Messenger Ribonucleic Acid Metabolism in Mammalian Mitochondria. Quantitative Aspects of Structural Information Coded by the Mitochondrial Genome†

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ABSTRACT: A procedure has been described to isolate polyribosomes from digitonin-washed mitochondria. Polyribosomes from *in vivo* labeled mitochondria exhibit a heterogeneity in size distribution. They are sensitive to EDTA, puromycin, and RNase, and insensitive to emetine. When isolated mitochondria are labeled with [^{3}H]ATP and [^{3}H]CTP, about 3–5% of the total acid insoluble counts are seen associated with polyribosome structures providing direct evidence for intramitochondrial mRNA transcription. RNA released from *in vitro* labeled mitochondrial polyribosomes resolve as three distinct peaks in the region of 11 S to 12 S. RNA from *in vivo* labeled mitochondrial polyribosomes, on the other hand, resolves into 6–7 peaks of 11 S to 16 S. Somewhat over half of the *in vivo* labeled polyribosomal RNA and none of *in vitro* polyribosomal RNA contain 80–100 nucleotide long poly(A) stretches. The kinetics of appearance of labeled RNA in *in vitro* polyribosomes indicate no appreciable delay between the synthesis of RNA and its association with polyribosomal structures. DNA–RNA hybridization experiments show that *in vitro* polysomal RNA pulse labeled with [^{3}H]CTP and [^{3}H]ATP hybridizes with mtDNA to the extent of 3.5%. Further, RNA released from unlabeled polyribosomes and labeled uniformly with ^{125}I hybridize with mtDNA to a maximum extent of 4.35% while ^{125}I labeled rRNA and tRNA together hybridize to the extent of 10.3%. These results indicate that the mitochondrial genome codes for 3–4 polypeptides.

The biogenetic contribution of the mitochondrion to organelle-specific metabolism and its role in cellular processes have been the subject of investigation in several laboratories (see Ashwell and Work, 1970; Borst, 1972; Mahler and Dawidowicz, 1973). The occurrence of intramitochondrial mRNAs and their translation by a unique organelle-specific protein synthesizing system have been well documented in various cell types (Kuntzel, 1969; Borst and Grivell, 1971; Beattie, 1971; Dawid, 1972a; Avadhani and Buetow, 1972a,b; Michel and Neupert, 1973) although there is no unanimous agreement on the genetic origin of intramitochondrial mRNAs (see Borst, 1972; Dawid, 1972a; Avadhani *et al.*, 1973a; Mahler and Dawidowicz, 1973; Gaitskhoki *et al.*, 1973a,b).

Observations by several groups of workers that ethidium bromide inhibits incorporation of labeled amino acids by isolated mitochondria (Kroon and DeVries, 1971; Schatz *et al.*, 1972) gave rise to the concept that intramitochondrial mRNAs are probably transcribed on the mt genome[1] (Ashwell and Work, 1970; Borst, 1972). Recent experiments of Grant and Poulter (1973) showed that rifamycin inhibited the synthesis of a rapidly turned over RNA species in an *in vitro* mitochondrial system with a concomitant decrease in [^{14}C]leucine incorporation, implying that the mt genome codes for mRNA. Using temperature sensitive *Saccharomyces cerevisiae,* and also using ethidium bromide as a probe to characterize intramitochondrial transcription, Mahler and Dawidowicz (1973) concluded that almost all the structural information present in intramitochondrial polyribosomes is coded by the mt genome. These studies provide indirect evidence for intramitochondrial mRNA transcription. Nevertheless, quantitative aspects of information contributed by the mt genome have not yet been reported. Additionally, most of the studies showing possible mRNA transcription on the mt genome use one or more inhibitors. This use of specific inhibitors has provided valuable guide lines on mitochondrial specific events; it should be recognized that many of these drugs, such as ethidium bromide, have as yet unknown manifold effects on the mitochondrial system (Grivell and Metz, 1973; Avadhani and Rutman, 1974b).

In this paper we show that isolated mitochondria from Ehrlich ascites cells synthesize 11S–12S RNA which can be recovered in the polyribosome fraction providing direct and unequivocal evidence for intramitochondrial mRNA transcription. Molecular hybridization experiments using polyribosomal RNA labeled with ^{125}I or labeled to equilibrium indicate that the mt genome contributes structural information for at least 3–4 polypeptides.

Materials and Methods

Chemicals. All the radioisotopes were purchased from New England Nuclear. Unlabeled nucleoside triphosphates, phosphoenolpyruvate, and pyruvate kinase were products of Sigma. Digitonin (80% pure) purchased from Sigma was washed with 25% ethanol and then crystallized from absolute ethanol before use. Thallium trichloride was a product of K & K Laboratories. Pancreatic and T_1 RNase were products of Worthington Biochemical Corp. Oligo(dT)-cellulose was purchased from Collaborative Research Inc.

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[1] Abbreviations used are: mRNA, messenger RNA; rRNA, ribosomal RNA; mtRNA, mitochondrial RNA; mtDNA, mitochondrial DNA; mt genome, mitochondrial genome; poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); nRNA, nuclear RNA, nDNA; nuclear DNA; hnRNA, nuclear heterogeneous RNA; BRS, 30 mM Tris-HCl (pH 7.5), 120 mM KCl, 18 mM $Mg(CH_3COO)_2$, and 5 mM 2-mercaptoethanol.

General. All glassware was acid cleaned and sterilized. Wherever possible sterilized reagents and sterile techniques were used.

