The Synthesis of Polyadenylic Acid-Containing Ribonucleic Acid by Isolated Mitochondria from Ehrlich Ascites Cells

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(Received 24 November 1975)

The synthesis of poly(A)-containing RNA by isolated mitochondria from Ehrlich ascites cells was studied. Isolated mitochondria incorporate [3 H]AMP or [3 H]UTP into an RNA species that adsorbs on oligo(dT)-cellulose columns or Millipore filters. Hydrolysis of the poly(A)-containing RNA with pancreatic and T_1 ribonucleases released a poly(A) sequence that had an electrophoretic mobility slightly faster than $4S_E$. In comparison, ascites-cell cytosolic poly(A)-containing RNA had a poly(A) tail that had an electrophoretic mobility of about $7S_E$. Sensitivity of the incorporation of [3 H]AMP into poly(A)-containing RNA to ethidium bromide and to atracyloside and lack of sensitivity to immobilized ribonuclease added to the mitochondria after incubation indicated that the site of incorporation was mitochondrial. The poly(A)-containing RNA sedimented with a peak of about $18S$, with much material of higher s value. After denaturation at 70°C for 5 min the poly(A)-containing RNA separated into two components of $12S$ and $16S$ on a 5–20% (w/v) sucrose density gradient at 4°C , or at 4° and 25°C in the presence of formaldehyde. Poly(A)-containing RNA synthesized in the presence of ethidium bromide sedimented at 5 – $10S$ in a 15–33% (w/v) sucrose density gradient at 24°C . The poly(A) tail of this RNA was smaller than that synthesized in the absence of ethidium bromide. The size of the poly(A)-containing RNA (approx. 1300 nucleotides) is about the length necessary for that of mRNA species for the products of mitochondrial protein synthesis observed by ourselves and others.

It is now well established that mammalian mitochondrial DNA codes for mitochondrial rRNA and at least 12 distinct species of tRNA (Borst, 1972). The nature and number of mRNA species encoded by mitochondrial DNA is still unclear. It has been demonstrated that mitochondria from human HeLa, Chinese-hamster ovary and insect cells contain a poly(A)-containing RNA which is distinct from that in the cytosol (Perlman *et al.*, 1973; Hirsch & Penman, 1973; Hirsch *et al.*, 1974; Ojala & Attardi, 1974*a,b,c*). The mitochondrial poly(A)-containing RNA from HeLa cells has a poly(A) tail that is approx. $4S$, or 50–80 nucleotides (Hirsch & Penman, 1973; Ojala & Attardi, 1974*b*). The poly(A)-containing RNA can be isolated from mitochondrial polyribosomes (Hirsch & Penman, 1974*b*; Ojala & Attardi, 1974*b*) and has been separated into eight species ranging between $24S$ and $16S$ (Hirsch & Penman, 1973; Hirsch *et al.*, 1974) or into two groups, one of about $12S$, containing seven distinct species, and one of $7S$ (Ojala & Attardi, 1974*c*). The nature of the association with mitochondrial polyribosomes (Hirsch & Penman, 1974*b*) and the presence of a poly(A) tail are properties consistent with those of a mRNA. The mitochondrial poly(A)-containing RNA is probably transcribed

from mitochondrial DNA, as its synthesis is inhibited by ethidium bromide, but not by camptothecin (Hirsch & Penman, 1974*a,b*), and it hybridizes with mitochondrial DNA (Hirsch & Penman, 1973; Ojala & Attardi, 1974*c*). Poly(A)-containing RNA also appears to be made in chick mitochondria (Groot *et al.*, 1974).

Avadhani *et al.* (1973, 1974) have obtained poly(A)-containing RNA from polyribosomes of Ehrlich ascites-cell mitochondria, but in contrast with the above results they conclude that this RNA is of nuclear origin. They also found an RNA that did not contain poly(A) associated with mitochondrial polyribosomes, that hybridized to mitochondrial DNA. The synthesis of poly(A)-containing RNA could not be shown in isolated mitochondria or mitochondrial digitonin particles from Ehrlich ascites cells. In contrast, a poly(A) polymerase has been isolated from rat liver mitochondria and Morris hepatomas (Jacob & Schindler, 1972; Jacob *et al.*, 1972, 1974; Rose *et al.*, 1975). Further, in HeLa-cell mitochondria it has been shown that the poly(A) tail is synthesized after transcription (Hirsch & Penman, 1974*a*). Gaitskhoki *et al.* (1973) and Kisselev *et al.* (1975) also observed poly(A)-containing RNA in mitochondria, but its transcriptional origin was unclear.

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In yeast, Cooper & Avers (1974) reported the existence of a poly(A)-containing RNA associated with mitochondrial polyribosomes, and Padmanaban *et al.* (1975) obtained a mitochondrial poly(A)-containing RNA which could be translated in an *Escherichia coli* ribosomal system. However, neither Groot *et al.* (1974) nor Eggitt & Scragg (1975) could detect any. The mitochondrial RNA extracted by Eggitt & Scragg (1975) could be translated in *Xenopus laevis* oocytes.

