expression of the selected *Pmr1* drug resistance marker on prD4-1 and for cell viability. Hence, an alternative explanation for failure to maintain a tandemly repeated wild-type 1.9-kb segment in Pm-R prD4 transformants was that the active upstream rRNA promoter repeat initiated transcripts which occluded the downstream, functional rRNA gene promoter, preventing expression of the *Pmr1* rRNA transcript.

The first model, that transcription through the origin interferes with its function, is more likely to explain the failure to maintain wild-type promoters upstream of origins seen in our subsequent work with $p1.9 \times 2$ derivatives, since no rRNA genes are on these constructs or are required for their maintenance (24). Independent support for this model is demonstrated in this work in which we placed a terminator downstream of the wild-type promoter, which allowed maintenance of tandem arrays of the origin-plus-promoter segments. The requirement for the terminator to be in its correct orientation suggests that this termination function is orientation specific. We also showed by direct analysis of transcript levels that the wild-type promoter in this construct is active, while the +G promoter produced no detectable transcript. Furthermore, the DNase I footprint seen on the wild-type rRNA promoter was specifically absent from the +G promoter. Together, these two findings provide strong evidence that the +G mutation, in a conserved string of G's located at −16 to −21 in the rRNA promoter, inactivates this promoter, relieving transcriptional inactivation of a downstream origin.

