Free ribosomal RNA genes in Paramecium are tandemly repeated

(gene amplification/electron microscopy of DNA/macronucleus/protozoa)

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ABSTRACT The genes coding for 17S and 25S rRNA in Paramecium tetraurelia were isolated. The macronuclear ribosomal DNA (rDNA) exists as relatively small, extrachromosomal molecules with both linear and circular forms. Electron microscopy and restriction endonuclease analysis revealed that the rDNA is arranged as tandem repeats with an average repeat size of 5.5 × 10^6 daltons. Some heterogeneity of repeat lengths was found both by electron microscopy and by restriction enzyme analysis. The rDNA does not snap back after denaturation. This study provides additional evidence that extrachromosomal rDNA may be a common feature among lower eukaryotes. However, in contrast to several other cases, the rDNA of Paramecium is not palindromic, but occurs as tandem repeats as in higher eukaryotes.

Among eukaryotic organisms the genes coding for rRNA generally exist as multiple, tandem copies integrated into the chromosomes at one or more locations. However, the ribosomal DNA (rDNA) may, in certain instances, occur as extrachromosomal copies, which in the oocytes of many animals are extensively replicated during the process of amplification (1, 2). Some of these extrachromosomal molecules are circular, and their sizes are integral multiples of a basic repeating unit. One unit encompasses the DNA coding for rRNA plus the transcribed and nontranscribed spacer regions (3, 4).

The rDNA of some lower eukaryotes also exists as extra- chromosomal molecules. This is true for the slime molds Physarum (5) and Dictyostelium (R. Grainger, N. Maizels, and A. Weiner, personal communication; A. Cockburn, W. Taylor, and R. A. Firtel, personal communication), and for the ciliated protozoans Oxytricha (6) and Tetrahymena (7, 8). Of these, Oxytricha represents a special case, since all of its macronuclear DNA exists as small molecules. In Tetrahymena the free rDNA copies found in the macronucleus represent a case of gene amplification, as only a single integrated rDNA gene occurs in the micronuclear genome (9, 10). Most interestingly, the free rDNA genes in Physarum (5), Dictyostelium, and Tetrahymena (11, 12) are palindromes.

In this paper we demonstrate that the rDNA of Paramecium is extrachromosomal, occurring in both linear and circular forms. However, the rDNA of Paramecium is not palindromic, but consists instead of multiple, tandem repeats.

MATERIALS AND METHODS

Organisms and Culture. Paramecium tetraurelia, stocks 51 and 148 (endosymbiont free), were kindly supplied by T. Sonneborn (Indiana University). The cultures were grown at 27° in Cerophyl infusion inoculated with Klebsiella aerogenes (13). In order to label Paramecium DNA, 0.1 ml of packed cells (1 × 10^6 cells) was added to 250 ml of Cerophyl infusion and [methyl-14C]thymidine (57.1 Ci/mmol, New England Nuclear) was added to 0.04 μCi/ml. After 24 hr of growth the culture was refed with Klebsiella. After an additional 24 hr the culture was in late logarithmic growth (14), and the organisms were collected by centrifugation at low speed. The cells were then washed and resuspended in Dryl's solution (15).

Tetrahymena thermophila, strain BIV, was grown axenically in 50 ml of 1% proteose peptone/0.003% sequestrin, containing 0.25 ml of antibiotic mixture (Gibco) at 27° with shaking. Cells were labeled by culturing for 24 hr with [methyl-14C]thymidine added to 0.01 μCi/ml, and were washed and harvested as above.

DNA Isolation and Alkaline Sucrose Gradients. Most DNA samples were isolated by a modification of the procedure of Kavenoff and Zimm (15), as described (7). Because of the large difference in ploidy levels between Paramecium micronuclei and macronuclei, the DNA used in these studies must be derived almost exclusively from macronuclei (16).

Alkaline sucrose gradients (30 ml, 15–30% wt/vol) were made up in 0.5 M NaCl/5 mM EDTA/0.1% Sarkosyl/50 mM KOH at pH 12.4 over a 1-ml cushion of 80% sucrose in the same buffer. DNA used for alkaline sucrose gradient sedimentation was not precipitated; after phenol extraction it was dialyzed in the cold against 5 mM EDTA/5 mM NaCl/10 mM Tris-HCl at pH 8.5. After dialysis, the DNA was denatured by adding 0.5 vol of 2.0 M NaOH, and an aliquot equal to 0.08 ml of packed cells (∼8 × 10^6 cells) was immediately layered on a gradient. The gradients were centrifuged at 20,000 rpm for 15 hr at 15° in a Beckman SW 25.1 rotor. Denatured λ b522 [32P]DNA (a generous gift of E. Casuto) was included in some gradients as a molecular weight standard. Its native molecular weight was assumed to be 23.6 × 10^6 (17).

