THE NATURE AND PROCESSING OF RIBOSOMAL RIBONUCLEIC ACID IN A DINOFLAGELLATE

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ABSTRACT

Certain features of the dinoflagellate nucleus suggest that it represents a primitive form of eukaryotic nucleus. For this reason, it was of interest to characterize dinoflagellate ribosomal RNA (rRNA) and its mode of synthesis to determine if it also deviated from typical eukaryotic patterns. *Gyrodinium cohnii* was chosen for this examination. *Gyrodinium* ribosomal RNA species are 16 and 25s as judged by their sedimentation velocities in isokinetic sucrose gradients. These values are typical of higher plants. In addition, the RNA cosedimented precisely with rRNA from the ciliate *Tetrahymena*. Nucleotide ratio analyses revealed a GMP + CMP content of 46% for both species of rRNA. The kinetics of incorporation of a radioactive precursor into ribosomal RNA have also been studied, and it seems likely that the maturation of rRNA starts with the synthesis of a 38s molecule. This serves as precursor to the 16s species, and, after a 27s intermediate, the 25s ribosomal component. The process is similar to that in other eukaryotes. The structure of the nucleolus has also been examined, and is seen to be typically eukaryotic.

INTRODUCTION

Members of the order Dinoflagellata are considered to be eukaryotes that have retained certain prokaryotic features with respect to nuclear structure and composition (24). They are unique among higher organisms in that the conformation of each of the DNA-containing bodies in the nucleus, referred to as chromosomes, is similar to that exhibited by the nucleoids of certain bacteria (7), and they appear to lack chromosomal basic proteins (5). Chromatid segregation in these organisms is also reminiscent of bacterial mechanisms, apparently involving attachment to the nuclear envelope and separation by membrane flow (10). The nuclear envelope itself shows structural variation within the order, from a smooth-surfaced bilaminar envelope with an unusual vesicle-mediated form of nucleus-to-cytoplasm transport (1), to the annulated envelope typical of eukaryotes. For these reasons, the dinoflagellate nucleus is thought to be a contemporary manifestation of the primeval eukaryote nucleus, and it was of interest to examine the nature and processing of ribosomal RNA in these organisms so as to determine if it exhibited properties supporting this concept.

An important distinction between prokaryotes and all the eukaryotes studied to date lies in what transformations ribosomal RNA undergoes during the period between transcription and its incorporation into assembled ribosomes. In bacteria, although their cistrons are closely linked (29), the RNA's which contribute to the large and small subunits of the ribosomes are apparently synthesized as discrete molecules and undergo no change in molecular size before the ribosome is completed (31). In eukaryotes, however, a single large molecule is synthesized on the nucleolar organizer DNA (3, 25), and this serves as the precursor, after a series of maturation steps, to both

the large and small major RNA components (for a review, see reference 4). This mechanism has been described for all eukaryotes examined, from yeast (32) to HeLa cells (28, 37). Another difference is in the size of the ribosome and its RNA's. Ribosomes are described as being 70s in prokaryotes, and 80s in eukaryote cytoplasm (33). The RNA's they contain have undergone somewhat more divergence in size and are described generally as being 16 and 23s in bacteria, mitochondria, and chloroplasts, 16 and 25s in plant cytoplasm, and 18 and 28s in animal cytoplasm (30).

The nature and processing of ribosomal RNA have now been examined in the Dinophycean *Gyrodinium cohnii*. This report will demonstrate that ribosomal RNA's in this species are of the plant type, that they are similar to those of other unicellular eukaryotes (in particular the ciliate *Tetrahymena pyriformis*), and that the RNA undergoes a maturation process homologous with that in other higher organisms. In addition, the dinoflagellate nucleolus is essentially indistinguishable in its morphology from the nucleoli of other eukaryotes.

MATERIALS AND METHODS

An axenic culture of *Gyrodinium cohnii* (Schiller) was kindly provided by Dr. G. Holz, State University of New York, Upstate Medical Center. The cells were grown in AXM medium (21) at 25°C either in 200-ml Roux bottles (for electron microscopy) or as 1-liter "spinner" cultures in 2-liter Erlenmeyer flasks (for RNA studies). The doubling time under both these conditions is about 9 hr. The details of labeling are given in the text and figure legends. Adenine-8-^{3}H (sp. act. 19.3 Ci/mmole) was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y., and sodium phosphate-^{32}P from Abbott Laboratories, N. Chicago, Ill.