Similar inhibitory effects of transcription through a chromo-

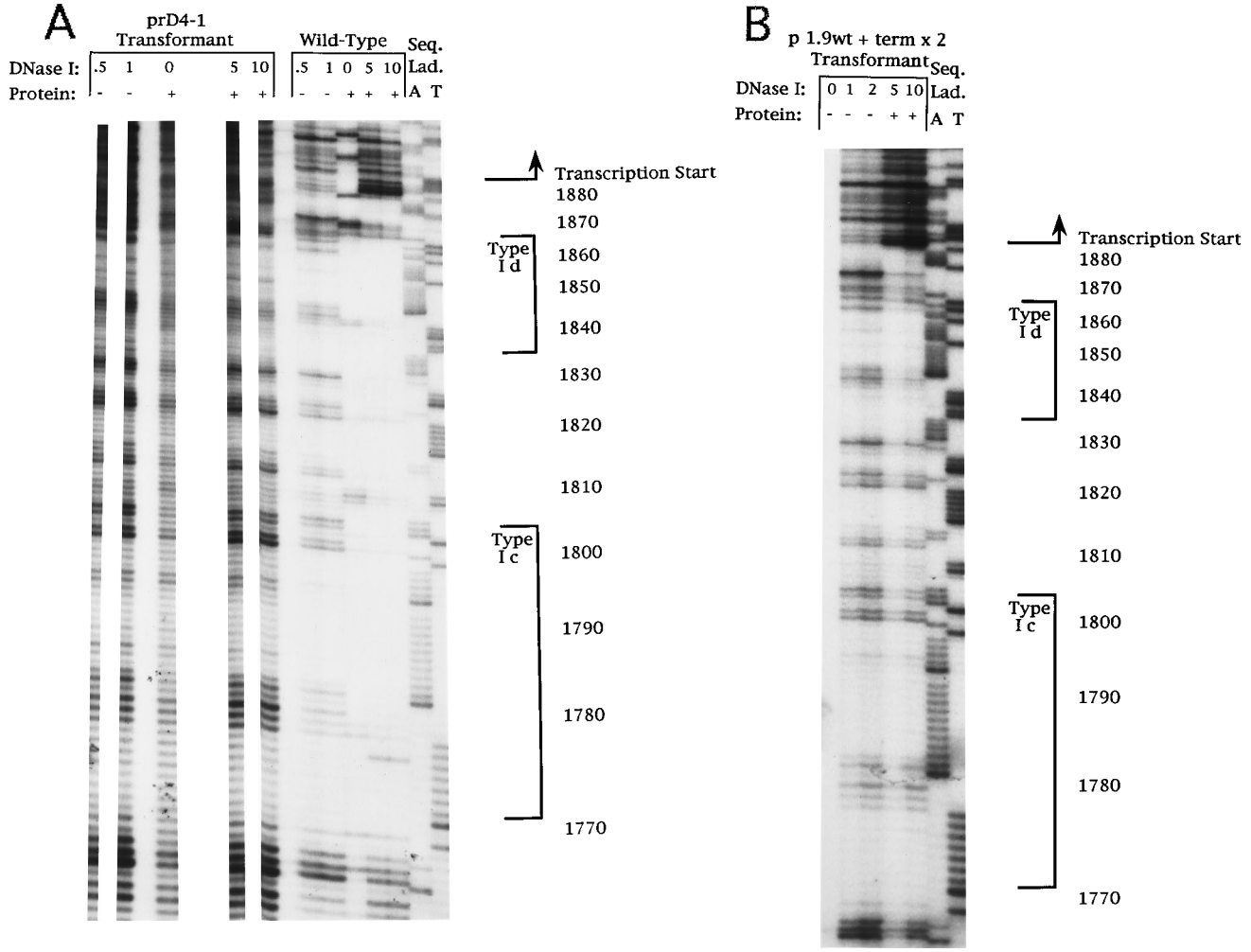

FIG. 5. Footprinting analysis of wild-type cells and transformants. (A) Nuclei were prepared from prD4-1 transformants and from control wild-type untransformed C3 cells and subjected to DNase I footprinting as described in Materials and Methods (+ Protein). Control naked DNA digestions (− Protein) were performed in parallel on DNA extracted from the same nuclei preparation but deproteinized before DNase I digestion, as described in Materials and Methods. Nucleotide numbers are shown at right. Transcription start site and type I repeats (7) are indicated. Final DNase I concentrations in the reactions (in units per milliliter) are indicated. (B) Nuclei and naked DNA samples were prepared from cells transformed with the $p1.9^{wt+term} \times 2$ plasmid and DNase I digested. The transformant was 42b; these are the same cells that were analyzed in the primer extension analysis. Lanes are as for panel A except that final DNase I concentrations in units per milliliter are actually one-half those indicated.

some element that requires establishment of a specific DNA-protein complex have been seen for other chromosomal elements. Human origin (14) and yeast centromere (16), ARS (26), and telomere (25) function are each blocked in vivo by forcing a transcript to run through the *cis*-acting sequences required for the function of these elements. In each of these cases, the transcript was produced by RNA polymerase II. Here we describe the first example of a situation in which transcription from an RNA polymerase I promoter impairs a chromosomal element, a downstream origin. Taken together with the work cited above, this result, obtained with a different RNA polymerase and origin in another eukaryote, suggests that the phenomenon of transcriptional inactivation of eukaryotic origins is a general property of these origins.

Various models can account for the inhibition of origin activity by transcription through the origin (14, 26). Movement of the transcription complex through the origin has been proposed to sterically prevent origin-specific factors from binding. This model was proposed to account for the disruption of ARS activity when transcription from a strong promoter proceeded through the ARS1 origin of *S. cerevisiae* in vivo (26). Promoter strength correlates with abrogation of function in the human system (14), suggesting that the act of transcription itself, and not simply open complex formation at the promoter, is required. A different model was proposed to account for the stimulation of plasmid R6K replication in *E. coli* when RNA polymerase was inhibited with rifampin. It was suggested that transcription through the origin induced a conformational change which prevented assembly of a functional DNA-protein complex at the origin (22, 23). Alternatively, an elongating RNA polymerase might interfere with the progress of a replication fork as it moves away from a nearby origin, instead of acting on initiation itself. However, electron microscopic analysis indicates that in the *E. coli* chromosome, movement of a replication fork is slowed but not blocked by rRNA gene transcription proceeding from the opposite direction (11). In addition, in vitro work in the bacteriophage T4 system has shown that while the replication fork pause caused by DNA polymerase colliding head-on with a stalled RNA polymerase is greater than that occurring when the polymerases are moving

