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A Small Number of Cistrons for Ribosomal RNA in the Germinal Nucleus of a Eukaryote, *Tetrahymena pyriformis*

(evolution of repeated genes/amplification/DNA·RNA hybridization/macro- and micronuclei)

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ABSTRACT **The percentage of DNA complementary to 25S and 17S rRNA has been determined for both the macro- and micronucleus of the ciliated protozoan, *Tetrahymena pyriformis*. Saturation levels obtained by DNA·RNA hybridization indicate that approximately 200 copies of the gene for rRNA per haploid genome were present in macronuclei. The saturation level obtained with DNA extracted from isolated micronuclei was only 5–10% of the level obtained with DNA from macronuclei. After correction for contamination of micronuclear DNA by DNA from macronuclei, only a few copies (possibly only 1) of the gene for rRNA are estimated to be present in micronuclei. Micronuclei are germinal nuclei. Macronuclei serve as somatic nuclei during vegetative growth but are destroyed every sexual generation and are reformed from products of meiosis, fertilization, and division of the micronuclei. Thus, the hybridization data suggest that the gene for rRNA must be amplified during macronuclear formation with each sexual generation. These observations also demonstrate that the multiple copies of a repeated gene in a somatic nucleus of a eukaryote can be generated from a small number of copies of that gene in a germinal nucleus.**

The cistrons coding for ribosomal RNA (rRNA) have been widely studied. It is now well established that these genes are present in multiple copies in eukaryotic organisms and it is likely that each of the copies in a particular organism is virtually identical (1, 2). In contrast to the homogeneity of the multiple copies of the rRNA gene within an organism, a portion of this gene, the transcribed and nontranscribed spacer, differs markedly in two closely related species (3). The portion of the gene which actually codes for rRNA also is demonstrably different in different species, although it appears to evolve more slowly than the spacer region (4). Similar intra-species homogeneity coupled with inter-species variability has been shown to occur for other repeated DNA sequences (5–7).

The identity of repeated copies of a gene within a species coupled with variability of the same gene between species raises important questions regarding the mechanism(s) by which these genes evolve, since mutation and selection alone seem inadequate to explain these observations. Several hypotheses have been put forward to explain the evolution of repeated sequences (5, 8). To our knowledge, little evidence exists to support any of these hypotheses.

The micro- and macronuclei of the ciliated protozoan, *Tetrahymena pyriformis* are analogous to the germinal and somatic nuclei of higher eukaryotes. While both nuclei are derived from the same zygotic nucleus during conjugation, only the micronucleus maintains the genetic continuity of this organism. The macronucleus is responsible for virtually all of the transcriptional activity during growth and division and is capable of dividing an indefinite number of times during vegetative growth. However, if *Tetrahymena* are allowed to undergo a sexual cycle (conjugation), the macronucleus breaks down. It is then formed anew from a division product of the zygotic nucleus which results from the fusion of gametic nuclei derived from the micronucleus (9).

The macronucleus of *Tetrahymena* has been shown to contain approximately 170–200 copies, per haploid DNA amount, of the genes coding for 25S and 17S rRNA (ref. 10, and this report). Recent studies show that the repeated copies of the gene for rRNA in the macronucleus are not physically linked to the rest of the genome (11). In this study, we have compared the number of genes for rRNA in macro- and micronuclear DNA by hybridization with homologous rRNA. DNA extracted from isolated micronuclei was found to contain approximately one-tenth to one-twentieth the number of genes for rRNA as did macronuclear DNA. Since most of the hybridization of rRNA to micronuclear DNA can be accounted for by macronuclear DNA which is known to contaminate the micronuclear preparation, we conclude that micronuclei contain a small number of genes (possibly only one) for ribosomal RNA. These observations suggest that, in at least one eukaryotic organism, the repeated copies of a gene found in a somatic nucleus are formed by expansion from a small number of copies of that gene which are present in the germinal nucleus.