Labeling of Mouse Ascites Cells. Ehrlich ascites hypotetraploid cells were used in all experiments reported in this paper. The growth of the tumors, collection of the cells, and other details were described before (Avadhani *et al.*, 1973a; Chun *et al.*, 1969). Labeling conditions were essentially as described before (Avadhani *et al.*, 1973a). Cells were diluted with Locke Ringer medium to a density of 10^8 cells/ml and incubated in a shaker water bath at 37°. After the cells had been acclimatized for 15 min, actinomycin D (Calbiochem) was added to a final concentration of 0.04 μg/ml and incubation was continued for 15 more min. ^{3}H-labeled cytidine (20–30 Ci/mmol) and adenosine (30–50 Ci/mmol) were added to final concentrations of 4 μCi/ml each and incubation was continued for 45 min before using the cells for the isolation of mitochondria.

Preparation of Mitochondria. Details of cell fractionation and separation of mitochondria by differential centrifugation were as described elsewhere (Avadhani *et al.*, 1973a). Crude mitochondria pelleted at 7500*g* for 15 min were purified by a two-step digitonin-washing technique as described elsewhere (Avadhani and Rutman, 1974a).

In Vitro Labeling of Mitochondria. Digitonin-washed mitochondria were used for *in vitro* labeling. The incubation mixture included 0.25 M sucrose, 25 mM Tris-HCl (pH 8.0), 7.5 mM $MgCl_2$, 20 mM KCl, 5 mM 2-mercaptoethanol, 2 mM phosphoenolpyruvate, 1 μg/ml of pyruvate kinase, and 12–15 mg of mitochondrial protein/ml. In addition, the incubation mixture contained [^{3}H]CTP (20 Ci/mmol) and [^{3}H]ATP (20–40 Ci/mmol) as described in the footnotes to the appropriate experiments. Unless otherwise mentioned, labeling was carried out for 30 min in a shaker water bath at 37°. Labeled mitochondria were washed once with a buffer containing 0.25 M sucrose, 25 mM Tris-HCl (pH 8.0), 20 mM KCl, 7.5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol before isolation of polyribosomes.

Isolation of Polyribosomes. Polyribosomes were prepared from either labeled or unlabeled mitochondria as required. The mitochondrial pellet was suspended in 4 volumes of BRS (30 mM Tris-HCl (pH 7.5), 120 mM KCl, 18 mM $Mg(CH_3COO)_2$, and 5 mM 2-mercaptoethanol) containing 150 μg/ml of emetine and homogenized with 15 strokes of a tight fitting Dounce homogenizer. The homogenate was centrifuged at 20,000*g* for 15 min to pellet the membrane fraction. The supernatant fraction mostly consisting of monomeric ribosomes, soluble proteins, and some RNA was discarded. The 20,000*g* pellet was washed once with 4–5 volumes of BRS and then suspended in 2 volumes of BRS containing 0.5% Triton X-100 and 0.5% Nonidet NP40 and homogenized gently with 3–4 strokes of a loose-fitting Dounce homogenizer. The homogenate was clarified at 15,000*g* for 10 min and then centrifuged over 0.5 volume of 1.5 M sucrose containing BRS (1 volume of sucrose:2 volume of homogenate) at 165,000*g* for 3 hr at 3–4°. The transparent pellet was rinsed with BRS to wash off sucrose, then dissolved in BRS and used immediately for further analysis.

Sucrose Density Gradient Centrifugation. Linear gradients of 10–35% sucrose containing 10 mM Tris-HCl (pH 7.5), 80 mM KCl, 10 mM $Mg(CH_3COO)_2$, and 1 mM 2-mercaptoethanol were prepared. The final volume of the gradient was 4.5 ml; 0.2–0.3-ml polyribosome samples were layered on the gradient and centrifuged at 165,000*g* for 2 hr at 3–5° in a Spinco Model L-2 ultracentrifuge. Gradients were fractionated using an ISCO density gradient fractionator and 4-drop fractions were collected and counted with a 10-ml Cab-O-Sil mixture. *Escherichia coli* 70S ribosomes and Ehrlich ascites cytoplasmic ribosomes were used as markers.

Release of Labeled RNA from Polyribosomes. RNA was released from the polyribosomes by treatment with puromycin. Polyribosomal preparations in BRS were incubated with 200 μg/ml of puromycin at 30° for 15 min. Ribosomes were then pelleted at 160,000*g* for 3 hr. The ribosome-free supernatant containing released RNA was carefully aspirated out and extracted with 2 volumes of phenol–chloroform (2:1) as previously described (Avadhani *et al.*, 1973a). The RNA was precipitated with 70% ethanol in the presence of 3% potassium acetate also as described before (Avadhani *et al.*, 1973a). In cases where the RNA content was too low for quantitative precipitation, the aqueous phase was washed with cold ether, and the ether was removed by a jet of nitrogen gas before analysis.

Polyacrylamide Gel Electrophoresis. The details of gel electrophoresis and radioactivity analysis were essentially as described before (Avadhani *et al.*, 1973a). Agarose–acrylamide gels containing 2.0% acrylamide and 0.5% agarose were prepared as described by Peacock and Dingman (1968). Sedimentation values of various RNA peaks were determined on the basis of relative migration as compared with *E. coli* rRNAs and Ehrlich ascites cytoplasmic rRNAs.

