MITOCHONDRIAL RNA FROM CULTURED ANIMAL CELLS

II. A Comparison of the High Molecular Weight RNA from Mouse and Hamster Cells

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ABSTRACT

Discrete RNA fractions sedimenting slightly slower than 18s ribosomal RNA have been found in mitochondrial preparations from both hamster (BHK-21) and mouse (L-929) cells. This RNA could be separated into two components, present in approximately equimolar amounts, by prolonged zonal centrifugation or acrylamide gel electrophoresis. The hamster components had sedimentation constants averaging 16.8 and 13.4, and molecular weights (estimated by gel electrophoresis) averaging 0.74 and 0.42 $\times$ 10^6 daltons. Mixed labeling experiments showed that the mouse components sedimented *and* electrophoresed 3–6% more slowly than the corresponding hamster components. The RNA from both cell lines resembled mitochondrial ribosomal RNA from yeast and *Neurospora* in being GC poor, and in addition the larger and smaller components resembled each other in base composition. These results, taken with those of other recent studies, are compatible with the idea that our high molecular weight mitochondrial RNA is ribosomal; such RNA would then constitute a uniquely small size-class of ribosomal RNA.

INTRODUCTION

We have recently described distinctive high molecular weight RNA associated with the mitochondria of cultured hamster (BHK-21) cells (1, 2). This RNA sedimented slightly slower than cytoplasmic 18s RNA and differed from the latter in that it had an unusually low GC content, was very poorly methylated, and was synthesized in the presence of a level of actinomycin D (0.1 μg/ml) that preferentially suppresses cytoplasmic ribosomal RNA (rRNA) synthesis.

We suggested that this might be the RNA of mitochondrion-specific ribosomes because (*a*) it was the only likely candidate for such a role (cf. 3) in hamster mitochondria; (*b*) its base ratios resembled those of mitochondrial ribosomal RNA from yeast (4) and *Neurospora* (5, 6); and (*c*) it was separable into a major and a minor component on prolonged centrifugation or on acrylamide gel electrophoresis. However, the RNA appeared to be unusually small for ribosomal RNA. Not only did both components sediment behind 18s RNA in density gradients, but their molecular weights as estimated by gel electrophoresis, 0.73 $\times$ 10^6 daltons and 0.41 $\times$ 10^6 daltons, were considerably lower than those of other ribosomal RNA's thus far examined (see reference 7).

The initial aim of the present study was to ascertain if this class of RNA was peculiar to our

hamster cell line or whether it was more widespread in nature. On finding analogous discrete high molecular weight mitochondrial RNA components in cultured mouse cells, we proceeded to further characterize these RNA species from both sources, and to compare the two in detail.

Our results indicate that the larger and the smaller components of this RNA fraction, as obtained from either mouse or hamster mitochondria, are present in approximately equimolar amounts and have similar base ratios, in agreement with the idea that the RNA may be ribosomal. In addition, small but definite centrifugal and electrophoretic differences between corresponding hamster and mouse high molecular weight mitochondrial RNA components were observed. Possible evolutionary implications of these results are discussed.

MATERIALS AND METHODS

Hamster (BHK-21; reference 8) and mouse (L-929; reference 9) cells were grown in spinner culture as previously described (10). Cells were fractionated in hypotonic buffer, and mitochondria were purified by differential and isopycnic gradient centrifugation (2). The procedures for purification and analysis of RNA are described in the legends to Figs. 1–3 and Table I. Radioactive chemicals were purchased from New England Nuclear Corp., Boston, Mass., medium components were obtained from Grand Island Biological Co., Grand Island, N. Y., and actinomycin D was the gift of Merck and Co., Inc., Rahway, N. J.

RESULTS

Preliminary Studies

L-cells were found to respond like BHK cells when subjected to extended treatment with actinomycin D at low concentrations. The only striking metabolic alteration observed was the cessation of cytoplasmic rRNA synthesis (cf. 2, 11, 12). In addition, as was found with BHK (2) and HeLa cells (13, 14), standard preparative methods did not yield L-cell mitochondria completely free of cytoplasmic ribosomes. Hence, in the experiments described below, mitochondria were prepared only from cultures labeled in the presence of actinomycin D (0.1 μg/ml, 22–24 hr). When uridine-^{14}C-labeled mitochondrial RNA prepared from such L-cell cultures was fractionated by density gradient centrifugation under conditions designed to display the cytoplasmic ribosomal RNA components, a "17s" peak resembling the unresolved high molecular weight RNA peak of BHK cells was observed (1, 2).