MATERIALS AND METHODS

Culturing of Cells. Tetrahymena pyriformis (Syngen 1, strain WH-6 or strain B-VII) was cultured axenically in enriched proteose peptone as described previously (12). For labeling nucleic acids with radioactive isotope, yeast extract was omitted from the culture medium and 1 ml of an antibiotic-antimycotic mixture (Grand Island Biological Co. New York) was added per 100 ml of culture at the time of addition of isotope.

Abbreviations: EDTA, ethylenediaminetetraacetate; SSC, standard saline–citrate solution (0.15 M sodium chloride–0.015 M sodium citrate, pH 7); 0.1 × SSC means that the concentration of the solution used is 0.1 times that of the standard saline–citrate solution.

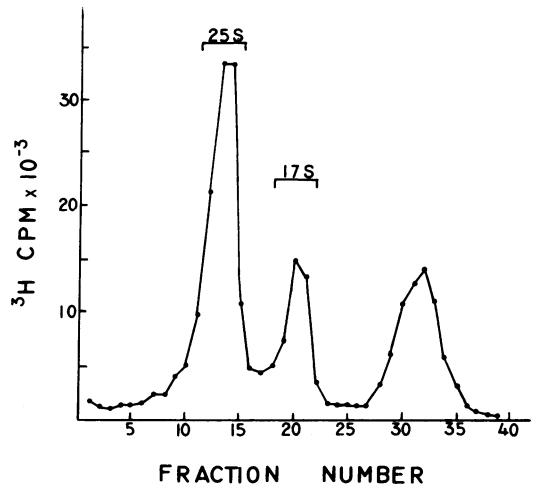

FIG. 1. Sucrose gradient profile of whole cell RNA labeled with [^{3}H]uridine. The fractions which were pooled for purification of 25S and 17S rRNA are indicated.

Isolation of Nuclei. One liter cultures containing 4 to 6 × 10^5 cells per ml were harvested, and macro- and micronuclei were isolated as described previously (13). Contamination of the micronuclear preparation by recognizable macronuclei was determined by counting the nuclei under the light microscope. One or less than one macronucleus was found for every 200 micronuclei.

Labeling of DNA. Cells were grown in proteose peptone containing 0.025 μCi/ml of ^{14}C-labeled thymidine ([*methyl*-^{14}C]-thymidine, 52.7 mCi/mM, 100 μCi/ml; New England Nuclear Corp., Boston, Mass.) for 20 hr. The specific activity of isolated DNA was about 500 dpm/μg.

Labeling of RNA. Cells in the log phase of growth (250,000 cells per ml) were harvested and resuspended in an equal volume of sterile 10 mM Tris·HCl at pH 7.4 containing 50 μCi/ml ^{3}H-labeled uridine ([5-^{3}H]uridine, 27.88 Ci/mM, 1.0 mCi/ml, New England Nuclear Corp.). In one experiment, cells were labeled for 1 hr and then were chased by the addition of an equal volume of 2× concentrated culture medium containing yeast extract. In a second experiment, cells were labeled for 30 min and chased for 30 min as described above.

Extraction of DNA. Freshly prepared nuclei or nuclei which had been stored frozen (−20°) for less than 2 days were incubated in 0.5% Sarkosyl, 0.1 M disodium EDTA (ethylenediaminetetraacetate) and 0.05 M Tris·HCl pH 8.4, at room temperature until lysed. Saturated CsCl was added to give a final density of 1.685 g/cm^3 and the lysate was centrifuged at 42,000 rpm for 20 hr at 20° in the 50 Ti rotor of a Spinco L3-50 ultracentrifuge. Fractions were collected and the A_{260} was measured for each fraction. Fractions containing DNA were pooled, diluted with 2 volumes of H_2O, and were pelleted by centrifugation at 42,000 rpm for 12 hr in the 50 Ti rotor. Pellets were dissolved in 0.1 × SSC (SSC = 0.15 M NaCl + 0.015 M Na-citrate) and were banded in a CsCl gradient as before. The DNA-containing fractions were pooled and dialyzed against 0.1 × SSC. DNA prepared by this method had an A_{260}/A_{280} = 1.85 or greater and an A_{260}/A_{230} greater than 2.50. Since the DNAs showed a sharp thermal denaturation profile with little or no early melting components when heated, and showed approximately a 40% increase in A_{260} after complete denaturation, there appeared to be little or no contamination by RNA.