Quantitative Estimation of Poly(A) Containing RNA. Poly(A) containing RNA was estimated by three different methods. One of the methods was the binding of labeled RNA to Millipore filters at high salt concentrations (Lee *et al.*, 1971). The details of this procedure have been described before (Avadhani *et al.*, 1973b). The second method involved the hybridization of labeled RNA with poly(U) and quantitative separation of triple-stranded structures using hydroxylapatite column chromatography as described by Greenberg and Perry (1972). This method was previously used to estimate poly(A) containing RNA from mitochondria (Avadhani *et al.*, 1973b). The third technique used to estimate poly(A) containing RNA was chromatography on an oligo(dT)-cellulose column. The 0.8 cm × 6 cm column was prepared using 1.0 g of oligo(dT)-cellulose essentially as described by Aviv and Leder (1972). The column was equilibrated with a solution of 10 mM Tris-HCl (pH 7.5) and 500 mM NaCl. RNA samples (0.2–0.5 ml) containing 500 mM NaCl were layered and washed with 25 ml of 10 mM Tris-HCl (pH 7.5) containing 500 mM NaCl. The column was then washed with a similar volume of 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl. Finally, poly(A) containing RNA bound to the column was eluted by washing with 25 ml of 5 mM Tris-HCl (pH 7.5). The elution rate was maintained at 0.2 ml/min. Two minute fractions were collected and counted as described for the hydroxylapatite chromatography technique (Avadhani *et al.*, 1973b).

Iodination of RNA. Labeling of RNA with ^{125}I was carried out essentially as described by Tereba and McCarthy (1973) in 0.1-ml final volumes. The reaction mixture contained 0.05 M sodium acetate buffer (pH 5.0), 0.08 mM potassium iodide, 1.2 mM thallium trichloride, 25–35 μg of RNA, and 100 μCi of $Na^{125}I$ (17 Ci/mg). The reaction was carried out for 30 min at 55°, and was terminated by adding sodium sulfite to a final concentration of 5 mM with

TABLE I: Control Experiment to Detect Cross-Contamination.[a]

Details	% of Total Radioactivity Detected in Polysomes	
	^3H	^{14}C
^3H membrane + ^{14}C soluble fraction	99.6	0.4
^{14}C membrane + ^3H soluble fraction	0.5	99.5

[a] Two batches of cells (10^9 each) were labeled *in vivo* as described in Materials and Methods. One batch was incubated with ^3H-labeled cytidine and adenosine and the other batch was labeled with [^{14}C]adenosine and [^{14}C]cytidine. Mitochondria were prepared from both batches and used for preparing soluble and membrane fractions as described in Materials and Methods. About 70,000 cpm each of ^3H membrane and ^{14}C soluble fraction and *vice versa* were mixed and incubated on ice for 15 min. The membranes were then sedimentated at 20,000*g* for 15 min, washed once with BRS, and used for preparing polyribosomes as described in Materials and Methods. ^{14}C and ^3H counts were determined in both the polyribosome preparations.

subsequent addition of 50 mM ammonium hydroxide–ammonium acetate buffer (pH 7.5) as described by Getz *et al.* (1972). The mixture was then heated at 60° for 45 min to release nonspecific and unstable ^{125}I bound to RNA. Free iodide was separated from RNA by chromatography on a Sephadex G-25 column (0.6 cm × 75 cm) as described by Tereba and McCarthy (1973).

DNA–RNA Hybridization. Preparation of mt DNA, fixing the DNA to filters, and estimation of the amount of bound DNA were carried out as described (Avadhani *et al.*, 1973a); 0.4 μg of DNA was fixed to S & S filters. The hybridization reaction was run at 45° for 18 hr. The final volume of the reaction mixture was 0.25 ml and contained 4 × SSC and 50% (v/v) formamide. After hybridization, filters were washed three times with 10 ml of 2 × SSC each. The first washing was carried out at 55° (for 10 min.), while subsequent washings were at 37° (10 min). Washed filters were incubated with 1.0 ml of 2 × SSC containing 40 μg of pancreatic RNase and 6 units of T_1 RNase at 37° for 45 min. The filters were finally washed two more times with 20 ml each of 2 × SSC at 37° and counted as described (Tereba and McCarthy, 1973).

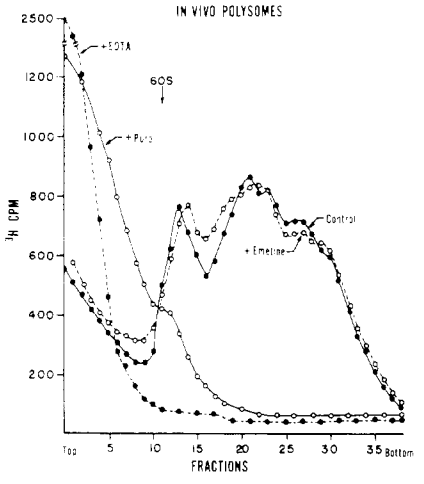

FIGURE 1: Polyribosomes from *in vivo* labeled mitochondria. 5 × 10^{10}–10^{11} cells were labeled with [^3H]cytidine and [^3H]adenosine, mitochondria prepared, and used to prepare polyribosomes as described in Materials and Methods. The resultant polyribosome fraction was divided into equal parts each containing about 15,000 cpm. One part was layered on the gradient without any treatment (control). The second portion was treated with 200 μg/ml of emetine (+emetine), the third part with 100 μg/ml of puromycin (+Puro) and last part with 10 mM EDTA (+EDTA). All of the treatments were carried out at 0–4° for 15 min. Details of density gradient centrifugation and radioactivity analysis were as described in Materials and Methods.

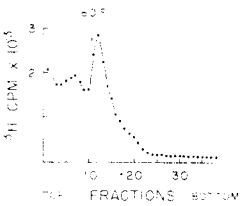

FIGURE 2: Effect of RNase on *in vivo* labeled polyribosomes. The polyribosome preparation from *in vivo* labeled mitochondria (19,000 cpm) was treated with 5 μg/ml of pancreatic RNase, incubated on ice for 10 min, and analyzed on density gradients as described in Materials and Methods and in Figure 1.