For electron microscopy, cells were pelleted at low speed and resuspended in Karnovsky's formaldehyde-glutaraldehyde (8). After 1–2 hr of fixation at 0–4°C, they were washed over a period of several hr with four to five changes of 0.1 M sodium phosphate buffer, pH 6.8, then postfixed with 2% OsO_4 in the same buffer. After several distilled water washes, the cells were exposed to uranyl acetate according to Ryter and Kellenberger (26), for preventing aggregation of the DNA. Pellets of cells were dehydrated in ethanol, embedded in Epon (16), and sectioned on a Sorvall MT-1 ultramicrotome equipped with a diamond knife. Sections were stained with 3% aqueous uranyl acetate and lead citrate (35), then examined with a Siemens Elmiskop I operated at 80 kv. Measurements were made relative to a carbon grating replica having 54,400 lines/in.

For RNA studies, cells from 1-liter cultures were centrifuged at 1000 g for 5 min, and the pellets were resuspended in 10 volumes of an extraction buffer (0.15 M sodium acetate, 0.1 M sodium chloride, 1% sodium lauryl sulfate) supplemented with 0.001% each of polyvinyl sulfate and heparin. The cells were disrupted by three rapid passes through a Logeman hand mill. RNA was extracted by Kirby's (9) phenol-cresol method: to the cell homogenate was added an equal volume of 6% 4-aminosalicylate and 2 volumes of phenol-cresol mixture (PCM: 500 g of phenol, 55 ml of distilled water, 70 ml of twice-distilled *m*-cresol, and 0.5 g of 8-hydroxquinoline). The first extraction was carried out for 15 min and at 0–4°C (as were all subsequent steps). After a 10-min centrifugation at 1000 g, the aqueous phase was made 2% in NaCl and extracted for 10 min with an equal volume of PCM. The aqueous phase was recovered, extracted once more, then the nucleic acids were precipitated by adding 2.5 volumes of 95% ethanol and storing at about −15°C for 2–3 hr. The flocculent precipitate was pelleted, washed with 95% and 100% ethanol, then dissolved in 0.1 M NaCl, 0.01 M Na acetate, pH 5.1, containing 0.001 M $MgCl_2$. Since the DNA is apparently not protein bound in these organisms, it remains in the aqueous phase during the extraction. To remove the DNA, the nucleic acid solution was subjected to a 30-min digestion at 37°C with DNase (Worthington Biochemical Corp., Freehold, N. J.; electrophoretically pure) at 50 µg/ml. This was followed by a PCM extraction, ethanol precipitation, and a 3 M Na acetate (pH 6.5) wash for purifying the ribosomal RNA from lower molecular weight contaminants (see Results). After ethanol dehydration, the RNA was dissolved in 0.1 M NaCl, 0.01 M Na acetate, pH 5.1, and this buffer was used in all subsequent procedures.

For analytical purposes, sucrose density gradients were designed according to Noll (18) to give constant velocity sedimentation. Convex exponential gradients (15–29.6% sucrose) were prepared for the SW 65 rotor by pumping 5 ml of 35% sucrose into an airtight mixing chamber containing 4 ml of 15% sucrose. The centrifuge tubes were filled from the bottom by displacing the contents of the mixing chamber. Details of the centrifugation conditions are given in the figure legends. Ribonuclease-free sucrose (Schwarz BioResearch, Inc.) dissolved in 0.1 M NaCl, 0.01 M Na acetate, pH 5.1 was used for all RNA gradients. The gradients were read continuously with a Gilford-Beckman recording spectrophotometer, and fractions were collected directly onto 2.4-cm filters (Whatman GF/A) for radioactivity determinations.