Centrifugal Properties of BHK and L-Cell Mitochondrial RNA

For a more precise comparison of hamster and mouse mitochondrial RNA, BHK, and L-cells were labeled with uridine-^{14}C and ^{32}P, respectively, and mitochondria were prepared from the cultures in parallel. The two mitochondrial bands were pooled, RNA was purified together with unlabeled L-cell cytoplasmic RNA, and this mixedly labeled RNA preparation was subjected to prolonged density gradient centrifugation (sufficient to partially pellet 28s rRNA). As shown in Fig. 1, the ^{14}C and ^{32}P patterns were quite similar; under these centrifugal conditions the discrete high molecular weight mitochondrial RNA from both

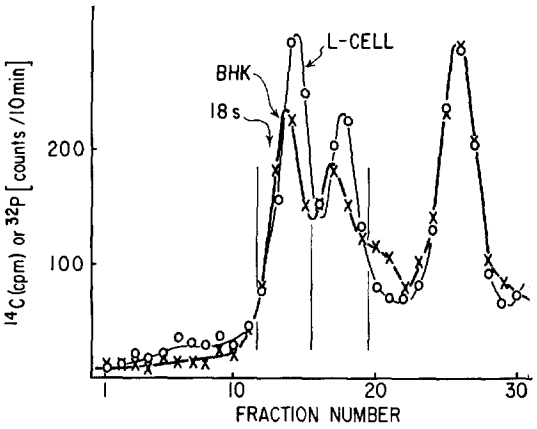

FIGURE 1 Velocity sedimentation analysis of mitochondrial RNA from BHK and L-cells. BHK-21 and L-929 cells (starting density 5 × 10^5/ml) were labeled for 22 hr with uridine-^{14}C (0.2 μCi/0.01 μmole/ml) and ^{32}P (10 μCi/1.2 μmoles/ml), respectively, in the presence of 0.1 μg/ml of actinomycin D. Mitochondria were purified in parallel by differential and isopycnic gradient centrifugation (2). The mitochondrial bands were pooled, unlabeled L-cell cytoplasmic RNA was added, and the RNA was extracted with 2% sodium dodecyl sulfate (SDS) and purified by shaking with phenol at room temperature as previously described (15). Velocity sedimentation was carried out in sucrose gradients (5–20% sucrose in 0.05 M NaCl, 0.01 M sodium acetate, pH 5.1) at 30,000 rpm, 5°C, for 16 hr (SW41 Spinco rotor [Beckman Instruments, Palo Alto, Calif.]). Portions of each fraction were precipitated with 5% trichloroacetic acid, and ^{32}P and ^{14}C were counted differentially as previously described (15). (-X-X-, ^{14}C; -O-O-, ^{32}P).

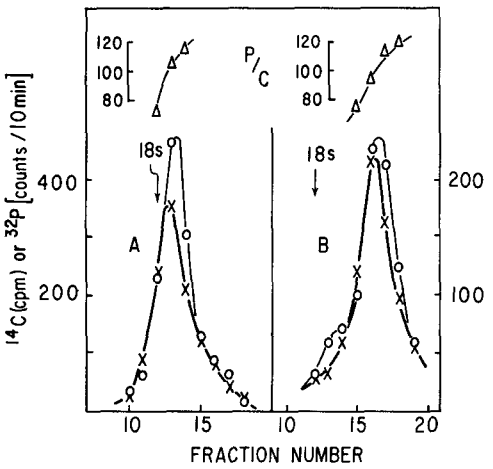

FIGURE 2 Velocity sedimentation analysis of the individual components of the high molecular weight mitochondrial RNA from BHK and L-cells. The peak fractions within the vertical lines of Fig. 1 were pooled, and the RNA was precipitated with ethanol and recentrifuged as in Fig. 1. Fig. 2 A represents the heavier RNA components, and Fig. 2 B the lighter. The $^{32}P/^{14}C$ ratios ("P/C") are plotted above the corresponding fractions. We have normalized these values by expressing them as percentages of the average value across each RNA peak. (-X-X-, ^{14}C; -O-O-, ^{32}P).

sources was partially resolved into two peaks, and there were in addition prominent 4s peaks and a small amount of rapidly sedimenting heterodisperse RNA, all as previously described for BHK cells (2). However, the positions of the ^{14}C-labeled high molecular weight components did not appear to coincide exactly with those of the corresponding ^{32}P-labeled peaks. To examine this discrepancy further, appropriate cuts from the gradient (Fig. 1) were recentrifuged, yielding discrete peaks of both ^{14}C and ^{32}P corresponding to both the heavy (Fig. 2 A) and the light (Fig. 2 B) RNA components. Analysis of these patterns confirmed the sedimentation discrepancies, as clearly demonstrated by the marked deflections of the ^{32}P:^{14}C ratios across each peak. Patterns such as those in Figs. 1 and 2 were used to determine the relative sedimentation constants according to the method of Martin and Ames (16) as applied to rRNA by Click and Tint (17). The values for the hamster mitochondrial components averaged 16.8 and 13.4 (four determinations; range 16.5–17.2 and 13.0–13.8)[1], and the corresponding L-cell values were always 3–4% lower.