Results

Isolation of Mitochondria-Specific Polyribosomes. Mitochondria-specific polyribosome structures have been isolated and analyzed from a variety of cell types (Avadhani and Buetow, 1972a,b; Mahler and Dawidowicz, 1973; Ojala and Attardi, 1972; Perlman and Penman, 1970; Michel and Neupert, 1973). Nevertheless, polyribosomes from mammalian mitochondria have been reported to behave in an unusual manner (for details see Dawid, 1972a). It was, therefore, imperative to standardize a technique to isolate intact polyribosomes sensitive to chelating agents such as EDTA and also to RNase action. Although a wide spectrum of conditions for lysis and recovery of polyribosomes were tried, solubilization of the membrane fraction from digitonin-washed mitochondria gave the best results. As described in the Materials and Methods section, this procedure was used throughout for the isolation of polyribosomes. In the initial experiments, detailed analysis of both *in vivo* and *in vitro* labeled mitochondria showed that 3–5% of total acid precipitable radioactivity is associated with ribonucleoprotein particles resembling polyribosomes.

In our experience, only about 6–7 A_{260} units (about 0.5 mg) of mitochondria-specific polyribosomes can be recovered from about 5 × 10^{11} Ehrlich ascites cells withdrawn from 100 to 125 tumor-bearing animals. The use of high specific activity labeling, both *in vivo* and *in vitro*, circumvents this problem. About 10^{10} cells (20–25 animals) yield enough mitochondria-specific polyribosomes for analysis and characterization using radioactivity measurements. Unless specifically mentioned, therefore, in all the experiments reported here polyribosomes were prepared from labeled mitochondria.

In order to verify the extent of nonspecific binding of labeled RNA to ribosome-like structures during various steps of isolation, control experiments were carried out. Soluble protein fractions and membrane fractions were prepared from *in vivo* labeled mitochondria, in one case with ^3H precursors and in another case with ^{14}C precursors (see Table I). The ^3H labeled soluble protein fraction was incubated with the ^{14}C labeled membrane fraction and *vice versa* at 0–4° for 15 min. Polyribosomes were then isolated from both membrane fractions and analyzed for ^3H and ^{14}C

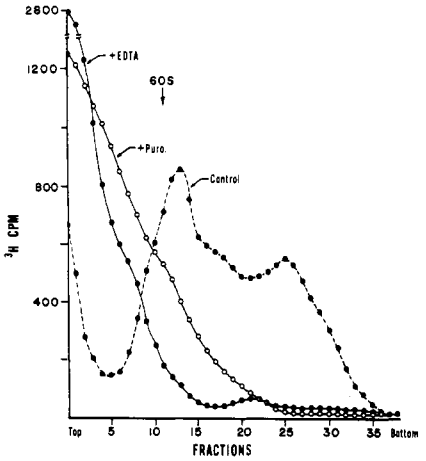

FIGURE 3: *In vitro* labeling of polyribosomes. Mitochrondria were prepared from 5 × 10^{10} to 10^{11} cells and labeled with 25 μCi/ml each of [^{3}H]CTP and [^{3}H]ATP as described in Materials and Methods. The polyribosome preparation from the resultant mitochondria was divided into 3 equal parts of 16,500 cpm each. One part was analyzed on a sucrose gradient without any treatment (control) and the other two parts were either treated with puromycin (+Puro) or EDTA (+EDTA) before analysis. Other details were as in Figure 1.

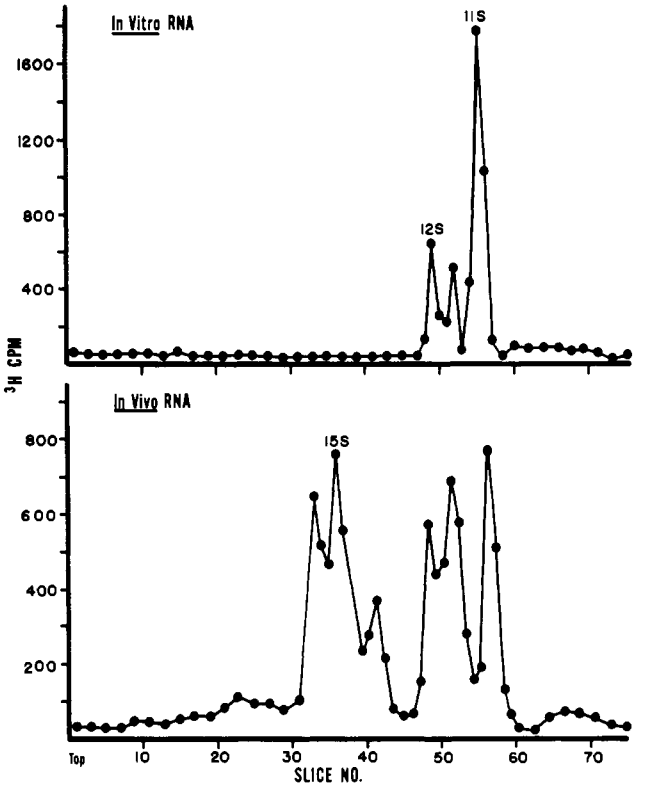

FIGURE 4: Electrophoretic patterns of RNA released from *in vivo* and *in vitro* polyribosomes. About 20,000–25,000 cpm each of RNAs released by puromycin treatment from *in vitro* and *in vivo* polyribosomes were electrophoresed on 0.6 cm × 7.5 cm agarose acrylamide composite gels containing 2.0% acrylamide and 0.5% agarose. Other details were as described in Materials and Methods.

counts. As shown in Table I, polyribosomes from the ^{3}H labeled membrane fraction contain less than 0.1% ^{14}C contamination. The same is true for polyribosomes isolated from ^{14}C labeled membranes. These results show that the amount of RNA, if any, nonspecifically bound to polyribosomes during isolation is negligible.