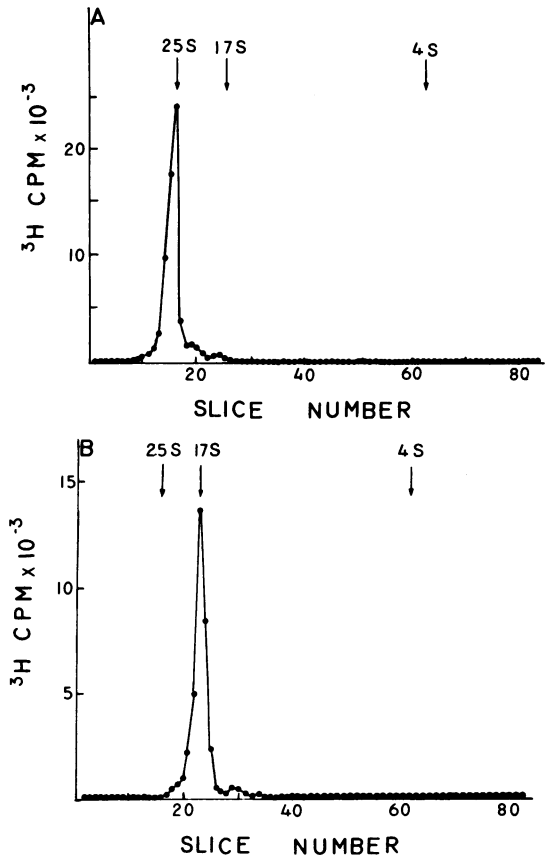

FIG. 2. Polyacrylamide gel electrophoresis of purified (*A*) 25S rRNA and (*B*) 17S rRNA. The positions of the major RNA species of carrier whole cell RNA run in the same gels are indicated.

In one experiment, DNA was isolated from strain B-VII by lysing nuclei in a solution containing 1% sodium dodecyl sulfate, 0.5 M ethylenediaminetetraacetate and 10 mM Tris·HCl, pH 9.5, digesting in Pronase (1 mg/ml), and deproteinizing with phenol (11).

Extraction and Purification of rRNA. Labeled cells were pelleted and fixed in acetic acid-ethanol (1:3). The cells were pelleted again and resuspended in NET buffer (0.1 M NaCl, 0.01 M Tris·HCl, pH 7.4, 0.001 M EDTA) containing 0.5% sodium dodecylsulfate. The RNA was deproteinized by shaking at 4° with an equal volume of chloroform: isoamyl alcohol (24:1) which had been saturated with NET buffer. The phases were separated by centrifugation. The aqueous phase was deproteinized again, collected, and the RNA was precipitated with 2 volumes of ethanol overnight at −20°.

The precipitated RNA was resuspended in NET containing 0.5% sodium dodecylsulfate, layered on a sucrose gradient (35 ml of 0.45 M–1.0 M sucrose in NET buffer), and centrifuged in the SW-27 rotor on a Spinco L3-50 ultracentrifuge at 26,000 rpm for 24 hr at 2°. Fractions (1-ml) were collected and 5 μl portions of each fraction were counted. Appropriate fractions were pooled and precipitated with ethanol as described (see Fig. 1).