For nucleotide ratio analyses, the two species of ribosomal RNA, uniformly labeled with ^{32}P, were separated on 15–30% linear sucrose gradients. To the pooled peak fractions were added 1 mg of cold

carrier RNA and 2.5 volumes of ethanol. The precipitates were washed thoroughly with ethanol and dried *in vacuo*. Each sample was then hydrolyzed with 0.3 ml of 0.3 N KOH for 18 hr at 37°C. The hydrolysate was neutralized by the addition of Dowex-50 (2×-100 mesh) in the pyridine form. The supernatant was removed after centrifugation, and the resin was washed three times with 0.7 ml of 0.1 N NH_4OH. The four supernatants were combined and dissolved in a small volume of distilled water and separated electrophoretically on Whatman 3 MM paper in 0.05 M Na citrate, pH 3.5, at 10 v/cm for 22 hr. The nucleotides were eluted from the paper with distilled water, and aliquots were counted in a Packard liquid scintillation spectrometer.

RESULTS

Electron Microscopy

Gyrodinium cohnii is an unarmored marine dinoflagellate. The cells have a trilaminar cell wall, and the cytoplasm is densely packed with ribosomes and has little endoplasmic reticulum. There are many mitochondria, with the tubular cristae

typical of unicellular eukaryotes. The general morphology of *Gyrodinium* has been described by Kubai and Ris (10).

Fig. 1 illustrates the essential features of the dinoflagellate nucleus. The nuclear envelope in this genus appears to be identical with that commonly seen in eukaryotic cells. "Chromosomes" in dinoflagellates remain condensed throughout the cell cycle, and become more condensed during division. Their structure has been discussed in detail by others (2, 7, 24). Several "chromosomes" are intimately associated with the nucleolus in an as yet undetermined fashion.

The nucleolus itself is bipartite, just as in other normal cell types, and has an extensive inner fibrillar component and a peripheral granular component (Fig. 1). The granules are about 250 Å in diameter and are ribonuclease sensitive. The intranucleolar paracrystalline structures illustrated by Kubai and Ris (10) have been seen frequently in these cultures also, but their nature has not yet been investigated.
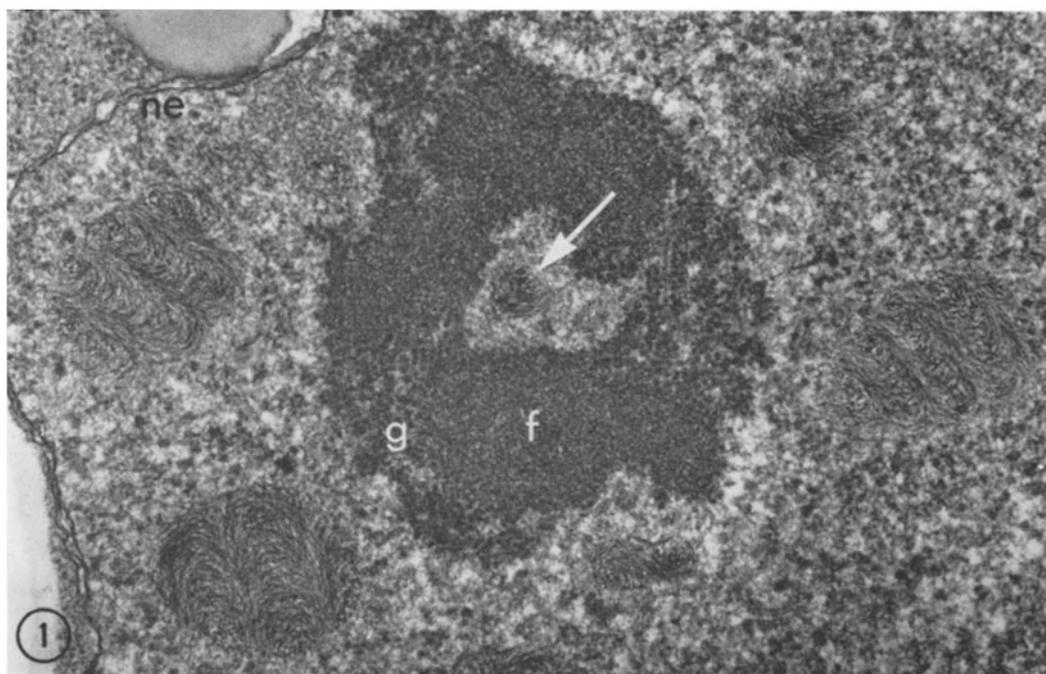

FIGURE 1   Part of the nucleus of *Gyrodinium cohnii*. A chromosome closely associated with the nucleolus is indicated by an arrow. *f* and *g*, fibrillar and granular components of the nucleolus; *ne*, nuclear envelope. Formaldehyde-glutaraldehyde and osmium tetroxide fixation; uranyl acetate and lead citrate staining. × 47,500.