A typical polyribosome pattern from *in vivo* labeled mitochondria has been presented in Figure 1. RNA counts on the gradient are distributed between the 60S and 300S region, indicating a heterogeneity in the size distribution of polyribosome particles. Puromycin, an antibiotic which is known to bind to the peptidyl site of the ribosome causing dissociation of polyribosome structures (Blobel, 1971), releases the radioactivity from high molecular weight particles (Figure 1). The similar release of labeled RNA from polyribosome-like structures isolated from HeLa mitochondria was observed in other studies (Perlman *et al.*, 1973; Ojala and Attardi, 1974). As seen in Figure 1, EDTA also causes the release of ^{3}H label from highly aggregated submitochondrial particles. In contrast, emetine, which is known to specifically inhibit cytoplasmic protein synthesis, by way of preventing tRNA binding (Pestka, 1971), has hardly any effect on the mitochondria-specific polyribosomes, indicating their purity.

Polyribosomes isolated from mitochondria of diverse origin have been reported to be resistant to RNase action (Ojala and Attardi, 1972; Michel and Neupert, 1973) probably because of contaminating proteins. Mitochondria-specific polyribosomes prepared from the membrane fraction in the present studies are quite sensitive to RNase as shown in Figure 2. Addition of RNase to *in vivo* labeled mitochondria-specific polyribosomes results in the degradation seen in the sedimentation pattern (Figure 2). The radioactivity peak at 60 S–65 S is the monomeric region. Thus, it is evident that the method described yields highly aggregated particles from Ehrlich ascites mitochondria having all the properties expected for polyribosomes.

In the experiment described in Figure 3, mitochondria labeled *in vitro* were used to prepare polyribosomes and the resultant preparation was analyzed on a sucrose gradient. *In vitro* labeled polyribosomes appear to be less aggregated as compared to *in vivo* polyribosomes; nonetheless radioactivity is distributed between 60 S and 300 S. Further, these particles are sensitive to puromycin and EDTA. Both *in vivo* and *in vitro* polyribosomes are also sensitive to RNase and resistant to emetine (results not shown).

Characterization of RNA. Electrophoretic patterns of RNA released from *in vivo* and *in vitro* polyribosomes by treatment with puromycin have been presented in Figure 4. The polyribosomal RNA labeled *in vivo* resolves into 6–7 distinct peaks ranging from 10 S to 16 S. *In vitro* polyribosomal RNA, on the other hand, resolves into three peaks in the region of 11 S to 12 S. This apparent difference in the number of peaks and their size distribution between *in vivo* and *in vitro* polyribosomal RNA can be interpreted as follows: (1) the *in vitro* system may not be efficient enough to synthesize all the RNA species seen in the *in vivo* system, or (2) additional RNA species seen in the *in vivo* labeled polyribosomes may be of extramitochondrial origin.

Recent studies in our laboratory (Avadhani *et al.*, 1973a,b) as well as others (Perlman *et al.*, 1973; Gaitskhoki *et al.*, 1973a) have shown the presence of poly(A) sequences covalently linked to intramitochondrial RNA of various animal cells. Ribonucleoprotein particles, presumed to be polyribosomes from HeLa mitochondria, have also been reported to contain poly(A) (Perlman *et al.*, 1973; Ojala and Attardi, 1974). In the present experiments, when the RNAs released from *in vivo* and *in vitro* polyribosomes were analyzed by three different techniques (see Table II), 60–65% of the former and none of the latter RNA contained poly(A). The ribonuclease digest of the *in vivo* labeled RNA migrates as a single peak slightly behind tRNA

TABLE II: Poly(A) Content of *in Vitro* and *in Vivo* Polysomal RNA from Mitochondria.[a]

Polysomal RNA	Acid Precipitable cpm Used	cpm Detected as Poly(A) Containing RNA		
		A	B	C
In vitro	42,000	<39	<20	<30
	168,000	<41	<24	<27
In vivo	12,760	6381	8926	7895
	6,380	3193	4350	3820

[a] *In vivo* and *in vitro* polysomes were prepared as described in Figure 1 and Figure 3, respectively, and incubated with puromycin as described in Materials and Methods. Puromycin-released RNA counts were analyzed by (A) Millipore filter technique; (B) poly(U) hybridization and hydroxylapatite chromatography method; and (C) oligo-(dT)-cellulose chromatography technique as described in Materials and Methods to determine poly(A) containing RNA.

TABLE III: Kinetics of Appearance of Radioactive RNA on *in Vitro* Polyribosomes.[a]

Time of Incubation (min)	Acid Precipitable Radioactivity (cpm/mg of protein)	
	Total	Polyribosomes
5	3850	126 (3%)
10	6472	256 (4.7%)
15	7492	375 (5.0%)
20	8470	431 (5.0%)
45	8395	166 (2.0%)

[a] Purified [^{3}H]mitochondria were labeled with 30 μCi/ml each of [^{3}H]CTP and [^{3}H]ATP as described in Materials and Methods. After intervals of time, samples were withdrawn and a small portion of each sample was used to estimate total RNA counts using cold Cl_3CCOOH (5%) precipitation method. Remaining portions were used to prepare polyribosomes. Values in parentheses indicate percentage of total counts found in polyribosomes.

(Figure 5), indicating that the poly(A) sequences are 80–100 nucleotides long. Similar RNase-treated RNA from *in vitro* labeled polyribosomes electrophoreses out of the gel leaving negligible radioactivity indicating no detectable poly(A) in these samples. The poly(A) sequence from cytoplasmic RNA, on the other hand, migrates as a 7S peak (Figure 5).

The two-step digitonin-washing technique has been previously shown to yield mitochondrial S-25 and mt ribosomes free of detectable cytoplasmic ribosome particles and rRNA (Avadhani and Rutman, 1974a). In the present experiments, digitonin-washed mitochondria from *in vivo* labeled cells yield polyribosomal RNA containing negligible 7S poly(A) characteristic of cytoplasmic mRNA. These observations, together with the DNA–RNA hybridization results (Avadhani *et al.*, 1973a), demonstrate the purity of mitochondrial preparations used in our studies.