RNA–DNA Hybridizations. DNA samples from either sucrose or CsCl gradients were attached to nitrocellulose filters according to the method of Gilsipie and Spiegelman (18). Hybridization with [3H]rRNA was carried out in 0.6 M NaCl/60 mM Na3 citrate at pH 7.0 and 40% formamide, at 37° for 20–24 hr.

Preparation of Labeled rRNA. To label rRNA, we grew a culture of Paramecium for 20 hr in Cerophyl infusion containing 50 μCi of [1H]uridine per ml (37.6 Ci/mmole, New England Nuclear). 17S and 25S [3H]rRNA was prepared by a modification of the procedure of Spradling et al. (19), and was purified on 5–20% sucrose gradients.

Isolation of rDNA. rDNA was isolated from stock 51 whole-cell DNA by equilibrium centrifugation. Approximately 250 μg of DNA per gradient was centrifuged in actinomycin D/CsCl (actinomycin D (Sigma) at 50 μg/ml, initial ρ = 1.640 g/cm^3) in a Beckman 50 rotor at 42,000 rpm for 24 hr at 18°. An aliquot of each gradient fraction was hybridized with Paramecium [3H]rRNA and the fractions that hybridized were pooled and rerun in actinomycin D/CsCl. The fractions that hybridized in the second gradient were again pooled and the

Abbreviation: rDNA, DNA coding for rRNA.
actinomycin D was extracted with isopropanol saturated with CsCl. After removal of the isopropanol, the DNA was centrifuged in CsCl (initial p = 1.690 g/cm³) in a Sorvall VT-865 rotor at 35,000 rpm at 18° for 24 hr. Fractions (0.1 ml) were collected from the bottom, aliquots were hybridized with [³H]rRNA, and the fractions that contained rDNA were pooled.

Electron Microscopy. DNA samples were prepared for electron microscopy as described by Davis et al. (20). The RF II form of φX174 (a generous gift of N. Godson) was included as an internal mass standard. Its molecular weight was assumed to be 3.4 × 10⁹ (20).

Restriction Endonuclease Digestion and Gel Electrophoresis. The restriction endonucleases EcoRI and Bgl II were gifts of T. Barnett and H. Erba, respectively. DNA was digested in an appropriate buffer with excess enzyme to ensure complete digestion. The conditions of agarose gel electrophoresis have been described (10).

RESULTS

The sedimentation pattern of Paramecium rDNA suggests that it occurs as free molecules in the macronucleus, not covalently integrated into the chromosomal DNA. This was demonstrated in the following manner. A culture of Paramecium was labeled with [¹⁴C]thymidine and whole-cell DNA was extracted, dehydrated, and sedimented in an alkaline sucrose gradient. Fractions were collected and loaded onto nitrocellulose filters, which were then hybridized with Paramecium 17S and 25S [³H]rRNA in order to demonstrate the location within the gradient of the genes coding for rRNA. As shown in Fig. 1, the highest molecular weight [¹⁴C]-labeled Paramecium DNA, constituting 70% of the [¹⁴C]radioactivity in the gradient, sedimented into the sucrose cushion. However, the fractions that hybridized with [³H]rRNA were broadly distributed across the middle of the gradient, showing that the rDNA had a relatively low molecular weight. The approximate single-stranded molecular weight of the rDNA, determined by comparison with λ[³²P]DNA, was 18 × 10⁶.

For comparison, Tetrahymena DNA was isolated and centrifuged in a similar fashion. The rDNA sedimented more slowly than the major DNA of Tetrahymena and showed a sharp hybridization pattern, as predicted from its known molecular weight and extrachromosomal location (7, 8; data not shown). Thus, Paramecium rDNA sedimented in alkaline sucrose gradients in a fashion similar to Tetrahymena rDNA, but Paramecium rDNA had a higher average molecular weight. In addition, the sedimentation profile of Paramecium rDNA was much broader than that of Tetrahymena rDNA or of λ DNA in the same gradient, which suggested that Paramecium rDNA was heterogeneous in size.

