Replication of the extrachromosomal ribosomal RNA genes of *Tetrahymena thermophila*

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ABSTRACT

Cultures of *Tetrahymena thermophila* were deprived of nutrients and later refed with enriched medium to obtain partial synchrony of DNA replication. Preferential replication of the extrachromosomal, macronuclear ribosomal RNA genes (rDNA) was found to occur at 40-80 min after refeeding. The rDNA accounted for one half of the label incorporated into cellular DNA during this period. Electron microscopy of the purified rDNA showed 1% replicative intermediates. Their structure was that expected for bidirectional replication of the linear rDNA from an origin or origins located in the central non-transcribed region of the palindromic molecule. Similar forms had previously been observed for the rDNA of a related species, *Tetrahymena pyriformis*. The electron microscopic data was consistent with an origin of replication located approximately 600 base pairs from the center of the rDNA of *T. thermophila*, in contrast to a more central location in the rDNA of *T. pyriformis*. One implication of an off-center origin of replication is that there are two such sequences per palindromic molecule.

INTRODUCTION

The macronucleus of the ciliated protozoan, *Tetrahymena*, contains multiple copies of the rRNA gene (rDNA). These linear, extrachromosomal molecules each have about 20,000 bp (base pairs) of DNA with an overall palindromic sequence symmetry (1,2). Each half of the molecule contains a transcription unit for the rRNA precursor, from which the 17S, 5.8S, and 26S RNA components of the ribosome are derived (3-7). The rDNA of different species of *Tetrahymena* are all extrachromosomal and palindromic, but differ from one another in the location of restriction endonuclease cleavage sites and in total length (8). They also differ with respect to the presence or absence of a 400 bp intervening sequence that interrupts the 26S rRNA coding region (5,8,9).

The rDNA of *Tetrahymena* presents an unusual case of a nuclear DNA molecule that is a single replicon. Because the genes are extrachromosomal and present in 10,000 copies per cell, they can be isolated with their associated proteins (10,11). These features make the rDNA a potentially useful system for the study of replication of non-viral genes in eukaryotes.

Replication of rDNA at a fixed time after nutritional shift-up of starved cells has been studied mainly in the amiconucleate species, *T. pyriformis* (12,13) and has proven valuable in the preferential labeling of rDNA (10). Truett and Gall (14) studied the structure of the replicative intermediates of the rDNA in *T. pyriformis*, and obtained evidence for bidirectional replication from an origin located near the center of symmetry of the molecule. We now extend these studies to *T. thermophila*, the species in which *Tetrahymena* genetics is being developed. We also present a new method for analyzing the structure of the replication forms, from which we conclude that the rDNA of *T. thermophila* has an off-center origin of replication.

MATERIALS AND METHODS

Growth and starvation of cells

T. thermophila BVII were grown at 32°C with shaking in 1% proteose peptone, 0.003% sequestrine, and 0.005% each of streptomycin and penicillin. Cells grown to a density of $1-2 \times 10^5$ cells/ml were starved by being washed in pre-warmed 50 mM Tris buffer [tris(hydroxymethyl)aminomethane, Sigma no. T1503], pH 7.5. They were resuspended in the initial volume of 50 mM Tris containing streptomycin and penicillin, and incubated for 16 hr at 32°C with shaking. One hour prior to refeeding, the cells were pelleted and resuspended in fresh Tris buffer to remove any cell debris. All centrifugation steps were performed at room temperature in a table top centrifuge. Refeeding was accomplished by addition of 10-fold concentrated medium to restore the original growth conditions (15). [^3H]thymidine (50-80 Ci/m mole, New England Nuclear) was added to a concentration of 1 $\mu\text{Ci/ml}$ at or subsequent to the time of refeeding.

Isolation of rDNA

Cells were collected by centrifugation at 4°C, washed once with cold water, and lysed at 50°C by gentle homogenization with an equal volume of 0.5 M EDTA, 2% sarkosyl, pH 9.5. After 20 min at 50°C, proteinase K (EM Biochemicals no. 24568) was added to a concentration of 1 mg/ml and the incubation was continued for at least 1 hr. The solution was then diluted with an equal volume of low salt buffer, extracted with chloroform:isoamyl alcohol (24:1 v/v), and ethanol precipitated. Following treatment with pancreatic RNAase (75 $\mu\text{g/ml}$) and T1 RNAase (250 units/ml, Sigma no. R8251), the rDNA was separated from bulk DNA by CsCl density gradient centrifugation (1.568 g/ml) in the presence of 10.5 μg Distamycin A (Sigma no. D6135) per 50 μg DNA (5).