In order to secure further evidence as regards intramitochondrial polyadenylation and subsequent processing of pre-mRNA, the kinetics of appearance of labeled RNA in polysomal complexes was studied. If the polyadenylation and processing of a pre-mRNA is a prerequisite for translation, it would be reasonable to expect a lag before newly snythesized mRNA is found in the polyribosome complex. The kinetic experiment reported in Table III indicated that within 5 min of labeling isolated mitochondria, 3% of the total radioactive RNA is found associated with the polyribosomes. This value reaches a plateau with a maximum of 5% in about 15 min. These results show that unlike the nuclear system, there is no apparent processing of mRNA in mitochondria in the *in vitro* system used in these experiments.

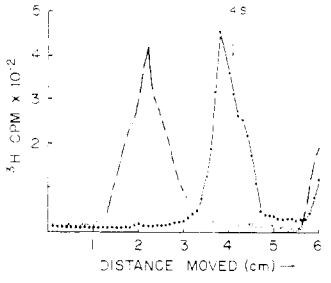

FIGURE 5: Analysis of poly(A) sequences. About 10^{10} cells were labeled with [^{3}H]adenosine as described in Materials and Methods. Purified mitochondria from these cells were used to prepare polyribosomes. RNA from puromycin-treated polyribosomes was digested with 3 μg/ml each of pancreatic RNase and T_1 RNase at 37° for 60 min. The post-mitochondrial supernatant fraction was clarified at 30,000*g* for 15 min and extracted with phenol–chloroform as described in Materials and Methods to obtain cytoplasmic RNA. Cytoplasmic RNA was also digested with RNases as above. RNase resistant sequences were precipitated with ethanol in the presence of 150 μg of carrier tRNA from *E. coli*. Precipitates were washed three times with ethanol, dissolved in 0.2 ml of 5 mM Tris-HCl, and electrophoresed on 7.5% polyacrylamide gels. (○) RNase resistant fraction from the cytoplasmic compartment; (●) the similar fraction from mitochondrial polyribosomes.

Extent of Complementarity with mt Genome. In order to estimate the quantitative representation of mt genomic information in *in vitro* labeled polyribosomes, molecular hybridization experiments between polyribosomal RNA and mtDNA were carried out. In the initial experiments, when ^{3}H labeled *in vitro* polyribosomal RNA was used, a maximum of 3.5% hybridization was observed (Figure 6). However, the quantitative significance of this result is limited because isolated mitochondria may not have the ability to synthesize all the mRNA species transcribed under *in vivo* conditions and because quantitation of hybridization data using pulse-labeled RNA is very difficult and often misleading (Dawid, 1972b). It was, therefore, decided to carry out the estimations using the steady-state population of mt polysomal mRNA by labeling with ^{125}I. Labeling with ^{125}I presents a particular advantage since it yields RNA with a very high specific activity (up to 3×10^7 cpm/μg) which can be used for hybridization (Getz *et al.*, 1972; Tereba and McCarthy, 1973). In the experiments shown in Figure 7, mitochondria-specific polyribosomes were prepared from about 10^{14} unlabeled cells and treated with puromycin to release mRNA as described in Materials and Methods. Released mRNA, RNA from the ribosomal pellet, and also purified tRNA were labeled with ^{125}I and used for hybridization with mtDNA. As shown in Figure 7, puromycin-released rRNA and tRNA together hybridize with mtDNA to a maximum of 10.3%, while mRNA hybridizes to the extent of 4.2%. Although not shown here, rRNA alone hybridizes with mtDNA to about 6.8%. A similar hybridization

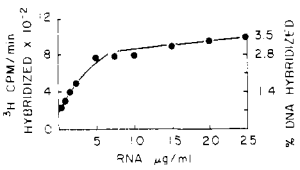

FIGURE 6: Extent of hybridization of *in vitro* polyribosomal RNA with mtDNA. *In vitro* polyribosomal RNA was prepared as in Figure 3 except that 40 μCi/ml each of [^{3}H]CTP and [^{3}H]ATP were used for labeling. Specific activity of the resultant RNA was 40,000–55,000 cpm/μg; 0.4 μg of mtDNA fixed on filters was used for hybridization. Appropriate blank filters were present in each reaction mixture and values from these filters were subtracted from experimental values. Other details were as given in Materials and Methods.

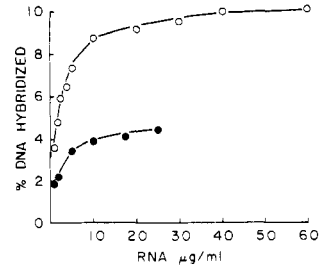

FIGURE 7: Hybridization of ^{125}I-labeled steady-state polyribosomal RNA with mtDNA. Polyribosomal RNA was prepared from 10^{13}–10^{14} unlabeled fresh cells as described in Materials and Methods. The RNA from ribosomes released after puromycin treatment was purified on Sephadex G-100 to eliminate small RNAs, etc. tRNA was prepared from mitochondria according to Dawid (1972b). All RNA fractions were labeled with ^{125}I and subsequently purified on Sephadex G-25 column to eliminate free ^{125}I as described in Materials and Methods. Specific activities of various RNA fractions were 1.07–1.27 × 10^7 cmp/μg. (○) The rate of hybridization of rRNA and tRNA added together in the system (ratio of rRNA/tRNA = 2:1); (●) the extent of hybridization with ^{125}I-labeled polyribosomal RNA. Other details were as in Figure 6.

rate was reported for mtRNA from *Xenopus* mitochondria methylated *in vitro* with [^{3}H]dimethyl sulfate (Dawid, 1972b). However, a much higher value was reported using ^{3}H pulse labeled RNA from HeLa mitochondria (Aloni and Attardi, 1971).

