Free Ribosomal RNA Genes in the Macronucleus of Tetrahymena

(gene amplification/repetitive DNA/electron microscopy of DNA/RNA-DNA hybridization/protozoa)

JOSEPH G. GALL

Department of Biology, Yale University, New Haven, Connecticut 06520

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ABSTRACT In the macronucleus of the ciliated protozoan, Tetrahymena pyriformis GL, the genes coding for 17S and 25S rRNA exist as free, extrachromosomal molecules. About 90% of the molecules are linear with a molecular weight of 12.6 x 10^6. Most of the remainder are circles of the same size, or lariats (circles with tails). A few dimers and oligomers are found. The extrachromosomal rDNA of Tetrahymena represents a kind of gene amplification that may be common among primitive eukaryotes.

The genes coding for ribosomal RNA (rRNA) undergo an extensive extrachromosomal replication or amplification in the oocytes of many animals, until in some cases they constitute the majority of the DNA in the nucleus (1, 2). The extrachromosomal ribosomal DNA (rDNA) contains circular molecules whose sizes are integral multiples of a basic unit (3–5). The basic unit is the DNA segment coding for one precursor rRNA molecule plus the accompanying nontranscribed "spacer" region. Extrachromosomal rDNA could be involved in other cases, as for example in the unusual behavior of the rDNA in both somatic cells and the germline of Drosophila (6, 7). However, aside from oocytes, the best evidence for extrachromosomal rDNA comes from the slime mold, Phy- sarum (8, 9), and the two ciliated protozoa, Styloschisma (10) and Tetrahymena (11, 12). In each of these organisms the rDNA is of low molecular weight even when extracted by gentle methods.

The present electron microscope study shows that the rDNA in the macronucleus of Tetrahymena occurs as free, extrachromosomal molecules, both linear and circular. Most of the molecules have a mass of 12.6 x 10^6 daltons and code for one precursor rRNA molecule. It is postulated that free rDNA genes are not limited to the macronucleus of ciliated protozoa, but occur widely among primitive eukaryotes. Such extrachromosomal rDNA represents a form of gene amplification that may have been developed to accommodate the greater cytoplasmic mass of eukaryotic cells as compared to their prokaryotic ancestors.

MATERIALS AND METHODS

Organisms and Culture. Tetrahymena pyriformis, amicronucleate strain GL, was supplied by M. Gorovsky (Rochester) from a stock originally obtained from J. Nilsson (Copenhagen). For most purposes the organisms were cultured with aeration in a medium consisting of 2% proteose peptone and 0.2% glucose.

Isolation of rDNA. Organisms from a 3-day culture were harvested by centrifugation and washed with distilled water. They were lysed at 65° in a solution containing 1% sodium lauryl sulfate, 0.5 M ethylenediaminetetraacetate, and 10 mM Tris·HCl, pH 9.5 (13). After 20 min the temperature was reduced to 50° and Pronase was added to a final concentration of 1 mg/ml. After a 4-hr incubation, the viscous solution was diluted 1:1 with water and extracted with an equal volume of water-saturated phenol. After centrifugation the aqueous supernatant was mixed with two volumes of 95% ethanol. The precipitate was redissolved and treated with pancreatic RNase, T1 RNase and α-amylase, each at 100 μg/ml. The DNA was again precipitated with ethanol and redissolved in 15 mM NaCl and 1.5 mM Na3 citrate, pH 7.0. The rDNA was isolated from approximately 4 mg of total DNA by successive isopycnic centrifugations. The first run was in the Beckman Ti 60 rotor at 35,000 rpm for 3 days at 17°, followed by two runs in the Ti 50 rotor under the same conditions (Fig. 1). The purified rDNA sample banded homogeneously in the Beckman model E Analytical Ultracentrifuge (Fig. 2).

Preparation of Labeled rRNA. For labeling with 32P, organisms were grown for 3 days in 100 ml of medium containing 1 mCi of carrier-free 32Porthophosphate. They were suspended in nonradioactive medium for 31/2 hr before harvesting. For labeling with 3H, organisms were grown 3 days in 100 ml of defined medium (14) containing 1 mCi of [3H]uridine (46.2 Ci/m mole) as the only pyrimidine source. Cells were chased with unlabeled medium for 4 hr before harvesting. Ribosomal RNA was prepared by the method of Leick and Plesner (15). The specific activity of the [32P]rRNA was initially 10^4 cpm/μg; that of the [3H]rRNA was 1.3 x 10^4 cpm/μg.

RNA-DNA Hybridization. DNA was attached to nitrocellulose filters according to the method of Gillespie and Spiegelman (16). Hybridization with radioactive rRNA was carried out for 16 hr at 65° in 0.3 M NaCl and 30 mM Na3 citrate, pH 7.0, or at 37° in 40% formamide, 0.6 M NaCl and 60 mM Na3 citrate, pH 7.0.