Centrifugation in the Beckman type 65 rotor proceeded at 20°C for 24 hr at 44,000 rpm followed by 30 hr at 34,000 rpm. Fractions (0.5 ml) were collected from the bottom of the tube and the radioactivity in a portion of each fraction was determined. The heavier density fractions containing rDNA were combined, extracted 10 times with isopropanol (saturated with a 1.6 g/ml solution of CsCl) to remove the Distamycin, and sequentially dialyzed against 100 mM NaCl, 10 mM Tris, 10 mM EDTA; 10 mM NaCl, 1 mM Tris, 1 mM EDTA; and 1 mM NaCl, 1 mM Tris, 1 mM EDTA, all at pH 7.5.

Electron microscopy

The rDNA in a solution containing 50% formamide, 100 mM Tris, pH 8.4, 10 mM EDTA, and 0.1 µg/ml ϕ X174 replicative form II DNA was mixed with cytochrome c and spread for electron microscopy as described previously (16). For aqueous preparations, DNA in 0.50 M $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$ and 100 µg/ml cytochrome c was spread on a hypophase of 0.25 M $\text{NH}_4\text{C}_2\text{H}_3\text{O}_2$. Contour lengths of molecules were measured from photographic prints (106,000x total magnification) with a Numonics electronic graphics calculator.

RESULTS

Replication of rDNA in starved-refed *Tetrahymena*

Tetrahymena thermophila were starved in Tris buffer and then refed with nutrient medium. As shown in Figure 1, incorporation of [^3H]thymidine occurred at a low rate immediately after refeeding. After 2.5 hr, the rate increased to approximately that of log-phase cells. Cells starved in 10 mM Tris buffer showed a similar response to those starved in 50 mM Tris buffer. In all subsequent experiments the starvation was done at the higher buffer concentration, which had been used in previous studies of ribosome and ribosomal RNA metabolism in *T. thermophila* (15,17,18).

DNA was extracted from cells that had been labeled with [^3H]thymidine during different time intervals after refeeding. The rDNA was separated from the bulk DNA by CsCl density gradient centrifugation in the presence of Distamycin. The ratio of [^3H]thymidine in the heavy density peak of rDNA to that in the main peak of DNA was undetectably low in the first 40 min after refeeding, was maximal in the 40-80 min interval, and diminished in the 80-120 min interval. In the 40-80 min interval, approximately one half of the radioactivity incorporated in cellular DNA was in the rDNA (Figure 2).

The rDNA isolated at various times after refeeding was prepared for electron microscopy by spreading from 50% formamide. A typical preparation contained 92% rDNA-size linear molecules, 8% longer linear molecules, and 0.3%

Figure 1. Incorporation of [^3H]thymidine into DNA after refeeding starved *T. thermophila*. Cells were starved in 10 mM Tris buffer (O) or 50 mM Tris buffer (Δ). At various times after refeeding, a 10 ml sample of the culture was removed. The cells were pelleted, lysed with sarkosyl, and digested with proteinase K. Nucleic acid was precipitated on ice with 5% trichloroacetic acid and collected by filtration on Whatman GF/C filters. The filters were washed with cold 5% trichloroacetic acid and then with 95% ethanol, dried, and counted in a liquid scintillation counter. Points are the average of duplicate determinations. The straight lines give the least squares fit of the combined data in the 0-2 hr and 2.5-6 hr time intervals.

Figure 2. Preferential incorporation of [^3H]thymidine into rDNA 40-80 min after refeeding starved *T. thermophila*. Cells were grown for 5 generations with $^{32}\text{P}_4$ and then starved. They were labeled with [^3H]thymidine from 40-80 min after refeeding. The isolated DNA was analyzed by Distamycin-CsCl density gradient centrifugation. Gradients were fractionated from the bottom, and 0.10 ml of each 0.50 ml fraction was taken for liquid scintillation counting. The peak at heavy density was identified as rDNA by the following criteria: the size of the major class of molecules observed in the electron microscope (21.4 ± 0.5 kb), the size of the DNA determined by 0.7% agarose gel electrophoresis (21 ± 2 kb) and the sizes of the fragments produced by treatment of the DNA with *Hind* III and *Bgl* II restriction endonucleases (sizes given in ref. 6). ($\square$) ^{32}P ; (O) ^3H .

rDNA-size circles. Depending on the time after refeeding, the samples also contained a low percentage of replication intermediates (Table 1). Replication forms were identified as branched molecules with a length measured along either of two paths equal to the length of rDNA. The frequency of replication forms at 50-80 min after refeeding was approximately 1%. No replication forms were observed in preparations of rDNA isolated at 20, 40, 100, or 120 min after refeeding (Table 1).