The precipitated purified rRNA was resuspended in NET buffer and was examined by electrophoresis in 2.8% acryl-

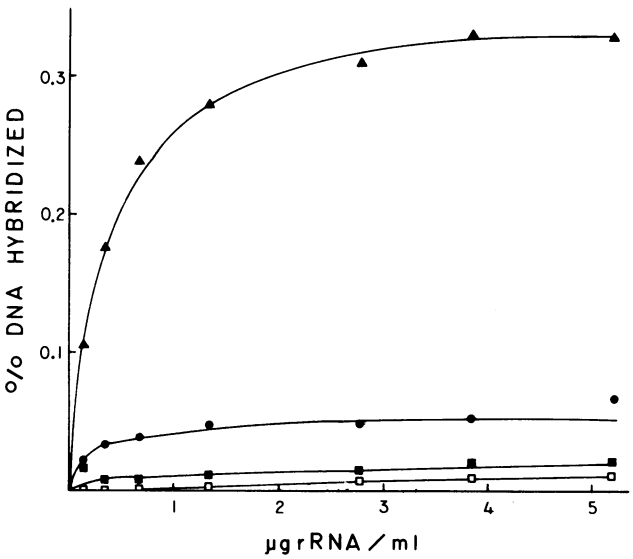

FIG. 3. Hybridization of radioactive 25S + 17S rRNA with macro- and micronuclear DNAs. DNAs bound to filters were incubated with increasing amounts of a mixture of 25S + 17S rRNA as described in the *text*. The percentages of DNA which formed hybrids were calculated by determining the amount of [^{14}C]thymidine labeled DNA and the amount of [^{3}H]uridine labeled RNA bound to each filter. ▲——▲, macronuclear DNA; ●——●, micronuclear DNA; ■——■, *Escherichia coli* DNA; □——□, blank filters.

amide gels (14). Whole cell RNA isolated from *Tetrahymena* was used as a marker (Fig. 2).

The specific activities of RNA labeled and isolated by these techniques were greater than 200,000 dpm/μg, and had a $A_{260}/A_{280} > 2$ and $A_{260}/A_{230} > 1.9$.

DNA·RNA Hybridization. DNA which had been alkali denatured was loaded onto nitrocellulose filter discs (Schleicher & Schuell type B-6) as described by Gillespie (15). About 6 μg of ^{14}C-labeled DNA was used for each filter disc, and approximately 80% of the ^{14}C radioactivity remained bound to each filter at the end of the hybridization. Hybridization was carried out in 1 × SSC containing 50% formamide at 37° for 16–20 hr with gentle shaking (10); 0.5 ml of RNA-containing solution was used per filter and each vial contained four filters (one each contained macro- and micronuclear DNA, a third contained DNA purified from *E. coli*, and a fourth was a blank filter). After hybridization, the filters were washed in 2 × SSC and digested with RNAse (10 μg/ml in 2 × SSC; 5 ml per vial at 37° for 30 min). The filters were then washed extensively, dried, and counted as described below.

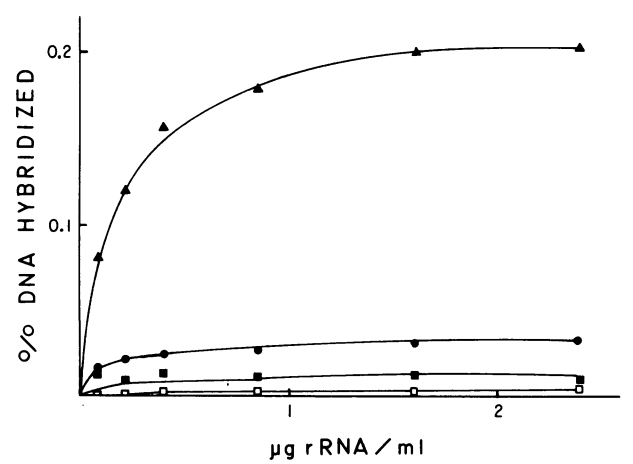

FIG. 4. Hybridization of radioactive 25S rRNA with macro- and micronuclear DNAs. Experimental procedures are the same as for Fig. 3 except that only 25S rRNA was used. ▲——▲, macronuclear DNA; ●——●, micronuclear DNA; ■——■, *E. coli* DNA; □——□, blank filters.