TABLE I

Base Ratios of High Molecular Weight Mitochondrial RNA from BHK-21 and L-929 Cells

	Mitochondria			
	Unresolved high molecular weight peak	Large component	Small component	Cytoplasmic 18s
BHK-21				
C	20.9 ± 0.1	22.3	20.8	25.8
A	34.0 ± 0.9	33.0	33.2	21.2
G	16.6 ± 0.8	17.7	18.4	31.1
U	28.5 ± 0.5	27.0	27.4	21.9
L-929				
C	20.3 ± 0.2	17.3	20.3	27.5
A	35.8 ± 0.9	37.7	38.3	19.8
G	16.3 ± 0.9	16.0	15.2	31.9
U	29.6 ± 0.1	28.8	26.1	20.8

All RNA preparations were labeled with ^{32}P for 22–24 hr and the results are expressed as mole %, plus or minus standard error where applicable. RNA samples were hydrolyzed with 0.5 N KOH for 16 hr at 37°C and brought to a pH of 3.5 with perchloric acid. High voltage electrophoresis was carried out in 0.05 M ammonium formate buffer, pH 3.5, for 90–100 min at 35 v/cm. The strips were counted either in a Nuclear Chicago strip counter (Nuclear-Chicago Corp., Des Plaines, Ill.) or, in the case of low levels of radioactivity, the spots were eluted with 0.05 N HCl, plated on stainless steel planchets, and counted in a low background end window counter. The separated mouse mitochondrial RNA components correspond to fractions 12–14 of Fig. 2 A and fractions 15–17 of Fig. 2 B and the separated hamster components are from an analogous preparation; these results represent one electrophoretic run each. For comparison, the values obtained for homologous cytoplasmic 18s RNA preparations are also given.

Base Composition

Base ratios were obtained on the ^{32}P-labeled (L-cell) RNA of each of the separated peaks of Fig. 2 as well as on a similarly labeled, unresolved L-cell high molecular weight mitochondrial RNA peak, and on corresponding RNA fractions from ^{32}P-labeled BHK cultures (Table I). Within the limits of precision of these assays, there was no difference between the hamster and the mouse

[1] Our original "17s" designation (10) of this RNA was strictly nominal, intended to indicate only that the RNA in question sedimented slightly slower than 18s rRNA.

RNA's, and in addition the larger and the smaller components resembled one another in base composition. The low GC content of these samples is in accord with results reported for putative rRNA of yeast and *Neurospora* mitochondria (4–6), and contrasts with the high GC content of animal cell cytoplasmic rRNA (a property that we have confirmed for our two cell lines).

Acrylamide Gel Electrophoresis

The mixedly labeled RNA preparation described in Fig. 1 was also fractionated by acrylamide gel electrophoresis and a representative pattern is illustrated in Fig. 3. The superior resolving power of this procedure allows clean separation of the BHK and L-cell high molecular weight RNA fractions into their large and small components. The apparent molecular weights of the BHK components, as determined by the method of Loening (18) using homologous cytoplasmic rRNA as standards, were previously found to be 0.73 $\times$ 10^6 daltons and 0.41 $\times$ 10^6 daltons

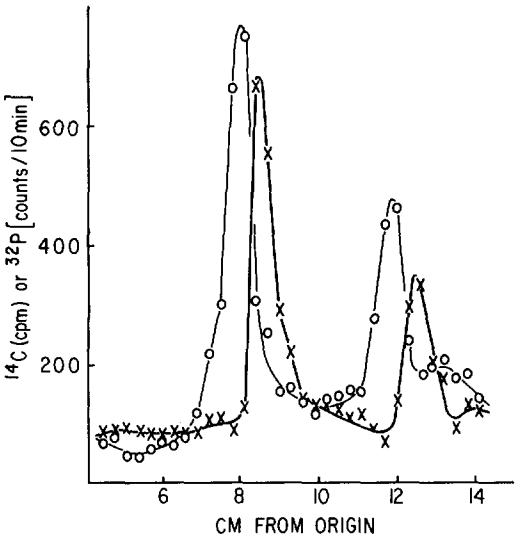

FIGURE 3 Acrylamide gel electrophoresis of mitochondrial RNA from BHK and L-cells. A sample of the mixedly labeled mitochondrial RNA preparation described for Fig. 1 was electrophoresed in 2.4% polyacrylamide gels for 5 hr at 10 ma/gel in buffer containing 0.04 M Tris, 0.02 M sodium acetate, 0.002 M EDTA, 0.5% SDS, brought to a pH of 7.4 with acetic acid. The gel was fractionated by means of a Savant gel divider (Savant Instruments, Inc., Hicksville, N. Y.). Each fraction, equivalent to 2.5 mm of gel, was collected on aluminum planchets and counted as for Fig. 1.