Structure of the replicative intermediates

In several preparations of rDNA isolated 50-80 min after refeeding, a total of 69 replicative intermediates were found. One of these was a replicating rDNA circle (θ form), while the remainder were replicating linear molecules as shown in Figure 3. Short single-stranded gaps were visible at 52% of the forks, and when they occurred at both forks of a replication bubble they were always in the trans configuration as defined by Blumenthal et al. (19).

The 50% formamide solution used in the sample preparation greatly reduced the frequency of relaxed circular rDNA molecules, an observation made previously by Karrer and Gall (1). To see whether non-covalently closed circles were involved in rDNA replication, one sample of rDNA was prepared for electron microscopy by the aqueous technique. The frequency of circular molecules increased from 0.3% to 2.4% and the frequency of replication forms remained at 1%, but the 7 replication forms observed all involved linear molecules.

The segment lengths of those molecules that could be measured unambiguously are portrayed in Figure 4. Because the molecules are oriented with the shorter unreplicated segment always to the left, the origin of replication (taken as the center of the replicated segment) will necessarily appear to be

Time after refeeding (min)	n	Replication forms	% of rDNA replicating
20-40	800	0	0
50-80	8491	69	0.8
100-120	842	0	0

Table 1. Replication forms observed by electron microscopy in samples of rDNA isolated at various times after refeeding of starved *T. thermophila*. Data from 4 separate time-course experiments are combined. n, the number of rDNA-size molecules that were scored.

Figure 3. An example of a replication form of *T. thermophila* rDNA. The bar has a length of 1.0 kb, based on length measurements of ϕ X174 RFII DNA on the same grid.

located to the left of the center of the molecules, a problem noted previously by Truett and Gall (14). An apparent off-center origin might simply reflect the random error of measurement that is intrinsic to electron microscopy of DNA. As seen in Table 2, however, the unreplicated arms of the replication forms differ in length by much more than the two halves of the replication bubble of the same molecules, and by much more than any two randomly paired ϕ X174 DNA or nonreplicating rDNA molecules. We therefore conclude that the two arms of each replicating molecule are different in molecular weight and that the replication bubbles are in fact off-center.

A molecule with a centrally located origin of replication would still show off-center replication bubbles if the two replication forks had different velocities. If this were the case, the distance between the center of the replication bubble and the center of the molecule would be close to zero in molecules that had just begun to replicate and would increase as a function of the extent of replication. As seen in Figure 5a, the data for the distance between the apparent origin of replication and the center of the rDNA $[(A-C)/2L]$, as defined in the legend to Figure 5] show considerable scatter, but the slope of the linear least squares fit does not increase with the extent of replication. When the data of Truett and Gall (14) for *T. pyriformis* rDNA were analyzed by the same method, the least squares fit similarly had a slope near zero (Figure 5b).

Because $(A-C)/2L$ is constrained to positive numbers, its average value is expected to overestimate the distance between the origin and the center of the rDNA. A better estimate of this distance can be obtained by plotting the frequency with which various values of $(A-C)/2L$ occur (Figure 6). For a centrally located origin, the interval near $(A-C)/2L = 0$ should be most

Figure 4. Structures of individual replicating molecules. Molecules are aligned with the shorter unreplicated arm to the left. Lengths are normalized; any molecule with total length > 2 SD from the mean length of rDNA was excluded. The three molecules shown at the bottom of the diagram had structures consistent with one replication fork having proceeded to the end of the molecule. The data for these molecules were excluded from the analysis of Figures 5 and 6.