Isotope Counting Techniques. Aqueous samples were counted in 5–10 ml of Spectrafluor (Amersham-Searle, Arlington Heights, Ill.) containing 10% Nuclear Chicago Solubizer (NCS) (Amersham-Searle). Acrylamide gel slices were incubated in Spectrafluor-NCS at 50° with shaking overnight. Filters containing DNA·RNA hybrids were dissolved in 10 ml of Phase Combining System (PCS) (Amersham-Searle) at 50° with vigorous shaking for 8–12 hr.

All counting was performed with a Nuclear-Chicago Isocap 300 scintillation counter; the automatic external standard was used to correct for quenching and spillover. A Nova 2 computer (Data General Corp.) was used to calculate dpm for dual isotope counting; the program used was kindly prepared for us by Dr. George Hoch, Department of Biology, University of Rochester.

RESULTS AND DISCUSSION

The sedimentation pattern of the labeled RNA isolated from whole cells is shown in Fig. 1. As is expected, the radioactivity appears mainly in the 25S and 17S peaks of rRNA and in a slower sedimenting peak, presumably 4S and 5S RNA. Fractions from the appropriate peaks were pooled as shown in Fig. 1; the RNA was precipitated and analyzed on polyacrylamide gels. As can be seen in Fig. 2, the RNA used for hybridization consisted almost entirely of the appropriate rRNA species.

When DNAs bound to nitrocellulose filters were incubated with increasing concentrations of purified 25S + 17S rRNA, typical hybridization-saturation curves were obtained (Fig. 3). The blank filters and the filters containing *Escherichia coli* DNA showed little binding of the radioactive *Tetrahymena* rRNA. After correction for nonspecific binding of the RNA to *E. coli* DNA, the saturation values were found to be 0.31% for macronuclear DNA and 0.03% for micronuclear DNA of strain WH-6. In a second experiment with independently isolated macro- and micronuclear DNAs and purified 25S rRNA alone, saturation values were approximately 2/3 the value obtained for 25S + 17S rRNA (Fig. 4). Again, a 10-fold difference was observed between the saturation values of macro- and micronuclear DNAs. In a third experiment the saturation values for macro- and micronuclear DNAs isolated from strain B-VII were found to be 0.30% and 0.014% respectively when hybridized with radioactive 25S + 17S rRNA.

Using the values obtained for the hybridization-saturation levels (about 0.30%) and knowing both the size of the haploid genome in *Tetrahymena* micronuclei (1.3×10^{11} daltons, ref. 16) and the molecular weights of 25S and 17S rRNA (25S = 1.3×10^{6}, 17S = $.69 \times 10^{6}$, ref. 17), we calculate that there are approximately 200 copies of the sequences coding for rRNA per haploid genome in the macronucleus. This value is similar to that observed for other eukaryotic cells and agrees with the value reported for an amicronucleate strain of *Tetrahymena* (10).

By the same calculation, we estimate that only about 10–20 copies of the gene for rRNA per haploid genome are present in the DNA isolated from the micronuclear preparation. However, this estimate does not take into consideration possible contamination of the isolated micronuclei by macronuclei; since macronuclei contain, on the average, 20 times as much DNA as micronuclei, contamination of the isolated macronuclei by micronuclei need not be considered. While completely accurate values for the amount of macronuclear contamination in micronuclear DNA cannot be easily determined for any particular experiment, we have estimated by three independent means that about 5–15% of the DNA in the micronuclear preparations isolated by our methods is macronuclear DNA (13). Thus, at least half, and probably more than half, of the hybridization to micronuclear DNA can be accounted for by macronuclear contamination. The true number of copies of the gene for rRNA in the micronucleus is thus probably less than 10 and is quite possibly as low as 1.