Electron Microscopy. DNA samples were prepared for electron microscopy by means of the aqueous and formamide modifications of the Kleinschmidt procedure described by Davis, Simon, and Davidson (17). DNA from ϕX 174 was included in all preparations as an internal mass standard. Its molecular weight was assumed to be 3.4 x 10^8.

RESULTS

The rDNA of Tetrahymena pyriformis strain GL has a higher buoyant density in CsCl gradients than the bulk macronuclear DNA, as shown originally by the RNA-DNA hybridization experiments of Engberg et al. (11). The difference in buoyant density, 1.699 g/cm³ for the rDNA versus 1.689...
an alkaline in for the material by three showing approximately 1.756 pooled were main contaminating DNA 2 hr at HC1 gradient fraction hybridized fractions CsCl. The in CsCl [3'2P]rRNA to Tetrahymena used for electron microscopy by both the aqueous and formamide modification of the Klein-schmidt technique (17). About 90–95% of the rDNA consisted of linear molecules of uniform length (Figs. 4 and 5). The average molecular weight of the most frequent size class was 12.6 ± 0.1 × 10^6 (n = 103) determined by comparison with DNA from φX 174 on the same grids. A few linear molecules of twice this unit size were found. Circular forms were

g/cm^3 for the main peak, permitted purification of the rDNA by three rounds of isopycnic centrifugation (Fig. 1). The material so isolated was essentially homogeneous when banded in neutral CsCl in the analytical ultracentrifuge (Fig. 2). In an alkaline CsCl gradient, only a single band was formed (\( \rho = 1.756 \) g/cm^3) indicating that the two strands of the rDNA are similar in G+T content. The purified rDNA hybridized with approximately 12.8% of its mass of Tetrahymena rRNA (Fig. 3). Mitochondrial DNA of strain GL has a buoyant density of 1.684 g/cm^3 (18), less dense than the main peak nuclear DNA. Therefore, it did not interfere with the isolation of rDNA from total cellular DNA.

Purified rDNA was prepared for electron microscopy by both the aqueous and formamide modification of the Klein-schmidt technique (17). About 90–95% of the rDNA consisted of linear molecules of uniform length (Figs. 4 and 5). The average molecular weight of the most frequent size class was 12.6 ± 0.1 × 10^6 (n = 103) determined by comparison with DNA from φX 174 on the same grids. A few linear molecules of twice this unit size were found. Circular forms were

Fig. 2. Purified *Tetrahymena* GL rDNA (Fig. 1b) centrifuged to equilibrium in CsCl in the analytical ultracentrifuge at 44,770 rpm, 25°, 20 hr. The density standard was *Micrococcus lysodeikticus* DNA (\( \rho = 1.731 \) g/cm^3). The rDNA has a buoyant density of 1.699 g/cm^3.

Fig. 3. Hybridization of purified *Tetrahymena* GL rDNA with [3H]rRNA. Purified rDNA was denatured with 0.1 N NaOH, neutralized with HCl, and applied to nitrocellulose filters by slow filtration (0.34 μg per filter). The filters were then incubated for 16 hr at 37° in 40% formamide, 0.6 M NaCl, 60 mM Na-citrate, pH 7.0. The [3H]rRNA (1.32 × 10^4 cpm/μg) was used at five concentrations from 0.25 to 4.0 μg/ml. The graph shows the ratios of RNA to DNA where the RNA is the amount on the filter after hybridization and the DNA is the amount initially applied to the filters. Control filters without DNA bound negligible counts (≤ 2.0% of the rDNA filters).
also present in the rDNA. These constituted 5.4% of the molecules in one sample and 11.1% in another (17/314 and 42/379 respectively). The frequency of circular molecules was difficult to estimate accurately since some of the linear molecules were tangled and could not always be distinguished from circles. The mean molecular weight of 35 circles was 12.7 ± 0.2 × 10^6, essentially the same as that of the major linear class. Some of the circular molecules were supercoiled, indicating covalent closure of both polynucleotide strands, but most were relaxed circles that presumably contained at least one single-strand nick.

In addition to the circular and linear molecules, more complex figures were found. The most common of these were lariats, i.e., circles with tails. Unlike the lariats in the amplified rDNA of Xenopus (3, 4) whose tails are often longer than the circle to which they are attached, the Tetrahymena lariats usually had tails the same length as the circles (five examples). A monomer circle with a dimer tail, and a dimer circle with a monomer tail, were also seen.

Among the several thousand molecules scanned a few other forms were found. Interestingly, these could usually be analyzed into components of monomer length. For instance, a Y-shaped molecule was seen in which each of the three arms was monomer length; in one case two monomer circles were joined by a linear segment of monomer length; and several lariats were found whose tail + circle length equaled one monomer.