Biochemistry

When RNA is extracted from *Gyrodinium* and analyzed by density gradient centrifugation, a very prominent peak of ultraviolet-absorbing material appears at 9.4s in addition to the faster sedimenting peaks characteristic of ribosomal RNA. This material is completely sensitive to DNase and contaminates the RNA because the DNA is apparently not bound to basic proteins in these organisms and, therefore, is not retained in the interphase during the phenol-cresol extractions. To minimize this contamination, all preparations were treated with DNase and washed with 3 M Na acetate after ethanol precipitation. This treatment solubilizes the low molecular weight nucleic acids; the material larger than 8–10s remains as a precipitate. Such treatment has no effect on the relative amounts or sedimentation properties of the large mol wt RNA components (9).

To establish approximate s values for dinoflagellate ribosomal RNA, purified RNA, labeled for three generations with 2.5 μCi/ml of adenine-^{3}H, was cosedimented in isokinetic sucrose gradients with unlabeled *E. coli* RNA which had been phenol extracted from ribosomes prepared by the method of Tissières et al. (34). As can be seen from Fig. 2, the peaks of the more slowly sedimenting components were superimposed. The larger dinoflagellate RNA, however, sedimented somewhat faster than the corresponding bacterial molecule. If the s values for bacterial ribosomal RNA are taken to be 16 and 23s (12), then the *Gyrodinium* RNA species are calculated to be 16 and 25s. The gradients were carefully calibrated for matching optical density and collected fractions, and the values have proven to be reproducible regardless of the centrifugation time.

For the purpose of comparison of dinoflagellate ribosomal RNA with that of another unicellular eukaryote, a similar experiment was done using unlabeled RNA from the ciliate *Tetrahymena pyriformis*. Although there is some variation in the reported s values for ribosomal RNA from protozoa and protophyta (see Discussion), Fig. 3 illustrates that RNA from these two distantly related species sediments at the same rate.

Gyrodinium ribosomal RNA was further characterized by nucleotide ratio analysis using RNA that had been labeled for several generations with ^{32}P at 1 μCi/ml. The two species of ribosomal RNA were separated on sucrose gradients, alkali hydrolyzed, and the proportion of radioactivity in
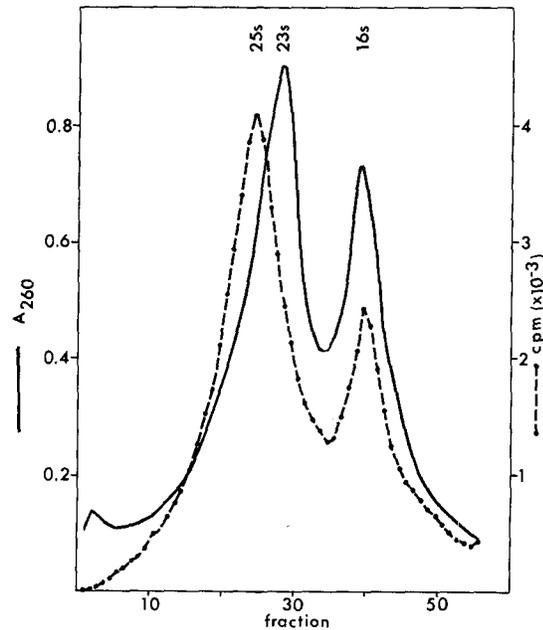

Figure 2 Cosedimentation of adenine-^{3}H–labeled *Gyrodinium* rRNA with unlabeled *E. coli* rRNA. In this and Fig. 3, radioactive RNA contributed less than 5% of the total optical density. Sucrose gradients in this and the following figures were 15–29.6% convex exponential as described in Materials and Methods. Sedimentation is from right to left. Centrifugation was at 65,000 rpm for 4 hr at 5°C.

each of the nucleotides was determined after separation by paper electrophoresis. Table I indicates the percentage of radioactivity in each of the four nucleotides for 16 and 25s RNA. The over-all %(GMP + CMP) is relatively low, about 46%, and there is little difference between the two RNA's in this regard. They do exhibit differences, however, when the percentages of each nucleotide are compared.