(2). Similar results were obtained in the present series of experiments (0.75 $\times$ 10^6 and 0.42 $\times$ 10^6) using L-cell rRNA as standard (assumed molecular weights 1.76 $\times$ 10^6 daltons and 0.70 $\times$ 10^6 daltons, see next section). As shown in Fig. 3, each of the L-cell high molecular weight mitochondrial RNA components migrated slightly slower than the corresponding BHK component. These differences were 5–6% for both components in each of two runs, leading to the estimated molecular weights of 0.79 $\times$ 10^6 daltons and 0.45 $\times$ 10^6 daltons for the L-cell components.

The acrylamide gel results were used to estimate the molar ratios of the two components, both because of the better separation of the components in gels and because of the evidence that electrophoretic mobility of large RNA molecules is a more reliable indicator of molecular weight than is sedimentation constant (7, 19). When the ratios of counts in the larger, to the smaller, components were calculated from patterns such as that of Fig. 3 (correcting for estimated contributions of heterodisperse RNA), the values ranged between 1.7 and 2.3 for both BHK and L-cells (three determinations each), averaging 2.1 for BHK and 2.0 for L-cells. These results lead to molar ratios of 1.2:1 and 1.1:1, for the hamster and the mouse mitochondrial RNA components, respectively, close to the 1:1 expected for ribosomal RNA components.

Centrifugal and Electrophoretic Properties of BHK and L-Cell Cytoplasmic RNA

Cytoplasmic RNA was purified from cultures labeled for 22 hr of exponential growth (i.e., in the absence of actinomycin D) and was examined by density gradient centrifugation and gel electrophoresis. Small but distinct differences, estimated to be 3–4%, were found in the sedimentation velocity and the electrophoretic mobility of the two 28s RNA's. As with the L-cell high molecular weight mitochondrial RNA components, the 28s rRNA from the mouse both sedimented and electrophoresed slower than the corresponding hamster material. Such a combination of differences must involve conformational effects, as suggested by Eliceiri and Green (20), who obtained similar results with human and murine 28s rRNA. A more precise explanation must await further physicochemical studies. Nevertheless, taking the electrophoretic results as the better measure of molecular weight, a value of 1.76 $\times$ 10^6 daltons

was calculated for L-cell 28s RNA (compared to 1.72×10^6 daltons for the BHK 28s). It is this value for L-cell 28s RNA which we used in determining the molecular weights of the mitochondrial RNA components.

A similar comparison of the 18s RNA's from the two rodents showed no detectable differences. Even after gel electrophoresis, or prolonged sedimentation as described for Fig. 1, the ^{32}P:^{14}C ratios across the 18s peaks varied by less than 6% of the average values, compared to a 40–120% variation across the 28s peaks.

DISCUSSION

The present results indicate that the high molecular weight mitochondrial RNA previously described in BHK cells (1, 2) is not peculiar to this cell line. In addition, they are in accord with our earlier suggestion that this RNA might be ribosomal RNA (2). Extensive studies have shown that ribosomal RNA's vary widely in size (7, 17, 21) and in nucleotide composition (22) depending upon the source of the ribosome. However, characteristically, all rRNA consists of two high molecular weight RNA components that are present in equimolar amounts (cf. 23) and that resemble each other in nucleotide composition (22). Our high molecular weight mitochondrial RNA satisfies these criteria. Furthermore, current studies with BHK cells indicate that our RNA is metabolically stable (Dubin and Czaplicki, unpublished observations) and occurs largely in particles sedimenting at 45–50s (24). It therefore seems reasonable to consider this RNA, at least for the sake of discussion, as ribosomal.