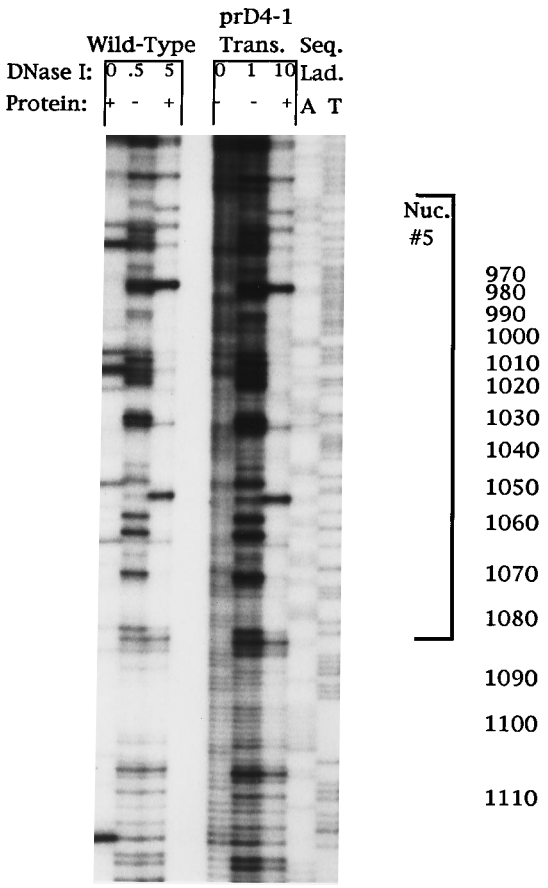

FIG. 6. In control primer extension analyses the same DNase I-treated samples primer extended with primer 11 for Fig. 5A were primer extended with primer 36. This primer detects a positioned nucleosome approximately 800 nucleotides from the transcription start site (nucleosome 5 [12a]). Nucleotide numbers are indicated at right.

in the same direction, DNA polymerase can proceed past a stalled RNA polymerase (20). Finally, although it had been proposed that a replication fork originating from an origin in the rDNA spacer of *S. cerevisiae* is arrested by transcription in the opposite direction (2), subsequent work has shown that the replication fork barrier (RFB) is not dependent on transcription. Instead, the barrier, which acted in a polar fashion, was interpreted to be a protein complex bound on the DNA, probably at the transcriptional termination site (3). These observations support the hypothesis that transcription interferes significantly with the initiation of DNA replication and not with the progress of an elongating replication fork.

There is no information concerning the existence of an RFB in the *Tetrahymena* rDNA transcription termination region. If there were an RFB and this functioned in a polar fashion, as in *S. cerevisiae*, the plasmid $p1.9^{wt+term} \times 2$ would be replicated unidirectionally from the two initially bidirectional origins. Inverting the terminator fragment would still permit replication in the opposite direction. Therefore, it is unlikely that an RFB, if its exists, is responsible for the difference in plasmid maintenance seen with $p1.9^{wt+term} \times 2$ and $p1.9^{wt+inv\text{-}t} \times 2$.

DNA replication origin activity has been found to be functionally linked in a variety of ways to transcriptional activity in the vicinity of the origin. In various eukaryotic viral and chromosomal origins, origin use is stimulated by the binding of transcription factors in the vicinity of the origin (reviewed in references 8 and 9). Certain prokaryotic origins appear to be activated by transcription. The lambda bacteriophage origin and the *E. coli* chromosomal origin on minichromosomes are positively regulated by transcription itself near the origin (1, 15). For the *E. coli* origin OriC, models for positive regulation by transcription include a direct and an indirect effect. In the direct effect, a transcript RNA supplies a primer for DNA synthesis (1). In the indirect effect, at least in minichromosome assays, the stimulatory effect is mediated through DNA conformational effects (1, 15, 21). This work shows that rRNA transcription is not a requirement for replication and high-copy-number maintenance, since the +G promoter in $p1.9^{+G} \times 2$ was inactive, but the plasmid was maintained. However, this result pertains specifically to the particular situation in which tandem arrays of 1.9^{+G} origin-plus-promoter regions accumulate on replicons. In normal rDNA palindromes, promoter sequences may play a positive role in stimulating origin function, because we have identified point mutations in the rRNA promoter which impose a replication disadvantage on palindromic rDNA (12a, 18).

The rDNA replicon of *T. thermophila* is subject to both cell cycle control and copy number control. Here we have demonstrated that transcription through the rDNA origin region inhibits origin function, and we suggest that transcription interferes with the assembly of factors at the origin. Further studies of the origin region in the rDNA will likely yield further insights into its structure and the control of origin function.

ACKNOWLEDGMENTS

We thank Guo-Liang Yu for help in the early stages of this work and Catherine Strahl and Tom Porter for help with manuscript preparation.

This work was supported by National Institutes of Health grant GM36252 to E.H.B. Support from the Lucille Markey Foundation to E.H.B. for laboratory facilities is gratefully acknowledged.

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