The kinetics of incorporation of a radioactive precursor into *Gyrodinium* ribosomal RNA was studied by incubating cultures with adenine-^{3}H at 10 μCi/ml and removing aliquots at specified times. The cells were quickly chilled by pouring into a slurry of 4 volumes of frozen medium, then centrifuged in the cold. The RNA was extracted and analyzed in sucrose gradients. In these experiments, the extracted nucleic acids were subjected to only a brief digestion with DNase for the purpose of a separate study of the timing of DNA synthesis. As a result, the subsequent 3 M Na acetate wash removed, in addition to the low mol

wt RNA, only about half of the DNA. The treatment did, however, convert the DNA to a lower average molecular weight shifting the peak to a position further away from the 16s ribosomal RNA.

Fig. 4 (*a* and *b*) illustrates that after brief periods of incorporation the label is distributed heterogeneously. The radioactivity in the upper part of the gradients is contributed by both RNA and DNA. By 2.5 min, a small peak of labeled RNA appears, sedimenting at 38s. At 5 min (Fig. 4 *c*), this peak becomes prominent and an additional peak forms at 27s. Activity in the 16s region is obscured by the incorporation of adenine into DNA. However, by 10 min (Fig. 4 *d*) there is a clear increase in radioactivity in both the 16 and 27s species, and a relative decline in the 38s peak. At this time, there is also some indication of in-

corporation into 25s RNA. At 20 min (Fig. 4 *e*), the radioactivity peaks are coincident with those of the optical density profile and are also now in approximately the same relative proportion; the activities in the higher molecular weight regions appear as a broad shoulder to the 25s peak. Finally, at 40 min (Fig. 4*f*), the radioactivity profile closely follows that of the optical density.

Although this sequence of incorporation distributions only indicates *possible* precursor-product relationships, it suggests that a series of events occurs in the production of ribosomal RNA in dinoflagellates that is similar to that observed in many other eukaryotes, i.e., in this case it is likely that a 38s molecule is synthesized and cleaved to yield 27 and 16s products. The 27s RNA then converts to the mature 25s species.
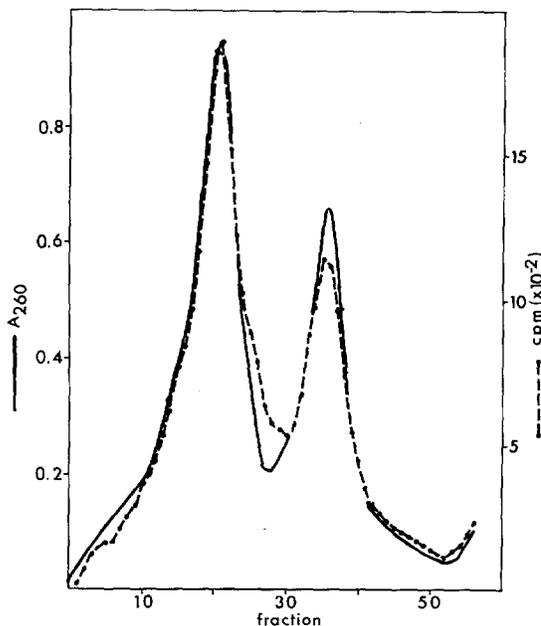
DISCUSSION

When one considers the low phylogenic status of dinoflagellates and the indications that their nucleus may, in some respects, represent a form intermediate between that of prokaryotes and that of eukaryotes (10, 24), the possibility existed that their ribosomal RNA, or the mode of its synthesis, might also reflect the transition from the lower to higher forms. This now appears not to be the case.