In order to evaluate the extent of contaminating rRNA in the puromycin-released mRNA fraction, hybridization was carried out using saturating concentrations of unlabeled rRNA and tRNA. As shown in Table IV, addition of 60 μg/ml of unlabeled rRNA and tRNA (2:1) did not affect the hybridization values of ^{125}I-labeled mRNA with mtDNA. Also, the extent of hybridization of ^{3}H labeled *in vitro* as well as *in vivo* polysomal RNA was not affected, suggesting no competition between these RNAs and unlabeled rRNA. These results demonstrate that puromycin released mt mRNA has completely different sequence properties as compared to rRNA and tRNA. Electrophoretic patterns showing the absence of "21S" and "13S" rRNA peaks would not only be very convincing but also much easier to prove the purity of mRNA. However, in our hands the iodination reaction invariably produced degradation, making it impossible to characterize the migration patterns of RNA species.

Discussion

Although there have been several attempts to isolate and characterize mitochondria-specific polyribosomes from various cell types (Perlman and Penman, 1970; Avadhani *et al.*, 1972a,b; Ojala and Attardi, 1972; Allen and Suyama, 1972; Michel and Neupert, 1973), very few of them have been completely satisfactory. In some cases, mitochondria-specific ribonucleoprotein particles have been reported to possess unusual properties (for details, see Dawid, 1972a). Mammalian systems, in particular, present problems because of the contaminating endoplasmic reticulum. The only mammalian system investigated so far is HeLa mitochondria. Ojala and Attardi (1972) isolated polyribosome-like structures having heterogeneous size distribution. However, their preparation was resistant to both RNase and EDTA. Perlman and Penman (1970), on the other hand, obtained only 74 S–95 S particles. Polyribosomes prepared from the membrane fraction in the present studies not only contain highly aggregated particles, but also exhibit sensitivity to puromycin, EDTA, and RNase, as expected (see Figure 1). Two important points were taken into consideration in developing the procedure to isolate polyribosomes: (1) rapid isolation and purification of mitochondria to avoid degradation; (2) prevention of the nonspecific binding of hydrophobic proteins to polyribosomes during lysis and isolation. If intact mitochondria were lysed directly with detergents, the resultant ribonucleoprotein preparation invariably aggregated and pelleted at the bottom of the gradient during analysis. Also, such preparations showed insensitivity toward RNase and EDTA. These observations indicate that separation of membranes before treatment with detergents probably eliminates the problem of nonspecific binding of proteins to polyribosome structures. Our results also indicate that almost all polyribosomes in mitochondria exist in membrane-bound form.

In vivo labeled polyribosomes contain much more highly aggregated particles as compared with *in vitro* labeled polyribosomes (Figures 1 and 2). This observation is consistent with the electrophoretic patterns of RNAs released from polyribosomes since *in vitro* polyribosomal RNA resolves into fewer and smaller species as compared with *in vivo* polyribosomal RNA. Also, in keeping with our previous finding with total RNA isolated from *in vitro* labeled mitochondria (Avadhani *et al.*, 1973a), no detectable poly(A) sequences could be found associated with RNA released from *in vitro* labeled mt polyribosomes (Table II). As pointed out before (Avadhani *et al.*, 1973a), the observed

TABLE IV: Extent of Hybridization of mRNA in Presence of Large Excess of rRNA and tRNA.[a]

Type of RNA	Unlabeled RNA Added	% Hybridization
^{125}I-labeled rRNA		6.76
^{125}I-labeled tRNA		4.17
^{3}H-labeled *in vivo* RNA		3.13
^{3}H-labeled *in vivo* RNA	rRNA + tRNA	3.08
^{3}H-labeled *in vitro* RNA	rRNA + tRNA	3.46
^{125}I-labeled polyribosome RNA		4.25
^{125}I-labeled polyribosome RNA	rRNA	4.31
^{125}I-labeled polyribosome RNA	tRNA	4.36
^{125}I-labeled polyribosome RNA	rRNA + tRNA	4.32

[a] Details of hybridization experiments were as described in Figures 6 and 7. rRNA and tRNA when added together were 40 and 20 μg (2:1), respectively. When added separately, they were at the level of 40 μg/ml.

reduction in size and number of RNA species and also our unsuccessful attempts to find poly(A) sequences in these RNAs (Figure 4 and Table II) could be due to inadequacies of the *in vitro* system, especially in view of recent reports claiming mitochondrial origin for RNAs containing short poly(A) sequences (Attardi and Ojala, 1974; Hirsch and Penman, 1973, 1974). We are presently reluctant to give any interpretation to the absence of poly(A) from *in vitro* synthesized mt mRNA in view of recent suggestions that both polyadenylation and deadenylation can occur in the cytoplasm (Slater and Slater, 1974; Brawerman, 1974). It is, however, clear that polyadenylation is not an obligatory requirement for the recognition of mRNA and the assembly of polyribosomes in this system.

Although several studies have presented indirect evidence suggesting that the mitochondrial genome codes for mRNA (Kroon and DeVries, 1971; Schatz *et al.*, 1972; Grant and Poulter, 1973; Mahler and Dawidowicz, 1973; Perlman *et al.*, 1973; Borst, 1972), to date there is no unequivocal proof in support of this hypothesis. However, experiments reported in this paper, demonstrating the ability of isolated mitochondria free of cytoplasmic components to synthesize labeled RNA which subsequently associates with polyribosomes (Figures 3 and 4), provide an unambiguous proof for intramitochondrial mRNA transcription.