highly populated, with a decreasing number of points falling in higher intervals. The histogram should approximate half of a normal distribution. This expectation was fulfilled when data for polyoma DNA replication (Figure 6d) and phage λ DNA replication (Figure 6e) were treated to represent linear molecules with origins of replication at their center. In contrast, our data for *T. thermophila* rDNA showed a clear absence of replication forms with (A-C)/2L near zero (Figure 6a). The data are those expected for a molecule with an origin of replication located approximately 600 bp from its center. The data for *T. pyriformis* rDNA (Figure 6b) had a distribution consistent with

Comparison	Length Ratio + SD	Length Difference + SD (bp)
Halves of replication bubble (B1, B2)	1.04 ± 0.03	266 ± 210
Unreplicated arms (A,C)	1.56 ± 0.58	1438 ± 772
φX174 DNA molecules, randomly paired	1.03 ± 0.03	177 ± 139
rDNA molecules, randomly paired	1.02 ± 0.02	408 ± 301

Table 2. Comparison of the lengths of various regions of rDNA replication forms, showing that the off-center replication bubbles cannot be attributed to measurement error. Length ratios were determined by dividing the length of the longer segment by that of the shorter segment for each molecule. Length differences were calculated as the larger minus the smaller length. For example, B1 is the longer half of a replication bubble and B2 is the shorter half, the length ratio is B1/B2, and the length difference is B1 - B2. The longer of the two unreplicated arms is designated A, the shorter is C. The φX174 and rDNA molecules were randomly paired and the length of the longer molecule of each pair was compared to that of the shorter.

an origin of replication located within 200 bp of the center of the molecule. Finally, we replotted the replication data of Vogt and Braun (20) for the extrachromosomal, palindromic rDNA of *Thyosarum*. From Figure 6c it appears that replication origins are located at fractional distances of 0.7 and 0.17 from the center of the molecule, a conclusion similar to that reached by the authors (20).

DISCUSSION

We have used starvation and refeeding to enrich for replicative intermediates of *T. thermophila* rDNA. Under our experimental conditions, preferential synthesis of rDNA took place from 40-80 min after refeeding. Both the maximum frequency of replication forms and the maximum incorporation of thymidine into rDNA compared to total DNA occurred in this period, prior to the major increase in cellular DNA synthesis (Figure 1). In *T. pyriformis*, preferential synthesis of rDNA has been found to occur 75-150 min after refeeding starved cells (12,13). Although this is somewhat later than the time we have determined for *T. thermophila* rDNA, the timing could be affected by the cell growth temperatures, starvation-refeeding protocols, and methods of assessing rDNA replication used in the various studies, and is not necessarily intrinsic to the species (cf. ref. 23). The significance of this early replication of rDNA in refed cells has not been established, though it has been suggested that some replication of rDNA could be a prerequisite for its transcription (12,24).

Figure 5. The distance between the center of the replication bubble and the center of the DNA molecule, $(A-C)/2L$, as a function of the fraction of the molecule replicated, $(B1 + B2)/2L$. (a) *T. thermophila* rDNA. (b) *T. pyriformis* rDNA, data of Truett and Gall (14). A and C are the lengths of the longer and shorter unreplicated DNA segments, respectively. B1 and B2 are the lengths of the longer and shorter halves of the replication bubble. The total length $L = A + C + (B1 + B2)/2$. Each symbol (+ or *) represents an individual replication form. The dashed line represents the linear least squares fit to the (+) data points. The (*) data points had values of $(A-C)/2L$ greater than 2 SD from the mean value, perhaps due to a stalled replication fork. They were excluded from the least squares analysis.

The preponderant type of rDNA replication form we observed involved a linear molecule. Although the population of rDNA molecules contained a small fraction of circles, these were not preferentially involved in replication. Even when the rDNA was prepared for electron microscopy by the aqueous tech-

Figure 6. The frequency of replication forms with different distances between the center of the replication bubble and the center of the DNA molecule. Distance is expressed as a fraction of the length of the molecule. (a) *T. thermophila* rDNA. (b) *T. pyriformis* rDNA, data of Truett and Gall (14). (c) *Physarum* rDNA, data of Vogt and Braun (20). (d) Polyoma DNA, data of Crawford et al. (21). (e) Phage λ DNA, combined data of Schnös and Imman (22) and D. Bastia and N. Sueoka (pers. commun.). The data for polyoma and λ DNA were manipulated to convert each molecule to a linear form with the origin of replication at its center. The breadth of these distributions must therefore be due to asynchrony of the replication forks. Note that the most probable value of $(A-C)/2L$ is near zero and that the shape of the two histograms approximates half of a normal distribution.

nique to preserve noncovalently closed circles, the replicating molecules appeared as linear forms. It remains possible that the ends of the replicating molecules could be involved in a noncovalent interaction *in vivo* that was disrupted by the DNA purification procedure.