The nucleolus, in which most of the steps in the maturation of ribosomes takes place (19, 20), appears in *Gyrodinium* to be structurally identical with nucleoli seen in all other eukaryotes. Even in the more primitive Dinophyceae *Prorocentrum* and *Exuviella*, which had been described as not having clearly demonstrable nucleoli (6), electron microscopy has revealed the presence of completely typical nucleoli (unpublished observations on *Prorocentrum* sp. and *Exuviella* sp. obtained from Dr. R. Guillard of the Woods Hole Oceanographic Institution).

A maturation of ribosomal RNA from a precursor molecule to the products in active ribo-

FIGURE 3 Cosedimentation of adenine-³H–labeled *Gyrodinium* rRNA with unlabeled *Tetrahymena pyriformis* rRNA. Conditions were as in Fig. 2.

TABLE I

Nucleotide-³²P Ratios in Ribosomal RNA of Gyrodinium cohnii

(Values are the averages of three determinations ± the standard deviations)

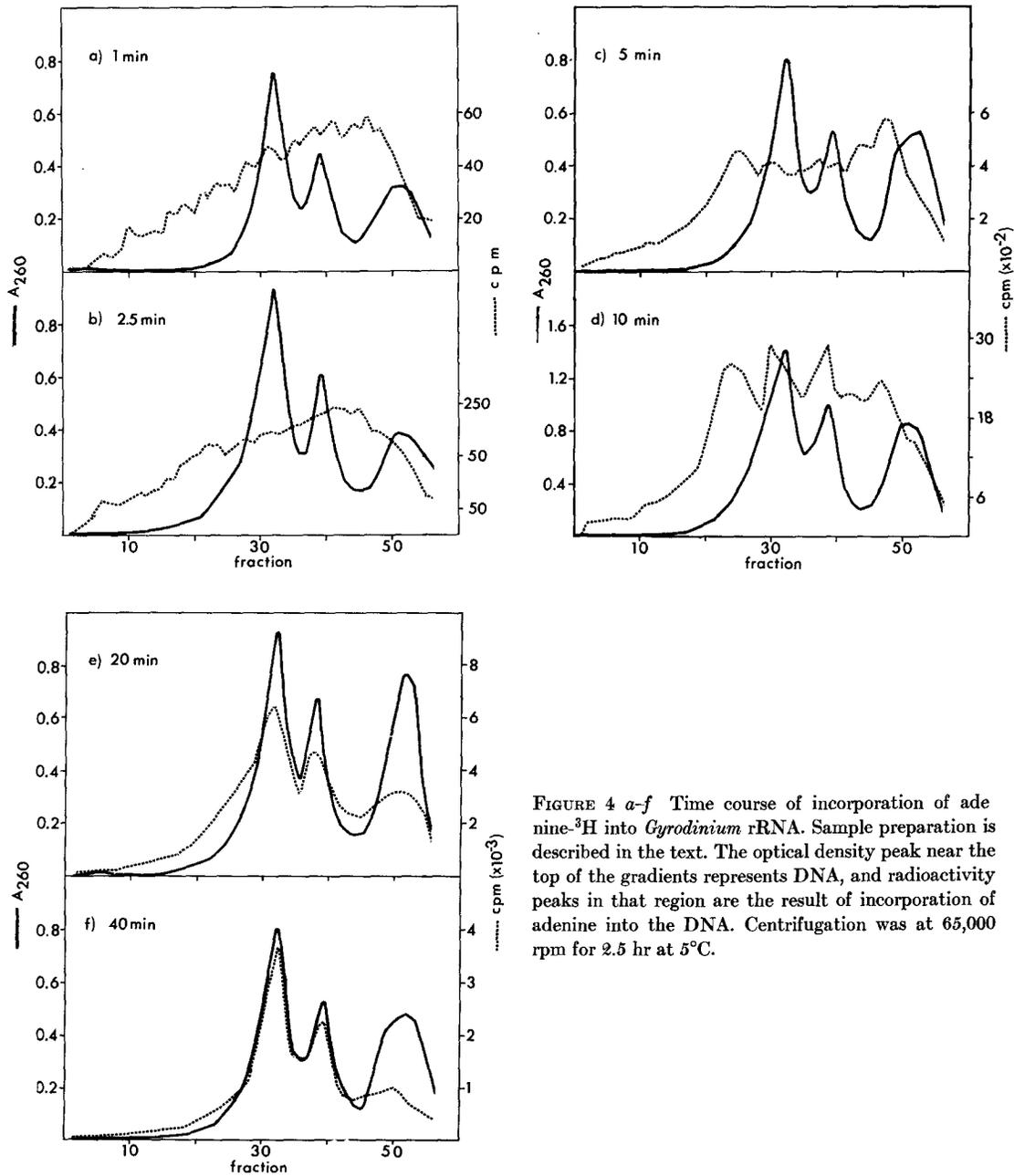| s	AMP	CMP	GMP	UMP	(G + C) %
16	26.8 ± 0.06	19.5 ± 0.06	26.5 ± 0.00	27.2 ± 0.06	46.0
25	25.1 ± 0.07	18.5 ± 0.00	28.0 ± 0.07	28.4 ± 0.00	46.5