Paramecium rDNA was purified from whole-cell DNA by equilibrium centrifugation. The rDNA and mtDNA were difficult to separate from one another since they have similar buoyant densities in neutral CsCl and similar molecular weights. Separation was achieved by centrifugation in actinomycin D/CsCl gradients, as shown in Fig. 2A. In neutral CsCl the purified rDNA has a buoyant density of 1.699 g/cm³ (Fig. 2B). Endonuclease digestion of the rDNA revealed the same pattern as had been previously determined with DNA from isolated macronuclei. Such macronuclear DNA lacks mtDNA but is usually of low molecular weight (21).

To confirm that we had purified rDNA, and not mtDNA, we isolated Paramecium mitochondria from stock 51 and purified their DNA. This mtDNA had the same buoyant density in neutral CsCl (1.702 g/cm³) as previously reported for stock 51 (22). Although the buoyant densities in neutral CsCl of the rDNA and mtDNA were almost identical, their properties differed in most other respects. As determined by electron microscopy, the linear mtDNA molecules occurred in discrete size classes with both monomer (25.6 × 10⁶ daltons) and dimer (51.6 × 10⁹ daltons) forms. Both the size and the partial denaturation pattern of the mtDNA agreed with the results of Goddard and Cummings (23, 24). The restriction pattern of the mtDNA differed appreciably from that of the rDNA (Fig. 3). Moreover, certain restriction fragments of the rDNA, when eluted from gels by the Southern technique (25), hybridized with Paramecium [³²P]rRNA, while those from mtDNA did not. These results confirmed that the rDNA had been separated from the mtDNA.

Purified rDNA was examined by electron microscopy. Measurement of molecules from enlarged photomicrographs showed a broad distribution of sizes, with some clustering around molecular weights of 5–7, 9–11, 20–23, and 29–30 × 10⁶. Both linear and circular molecules were observed, and one lariat form was seen.

When rDNA was spread under partially denaturing conditions in 84% formamide, its repetitive structure became apparent. Denaturation bubbles occurred at regular intervals in a repeating pattern with one native and one denatured region per repeating unit (Fig. 4). The mean center-to-center spacing...
of these bubbles was \(5.5 \times 10^6\) daltons (SD = 0.55 \(\times 10^6\) daltons, \(n = 169\)), determined by comparison with double-stranded \(\phi X174\) in the same sample. The single-stranded regions were assumed to have the same contour length as the double-stranded regions from which they were derived. Linear and circular molecules had the same partial denaturation pattern, although only a few circles were available for analysis. The smallest circles found were dimers. The largest linear and circular molecules had molecular weights of 77.3 \(\times 10^6\) (13 repeats), and 38.4 \(\times 10^6\) (7 repeats), respectively. A representative partially denatured molecule is shown in Fig. 5.

Electron microscopy was also used to search for snapback rDNA molecules. The rDNA was denatured either by heat or by alkali; in the first case it was cooled briefly (11), and in the second it was neutralized before being added to the spreading solution. The DNA was largely single stranded when viewed in the electron microscope. However, rare double-stranded circles were seen. These must have arisen from covalently closed circles, since a nick in either strand would have permitted the two strands to separate completely from one another. The circles were the same size as those seen in non-denatured preparations. No configurations were seen that could have arisen from intramolecular reassociations of the sort expected for repeats arranged in a head-to-head fashion (11).

The fact that circles were observed, especially the covalently closed circles, reinforced the conclusion that the rDNA was extrachromosomal. The double-stranded molecular weight of rDNA molecules determined by electron microscopy was approximately twice the single-stranded molecular weight calculated from alkaline sucrose gradients. Thus, the electron microscopic observations confirm that the sedimentation pattern accurately reflects the size of the rDNA, and demonstrate that the sedimentation profile was not the result of specific single-stranded nicks in the rDNA.

The repeat size obtained by digestion of the rDNA with the restriction endonucleases \(Bgl\) II or EcoRI agreed closely with that from electron microscopy (Fig. 3). The size of the rDNA repeat determined by digestion with \(Bgl\) II is 5.49 or 5.2 \(\times 10^6\) daltons, and with EcoRI is 5.46 or 5.2 \(\times 10^6\) daltons. The difference in the repeat lengths of approximately 0.3 \(\times 10^6\) daltons corresponds to the size difference between the two largest \(Bgl\) II fragments, or the 1.14 and 0.88 \(\times 10^6\)-dalton EcoRI fragments. The two large \(Bgl\) II fragments map to the same position within the rDNA repeat, and hybridization experiments revealed that they contain both 175 rRNA coding sequences and spacer sequences. Similar experiments showed that the 1.14 and 0.88 \(\times 10^6\)-dalton EcoRI fragments also map to the same position within the repeat and contain only spacer DNA (data not shown). These studies suggest that the 0.3 \(\times 10^6\)-dalton dif-
ference in repeat lengths represents the size heterogeneity due to variable length spacer sequences.