It was of interest to know whether the linear molecules were identical in sequence, or consisted instead of a population of cyclically permuted sequences. A sample of rDNA was spread in 80% formamide, a concentration high enough to produce partial denaturation of many of the molecules. Inspection in the electron microscope showed that a consistent denaturation pattern was present. A rather large, easily denatured region was found near each end of the linear molecule, as well as several shorter regions in the middle (Figs. 4b and 5). The uniform pattern of denaturation implied that all of the linear molecules had a similar or identical sequence, and could not have been produced by random breakage of circles. A few partially denatured circular molecules were found, and these had the same denaturation pattern as the linears. No partially denatured lariats were seen.

**DISCUSSION**

Electron microscopic observations showed that a purified rDNA fraction from the macronucleus of Tetrahymena consisted primarily of linear and circular molecules with a molecular weight of 12.6 × 10^6. It seems unlikely that such a popula-
tion could be produced as a preparative artifact, and it is concluded that the rDNA consists in vivo of free, extrachromosomal molecules. If the rDNA were chromosomally integrated, then it would be necessary to postulate specific cleavage during the isolation procedure to yield linear and circular molecules all of the same length.

It is probable that both linear and circular forms exist in vivo. If the circular forms arose during or after isolation by closure of "sticky" ends of linear molecules, then supercoiled forms should not exist. Also, the circles would not be stable, in 50% or 80% formamide unless very long terminal overlaps were involved. On the other hand, it is unlikely that all of the rDNA is circular before isolation. If this were true, then the linear forms would have to arise as broken circles. However, de-

The lariats are of special interest, since they resemble the rolling circle replicative forms of the amplified rDNA of Xenopus oocytes (3, 4). However, most of the Tetrahymena lariats had tails equal in length to the circle. It is possible that they arose by some kind of recombination process between a linear and a circular molecule. On the other hand, if they are a replication form, then replication must have stopped after one "roll" of the circle. One could postulate that the circular molecules are the nonreplicating form. These might cyclize and then form a new unit by the rolling circle mechanism. Such a scheme would lead to a simple doubling of the rDNA during the vegetative fission cycle. Electron microscope autoradiography should permit identification of the replicating molecules and thus clarify the mechanism of rDNA replication.

The number of coding units in the monomer rDNA mole-
cule was estimated by an RNA-DNA hybridization experiment. If each monomer rDNA molecule of mass 12.6 × 10^6 daltons hybridized with one 17S and one 25S RNA molecule of combined mass 2.0 × 10^6 daltons (19), then the ratio of RNA to DNA would be 15.9% (2.0/12.6 = 0.159). The hybridization of RNA to purified rDNA gave an RNA to DNA ratio, at saturation, of 12.6% (Fig. 8) which is slightly lower than the value expected if one coding unit per monomer is assumed. It is probable that the amount of DNA in the hybrids was overestimated, since the rDNA was unlabeled and its retention by the nitrocellulose filters could not be measured.

The existence of free ribosomal RNA genes in Tetrahymena is consistent with the recent data of Engberg et al. (12), who demonstrated that nucleolar DNA from gently lysed nuclei sedimented more slowly in a sucrose gradient than the bulk of the DNA. From their most recent sedimentation data (20) their calculation of a molecular weight of about 11 × 10^6 is only slightly lower than found here by electron microscopy. Similar observations have been made on the rDNA of the slime mold, Physarum by Newlon et al. (8) and by Zellweger et al. (9). Not only does the Physarum rDNA sediment slowly in a sucrose gradient, but it can be partially purified from bulk DNA by a differential salt precipitation. Newlon et al. (8) suggested that the rDNA in Physarum might be extrachromosomal. Finally, the rDNA of the ciliated Protozoan, Stylonychia also sediments slowly in a sucrose gradient (10). Stylonychia is unique, however, in that all of the macronuclear DNA exists as small molecules (0.2-2.2 μm by electron microscopy). These cases raise the interesting possibility that extrachromosomal rDNA is a common feature among simpler eukaryotes. One can imagine that primitive eukaryotes, with a much greater cytoplasmic volume than their prokaryote ancestors, required multiple rDNA templates to provide an adequate supply of rRNA. These copies might have been extrachromosomal early in evolution, and only later integrated into the chromosomes, as is presumed to be the case for present-day higher eukaryotes.

The existence of micro- and macronuclei in the ciliates, and the conversion of a micronucleus into a macronucleus after conjugation, offers an ideal system for studying the origin of the extrachromosomal rDNA in the macronucleus. Recently Yao, Kimmel, and Gorovsky (21) have found that the number of rDNA copies per genome is at least 10 times higher in the macronucleus than in the micronucleus. The micronucleus contains no more than 20 copies of rDNA per genome and possibly only a few, whereas the macronucleus contains approximately 200 copies per genome. It is not known whether the micronuclear rDNA is chromosomally integrated, but in any case, there must be a true extrachromosomal amplification of rDNA when the new macronucleus is formed after conjugation. Tetrahymena should prove to be a good organism in which to study the biochemistry of rDNA amplification.

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