FIGURE 4 a-f Time course of incorporation of ade nine-^{3}H into *Gyrodinium* rRNA. Sample preparation is described in the text. The optical density peak near the top of the gradients represents DNA, and radioactivity peaks in that region are the result of incorporation of adenine into the DNA. Centrifugation was at 65,000 rpm for 2.5 hr at 5°C.

somes has been described for several eukaryotes. The process is exceptionally well characterized for mammalian cells (20, 28, 37) and may be summarized as follows (4): a large molecule, nominally 45s, is transcribed, then rapidly methylated, and associated with protein. Still within the nucleolus, the RNA molecule is cleaved,

yielding the 18s rRNA component (possibly after a 20s stage), which rapidly enters the cytoplasm, and a 41s molecule which results, after a 36s intermediate, in a 32s molecule. This yields finally the 28s ribosomal RNA. About half the 45s precursor is degraded in this process, but the methylated regions are conserved.

The sequence of events in the maturation of ribosomal RNA in higher plants is not so clear as in animal cells (14). Waters and Dure (36) have detected the presence of a ribosomal precursor particle in cotton plantlings which they find to sediment between 40 and 50s, and which, upon treatment with anionic detergent, releases two RNA molecules; the more slowly sedimenting component has the same rate as the small ribosomal RNA, and the faster lags somewhat behind the larger ribosomal RNA. Evidence for a *large molecular weight* rRNA precursor in plants has been obtained in the water mold *Blastocladiella* (17), in lettuce (15), and most clearly by Loening (13) in peas.

In dinoflagellates, which are considered primitive when classified either with the algae or protozoa, it has been possible to detect the presence of a 38s molecule which apparently gives rise to a 16s and a 27s molecule. The 27s species seems to convert to the mature 25s ribosomal RNA component. The over-all sequence is directly comparable with that described for HeLa cells. Yet to be examined are such considerations as the extent of methylation and the degree to which the initial precursor molecule is conserved (in the ciliate *Tetrahymena* (11) and in plants (14), relatively less RNA is degraded than in HeLa).

A comparison of the sedimentation rates of dinoflagellate ribosomal RNA with that of other organisms confirms its evolved state. The values obtained for the larger and smaller components,

25 and 16s, are the same as those published for higher plants (30), and are similar to those for the unicellular alga *Chlamydomonas* (24 and 16s; 27). Although there appears to be some variation in sedimentation rates among the unicellular eukaryotes (e.g., 24 and 20s in *Euglena*; 22), *Gyrodinium* rRNA was found to cosediment exactly with that of the advanced ciliate *Tetrahymena*. These results suggest that distinctions made between protozoan and protophytan ribosomal RNA's may not be real, at least if relative sedimentation rate is used as the criterion, and that the scheme for ribosome evolution proposed by Reisner et al. (23) may be more elaborate than is actually the case.

In conclusion, it appears that although dinoflagellates have retained certain primitive features with respect to nuclear structure and function, they have acquired the eukaryotic form of ribosomal RNA and its processing.

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