The data from electron microscopy and restriction analysis are consistent with a sequentially repeated, nonpalindromic unit in Paramecium rDNA.

DISCUSSION

The extrachromosomal rDNA of Tetrahymena, Physarum, and Dictyostelium exists primarily as palindromic dimers and, hence, the isolated molecules are quite homogeneous in molecular weight. By contrast, the extrachromosomal Paramecium rDNA exhibits a wide range of molecular weights, as shown both by the broad sedimentation pattern on alkaline sucrose gradients and by the heterogeneous size distribution seen by electron microscopy. In this respect Paramecium rDNA more closely resembles the amplified rDNA of Xenopus oocytes (2, 3) than the rDNA of other lower eukaryotes.

Partial denaturation mapping revealed that the rDNA molecules were composed of tandem repeats, which were equal in length along most molecules. However, some molecules showed evidence of heterogeneity of repeat length that was probably not due to measuring errors. For example, in Fig. 4 the third and fourth molecules are both circles with the same number of repeats, but the fourth molecule is smaller and contains one repeat that is significantly shorter than average. This suggests that heterogeneity of adjacent repeats occurs in extrachromosomal rDNA molecules. The repeat size found upon restriction enzyme analysis of Paramecium rDNA agreed with the value from electron microscopy and also gave evidence of heterogeneity in the size of the spacer sequences. However, the restriction studies do not distinguish between intra- and intermolecular heterogeneity. Confirmation of the intramolecular heterogeneity will require an electron microscopic study of heteroduplex molecules prepared by annealing isolated rDNA with a homogeneous cloned rDNA fragment (26).

The observed heterogeneity has interesting implications with respect to amplification of the rDNA. Most ciliates contain a transcriptionally active polyploid macronucleus and an inactive diploid micronucleus which is responsible for the genetic continuity of the organism. The extrachromosomal rDNA in the Paramecium macronucleus presumably arises from micronuclear rDNA after conjugation. The developmental pattern of the macronuclear nucleoli suggests that rDNA amplification may occur during polyploidization of the new macronuclei (16). The heterogeneity seen in the restriction pattern of Paramecium rDNA shows that more than one repeat size must exist in the cell population used in this study. More interestingly, intramolecular heterogeneity was found in the extrachromosomal rDNA. The simplest explanation of this observation is that the Paramecium micronucleus contains integrated tandem copies of the rDNA of variable unit lengths, and that adjacent repeats serve together as templates during amplification of the chromosomal rDNA. The number and arrangement of the different classes of repeats and the mechanism of amplification are not known. The organization of Paramecium rDNA is thus sig-

![Figure 4](image-url)  
**FIG. 4.** Partial denaturation pattern of linear and circular rDNA molecules spread in 84% formamide. The sample includes molecules from two different rDNA preparations and indicates the range of sizes seen by electron microscopy. Native regions are represented by a single line, denatured by double lines. The molecules were arranged arbitrarily by aligning a denatured region near the left ends of the molecules. The five molecules with a denatured region at their extreme left end were circular.

![Figure 5](image-url)  
**FIG. 5.** A partially denatured linear rDNA molecule spread in 84% formamide, which has a molecular weight of $30.5 \times 10^6$ and contains five denatured regions. (X33,000).
significantly different from that of *Tetrahymena*, in which the extrachromosomal macronuclear rDNA arises from a single integrated copy in the micronucleus (9, 10). The heterogeneity of adjacent repeats seen in *Paramaecium* extrachromosomal rDNA suggests that it is organized more like *Xenopus* chromosomal rDNA, in which adjacent repeats can differ in length, than like *Xenopus* amplified rDNA, in which tandem repeats along a single molecule are equal in length (26).

This study provides additional evidence that extrachromosomal rDNA may be a common feature among lower eukaryotes (7). However, in contrast to the palindromic dimers found in *Tetrahymena*, *Dictyostelium*, and *Physarum*, the rDNA of *Paramaecium* is arranged as multiple tandem repeats in linear or circular molecules.

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