The biogenesis of mRNA in the nuclear system appears to occur in ordered steps (Darnell *et al*, 1973; LaTorre and Perry, 1973) including hnRNA synthesis, polyadenylation, and "processing" to yield mature mRNA. On the basis of kinetic analysis, it has been estimated that complete synthesis of mRNA and its transportation to cytoplasm require approximately 15 min (Adesnik *et al.*, 1972). In bacterial systems, on the other hand, where no apparent processing of mRNA has yet been reported, radioactivity appears in polyribosomes almost immediately after pulsing. If the mRNA coded by the mt genome is polyadenylated intramitochondrially as proposed (Ojala and Attardi, 1974; Hirsch and Penman, 1974), it might be reasonable to expect an appropriate time delay for processing. In a previous report from this laboratory (Avadhani *et al.*, 1973a), we demonstrated such a delay before appearance of labeled poly(A) containing mRNA in the mitochondria; the process was sensitive to high actinomycin D and resistant to ethidium bromide. Similar *in vivo* properties have also been reported by Hirsch and Penman (1974) and Gaitskhoki *et al.* (1973a). In contrast, in the current *in vitro* experiments no lag in the association of the newly synthesized mRNA with mt polyribosomes has been observed.

The data from this study is also of interest in another unresolved area, namely, that of the extent of complementarity of mtRNA to mtDNA. To arrive at a contribution to this question, we have measured the complementarity of ^{3}H *in vivo* mtRNA, ^{3}H *in vitro* mtRNA and, following the suggestion of Dawid (1972b), chemically labeled steady-state polyribosomal mtRNA. In agreement with Dawid (1972b), we find (Figure 7, Table IV) that mt rRNA and mt tRNA are complementary to 10–11% of the mt genome. The chemically labeled mt mRNA hybridizes to a maximum extent of 4.5%; the estimates based on *in vivo* (6–7 peaks 11 S–16 S) or *in vitro* (3 peaks 11–12 S) pulse labeled mRNA are somewhat lower but the differences may not be significant. On this basis, complementarity accounts for no more than a third of the expected polypeptides believed to be synthesized in mitochondria (Lederman and Attardi, 1973; Michel and Neupert, 1973; Weiss *et al.*, 1973). Our results differ from those of Hirsch and Penman (1974), and are also at variance with those of Ojala and Attardi (1974) since the single 7S peak reported by the latter authors could not code for the 50,000-dalton peptides expected to be synthesized in mitochondria (Lederman and Attardi, 1973; Michel and Neupert, 1973) particularly if allowance is made for the portions of the molecule not involved in coding (Weiss *et al.*, 1973; Gaskill and Kabat, 1971; Blobel, 1971). From the foregoing, it appears that two basic problems remain unsolved: the source(s) of the genetic information for the intramitochondrial synthesis of the majority of mt polypeptides and the nature or origin(s) of intramitochondrial poly(A) containing RNAs.

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Early Changes in Immunoglobulin M Synthesis after Mitogenic Stimulation of Bone Marrow Derived Lymphocytes†

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ABSTRACT: Small resting B-lymphocytes synthesize 10% of their total cellular pool of immunoglobulin M (IgM) molecules within 2–4 hr. They release these IgM molecules from the cells into the supernatant medium with a half-disappearance time between 20 and 40 hr, mainly as 7–8 subunit IgM_s. Bacterial lipopolysaccharides (LPS) stimulate small resting B-lymphocytes within the first hour to a two- to threefold increased rate of IgM synthesis. This stimulation is mitogen-dose dependent and can also be observed with certain batches of fetal calf serum and with the purified protein derivative of tuberculin, two other B-cell mitogens. IgM molecules synthesized with this increased rate are actively secreted from the cells with a median disappearance time of 2–4 hr, mainly as 19S IgM pentamers. The initial change in the rate and type of IgM synthesis after mitogenic stimulation can also be observed in the presence of 5 μg/ml of actinomycin D. IgM synthesis, which is more sensitive to inhibition by actinomycin D than the sum of all cellular protein syntheses in small unstimulated B-lymphocytes, is rendered more resistant to this inhibitor immediately after mitogenic stimulation. Stimulation of small, resting B-lymphocytes by mitogens, in the presence or absence of actinomycin D, leads to a redistribution of ribosomes from monoribosomes to polyribosomes within the first hour after stimulation. It appears, therefore, that stimulation of small, resting B-lymphocytes by mitogens leads to stabilization of RNA synthesis-dependent components of IgM synthesis, such as messenger RNA, from degradation through the formation of polyribosomes and re-programs IgM synthesis from a synthesis of surface membrane bound receptor IgM to a synthesis of actively secreted IgM.

Thymus-derived (T) and bone marrow derived (B) lymphocytes[1] cooperate in the immune response against most antigens (Miller and Mitchell, 1969). B-Lymphocytes produce and secrete immunoglobulins (Ig). Small, resting B-lymphocytes contain Ig, mainly of IgM class, in their surface membrane (Greaves and Hogg, 1971). These membrane-bound Ig molecules are thought to serve as receptors for antigen. Binding of antigen initiates reactions in the resting, so-called antigen-sensitive cells which lead to proliferation of clones of lymphocytes in which B-cells differentiate to Ig-secreting plasma cells.

Mitogens can mimic the action of antigen on lymphocytes. Bacterial lipopolysaccharide (LPS), the purified protein derivative of tuberculin, or certain batches of fetal calf serum (see articles in Möller, 1972) stimulate B-lymphocytes to proliferate and to differentiate into Ig-secreting plasma cells. While an antigenic determinant stimulates

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[1] Abbreviations used are: T-lymphocytes, T-cells, thymus-derived lymphocytes; B-lymphocytes, B-cells, bone marrow derived lymphocytes; IgM, immunoglobulin M; LPS, bacterial lipopolysaccharide from Gram-negative bacteria; PFC, plaque-forming cells.