The results presented above do not rule out the possibility that both nuclei contain a small number of rRNA genes but the sequence complexity of macronuclear DNA is only 5–10% that of micronuclear DNA, as recently described for another ciliate, *Stylonychia* (18). However, in experiments to be published elsewhere we have shown by kinetic analysis of the hybridization of macro- and micronuclear DNAs that 85–90% of the sequences contained in micronuclei are present in similar amounts in macronuclei.

Since only the micronucleus maintains the genetic continuity between sexual generations, it is immediately clear that only a small number of copies of the gene for rRNA (possibly only one) is transmitted genetically in *Tetrahymena*. It is also clear that an event analogous to gene amplification (19, 20) must take place each sexual generation to give rise to the multiple copies of the gene for rRNA which are present in the somatic (macro-) nucleus. This idea is further supported by studies showing that the genes for rRNA in *Tetrahymena* macronuclei are physically unlinked to the bulk of the DNA (11) and can be replicated quite independently of the remainder of the macronuclear DNA (21, 22). However, amplification in *Tetrahymena* shows distinct differences from amplification in oocytes. In *Tetrahymena*, amplification occurs from a small number of genes in a germinal (micro-) nucleus to a number of genes in the somatic (macro-) nucleus (200/haploid DNA amount) which is similar to the level of genes for rRNA found in the somatic nuclei of other eukaryotes. In oocytes, amplification results in a number of genes for rRNA greatly exceeding the number found in somatic tissues. Moreover, the amplified genes in *Tetrahymena* are capable of replicating indefinitely during vegetative growth whereas to our knowledge, there is little evidence to suggest that the amplified genes found in oocytes replicate subsequent to amplification.

In *Tetrahymena*, amplification of the gene for rRNA occurs from a small number of gene copies (in the micronucleus) similar to the number found in prokaryotes. In addition, the ribosomal genes of other lower eukaryotes may also replicate independently (23) and be physically unlinked to the bulk of nuclear DNA (18, 24). These observations suggest that the process of amplification may have arisen early in the evolution of eukaryotes, possibly as a mechanism to provide increased numbers of templates for the synthesis of ribosomes necessary for the formation of larger cells (11). It should also be noted that if amplification from a limited source occurs by a mechanism involving a rolling circle model of DNA synthesis (25, 26), it is possible to generate identical, tandemly repeated DNA sequences. These could be broken down to the size of one or a few genes (as are found in *Tetrahymena*, ref. 11) or, in the course of evolution, might have been retained in tandem configuration or possibly even have been integrated into larger chromosomal arrays.

The properties of the genes for rRNA in *Tetrahymena* macro- and micronuclei provide a simple mechanism for keeping the multiple copies of this gene identical within the organism while the gene evolves. Whether a similar mechanism occurs in other organisms or is unique to *Tetrahymena* remains to be elucidated. At first sight, it seems that a similar mechanism may not operate for the rRNA genes of higher organisms, since the number of cistrons for rRNA in the somatic and germinal tissues of higher organisms appears to be the same (27, 28). However, reduction to a few copies of a repetitive gene might be a transient event, occurring only briefly in the primordial germ line in higher organisms. It is also possible that expansion from a limited copy can occur without immediate loss of preexisting amplified copies.

A small number of genes for rRNA may be demonstrable in the micronucleus of *Tetrahymena* only because the micronucleus is unique among germinal nuclei inasmuch as it always occurs in the same cell as the somatic (macro-) nucleus. Therefore, the micronucleus need not form a nucleolus or synthesize ribosomes during vegetative growth. Germinal nuclei in other organisms must also act as somatic nuclei and, therefore, may require large numbers of genes for rRNA during most of their existence.

It should now be possible to examine other repetitive genes (for 5S RNA, for histones) to determine if a similar mechanism can account for the genetic transmission of all such genes in *Tetrahymena*, or if additional mechanisms must be invoked. It should also be possible to characterize the nature of the copy(ies) of the genes for rRNA in micronuclei and to study the process by which they are amplified during conjugation.

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