A number of recent reports have described discrete, high molecular weight RNA species, also putatively ribosomal, in mitochondrial fractions from other sources. In the best documented studies, the "ribosomal" RNA from *Neurospora* mitochondria has been shown to sediment like bacterial rRNA and more slowly than homologous cytoplasmic rRNA (5, 6). A similar situation appears to hold for yeast (4, 25–27). In addition these RNA components from either *Neurospora* (6) or yeast (4) contained substantially less GC than homologous cytoplasmic RNA, and the two mitochondrial components resembled each other in base composition. Suyama, working with *Tetrahymena* mitochondria, has observed discrete, high molecular weight species that, like ours, sediment somewhat more slowly than bacterial rRNA (28 and personal communication). These results, taken with ours, support the idea that mitochondria may have distinctive rRNA species; and that this putative mitochondrial rRNA may constitute, in the case of animal cells, a new size class of ribosomal RNA, smaller than the smallest class (bacterial or prokaryotic with molecular weights of approximately 1.1 and 0.55×10^6 daltons) in schemes recently proposed by a number of authors (7, 17, 21). Mitochondrial ribosomal RNA would then appear to be an exception to the generalization, best documented by Loening (7), that the higher the organism the larger the ribosomal RNA. Perhaps mitochondrial and cytoplasmic ribosomes have evolved in complementary, rather than parallel, fashion; i.e., perhaps with increasing complexity of cytoplasmic ribosomes, simpler mitochondrial ribosomes, with smaller structural RNA's, suffice.

The slight differences we find in electrophoretic and centrifugal behavior between the hamster and the mouse larger rRNA's (both cytoplasmic and putative mitochondrial) are in agreement with Loening's (7) survey, which indicates that evolutionary variation of the larger rRNA is a general characteristic of the animal kingdom. On the other hand, the differences between the hamster and mouse smaller mitochondrial RNA components contrast with our findings for 18s cytoplasmic RNA, as well as Loening's findings on 18s RNA from a number of other animal sources. The remarkable evolutionary conservation in the size (or at least the electrophoretic mobility) of the smaller animal cytoplasmic rRNA may not embrace mitochondrial rRNA.

We must emphasize that the designation of our high molecular weight mitochondrial RNA as ribosomal must remain tentative until these species can be shown to be an integral part of a ribonucleoprotein particle required for protein synthesis. This has not been demonstrated in our studies, nor in most of the other studies cited above. In fact, the results of two groups working with HeLa cells have led to interpretations different from ours. Vesco and co-workers (13, 29) have described two discrete mitochondrial RNA species, labeled in the presence of a low level of actinomycin, that electrophorese like our L-cell mitochondrial RNA species and that sediment like our unresolved "17s" peak. However, these workers proposed that their RNA was not ribosomal, since (*a*) it was not associated with struc-

tures sedimenting like cytoplasmic polysomes or ribosomes, and (*b*) the two species were approximately equally labeled (13). However, their RNA *was* associated with particles, sedimenting between 45s and 80s, that may represent the mitochondrial equivalent of polysomes (or derivatives thereof); furthermore, their failure to find equimolar amounts of the two RNA species may be the result of technical factors such as their use of cell suspensions concentrated to densities beyond those permitting normal growth, or their use of relatively short labeling periods. We have in fact found that the smaller BHK component is labeled slightly more rapidly than the larger component in a 2½ hr pulse (Dubin and Czaplicki, unpublished observations).

Vesco and co-workers have also reported that their RNA turns over, with a half-life of approximately 3 hr, as measured by "chasing" in the presence of ethidium bromide (29). This is in apparent conflict with their initial studies (13), as well as with our studies cited earlier (in which a high level of actinomycin was used to "chase"), and may reflect general mitochondrial damage produced by ethidium bromide.

Attardi and Attardi (30), using HeLa cells labeled in the *absence* of actinomycin, have reported discrete mitochondrion-associated RNA peaks sedimenting at 21s and 12s. Their 21s peak did not hybridize with mitochondrial DNA, and does not correspond to any of the mitochondrial RNA species observed in actinomycin-treated cells by us or by Vesco and co-workers[2]; it is possible that this peak represents altered cytoplasmic 28s RNA (cf. 2, 10). (Cytoplasmic 28s RNA is a prominent constituent of the mitochondrial fraction of cultured animal cells (2, 13, 14), but is not labeled in the presence of actinomycin.) In any event, the failure of Attardi and Attardi's 21s RNA to hybridize with mitochondrial DNA cannot be taken as evidence for the *absence* of distinctive rRNA of mitochondrial origin, especially in view of the fact that their "12s" peak (which was rather broad) *did* hybridize with mitochondrial DNA.

It is clear that more work will be required before the discrete high molecular weight mitochondrial RNA species can be assigned definitive functions or origins.

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[2] It should be noted that Vesco and Penman's "21s" designation (13) is purely an extrapolation of *electrophoretic* data; this species sediments with or slightly behind 18s RNA.

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