The replication bubbles were centered ~ 600 bp from the middle of the rDNA molecules in *T. thermophila* (Figures 5a and 6a). The center of a replication bubble should be the origin of replication unless (1) the two replication forks had different average velocities, or (2) there was a substantial time lag between initiation of replication in the two directions. The first possibility is difficult to reconcile with the data of Figure 5, which show that the distance between the center of the bubble and the center of the rDNA does not increase with the degree of replication. The second possibility would require a 12 sec time lag between initiation in the two directions,

based on a 3 kb/min fork rate (25). Because our sample contained few molecules with a very small extent of replication, we cannot rule out this possibility for the rDNA. Such a lag is clearly inconsistent, however, with data for polyoma and SV40 DNA replication, where small replication bubbles have been measured and found to be centered at the origin (21,26).

The rDNA molecules vary somewhat in length due to different numbers of repeats of the sequence C_4A_2 at their termini (27). While this terminal heterogeneity probably results in the center of palindromic symmetry being displaced from the physical center of the molecule, several lines of evidence show that any such displacement is minor. First, the difference in size between the longest and shortest rDNA molecules in a population has been estimated as 150-200 bp (28), so the average difference will be smaller. The standard deviation in length that we measured for our rDNA molecules was consistent with a population of molecules homogeneous in molecular weight, as judged by the criteria of Davis et al. (29). An average variation in the number of base pairs per molecule of < 100 bp would not have been detected. Second, the absence of visible single-stranded tails in intramolecular "snap-back" renaturation products of rDNA (5) precludes the two ends of a given molecule differing by 600 bp. Finally, the rDNA of *T. pyriformis* has the same type of terminal heterogeneity as that of *T. thermophila* (7,8,27), yet the origin of replication is significantly more off-center for *T. thermophila* (Figure 6a,b). Thus, the heterogeneity at the ends of the rDNA is not large enough to account for the observed displacement of the origin of replication from the center of the molecule in *T. thermophila*.

It therefore seems likely that the origin of replication is not at the axis of symmetry of the *T. thermophila* rDNA palindrome, although it is certainly located within the central nontranscribed spacer region. Nucleotide sequence analysis of the central region of the *T. thermophila* rDNA has confirmed that this region of the molecule is in fact a perfect palindrome except for a 29 bp nonpalindromic region at its very center (30). Therefore, if the origin of replication is not at the axis of symmetry, there must be two such sequences. Comparing the estimated 3.5 min required for bidirectional replication of a 21 kb DNA molecule with the 1-2 hr during which the rDNA is replicated in vegetative growth (31), it seems likely that only one of the two origin sequences would be used in any round of replication.

Our study does not directly relate to the process of rDNA amplification, in which multiple extrachromosomal palindromic genes are generated from a single integrated non-palindromic copy during formation of a new macronucleus

(32). Pan and Blackburn (33) have recently identified an 11 kb half-palindrome form of rDNA in conjugating *T. thermophila*. This molecule may be an intermediate in amplification, and it is also able to replicate itself. By our measurements, such a half molecule should still contain a single origin of replication.

The origin of replication appears to be < 200 bp from the center of *T. pyriformis* rDNA, compared to ~ 600 bp in *T. thermophila* rDNA (Figure 6a,b). Transcription of the rRNA precursor begins ~ 1030 bp from the center in *T. pyriformis* (E. Niles, pers. commun., and ref. 7) and ~ 1580 bp from the center in *T. thermophila* (N. Din and J. Engberg, pers. commun.). Thus, despite the difference in size of the nontranscribed spacers, the distance between the origin of replication and the initiation of transcription may be very similar in the rDNA of the two species.

Kiss et al. (34) have recently demonstrated that a *T. thermophila* rDNA fragment that extends from 38 bp to 1850 bp from the axis of symmetry (and therefore includes the region that we have mapped as the origin) allows autonomous replication of plasmids in yeast. An extension of this technique to smaller DNA fragments should allow a more precise localization of the origins of replication.

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