To resolve some of these conflicting results we have examined the synthesis of poly(A)-containing RNA by isolated mitochondria from Ehrlich ascites cells. Our results indicate that these mitochondria synthesize an RNA fraction that contains a poly(A) sequence of 4S_E. The poly(A)-containing RNA has been characterized.

Materials and Methods

Materials

All chemicals were reagent grade where possible. Atractyloside was purchased from Calbiochem., La Jolla, CA, U.S.A. Nucleoside triphosphates were obtained from Boehringer (Mannheim) Corp., New York, NY, U.S.A. Deoxyribonuclease I, ribonuclease A and immobilized ribonuclease were from Worthington Biochemical Corp., Freehold, NJ, U.S.A., and T₁ ribonuclease was from Sigma Chemical Co., St. Louis, MO, U.S.A. NCS solubilizer, PCS solubilizer, [5-³H]UTP (17 Ci/mmol), [2-³H]AMP (22 Ci/mmol), [2-¹⁴C]uridine (50 mCi/mmol) and [2-³H]-adenosine (25 Ci/mmol) were obtained from Amersham/Searle Corp., Toronto, Ont., Canada. Oligo-(dT)-cellulose (type T-2) was purchased from Collaborative Research Inc., Waltham, MA, U.S.A.

Cells. Ehrlich ascites cells were maintained in 8–10-week-old Swiss-Webster mice as described previously (Haldar & Freeman, 1968) and were collected from 7 to 10 days after the initial injection.

Methods

Isolation of mitochondria. Cells were washed and then disrupted in 0.3 M-sucrose/2 mM-EDTA/2 mM-Tris/HCl, pH 7.4, as described previously (Freeman, 1965). After disruption, nuclei and cell debris were removed at 2000g for 4 min, the pellet was resuspended and re-centrifuged as before. The combined supernatants were centrifuged first at 2000g for 4 min to remove further nuclei or nuclear fragments and then at 5000g for 10 min. The mitochondrial fraction was washed once with 0.25 M-sucrose/2 mM-EDTA, pH 7.4, before incubation.

Incubation of isolated mitochondria. Mitochondria were incubated in the hypo-osmotic medium described previously (Fukamachi *et al.*, 1972). Either [³H]UTP or [³H]AMP was used to label the RNA. The amount is indicated with each experiment. Incubations were

6 ml and contained about 3 mg of mitochondrial protein/ml. After the incubation mitochondria were recovered by centrifugation, washed once with 0.25 M-sucrose/2 mM-EDTA, pH 7.4, and the RNA was extracted.

Isolation of RNA. RNA was isolated by the cold-phenol method used by Fukamachi *et al.* (1972) to obtain total RNA, a modification of a method of Kirby (1965). About 1 mg of carrier rat liver rRNA was used in each extraction. The RNA was recovered by precipitation with 2.5 vol. of 95% (v/v) ethanol at -20°C overnight and the precipitated RNA was used directly.

Isolation of poly(A)-containing RNA. Poly(A)-containing RNA was recovered by adsorption on and elution from a column of 100 mg of oligo(dT)-cellulose as described by Hirsch & Penman (1973). Alternatively it was adsorbed on Millipore filters as described in method B of Lee *et al.* (1971).

Isolation of poly(A). RNA was dissolved in sterile water and digested in 1 ml Eppendorf plastic microtubes in 100 µl containing 0.45 M-KCl/15 mM-Tris/HCl, pH 7.4, 20 µg of pancreatic ribonuclease/ml and 10 units of T₁ ribonuclease/ml. [One unit is defined as that sufficient to produce acid-soluble oligonucleotides equivalent to an E₂₆₀ of 1.0 in 15 min at pH 7.5 and at 37°C (in a reaction mixture of volume 1.0 ml)]. After 30 min at 37°C, 1 ml of 0.1 M-NaCl/25 mM-sodium acetate (pH 8.5)/2% sodium dodecyl sulphate was added and the solution extracted with an equal volume of water-saturated phenol at 22°C. RNA was precipitated from the aqueous phase with 2.5 vol. of 95% (v/v) ethanol at -20°C.

Denaturation of poly(A)-containing RNA. Total RNA was dissolved in 0.1% sodium dodecyl sulphate/1 mM-EDTA/10 mM-Tris/HCl, pH 7.4, heated at 70°C for 5 min (Ojala & Attardi, 1974a) and placed at 0°C for rapid cooling. After denaturation, the solution was adjusted to 0.4 M-NaCl and the poly(A)-containing RNA adsorbed on an oligo(dT)-cellulose column. After elution with 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA, the poly(A)-containing RNA was analysed directly on polyacrylamide gels or denatured again before centrifugation on sucrose density gradients. Alternatively the poly(A)-containing RNA was precipitated with 95% ethanol, dissolved in 1 mM-Tris/HCl (pH 7.4)/0.25 mM-EDTA/0.2% sodium dodecyl sulphate with or without 1% (v/v) formaldehyde, heated at 70°C for 5 min and rapidly cooled to 0°C before centrifugation.

Gel electrophoresis. The 14% polyacrylamide gels were prepared and run with buffer E as described by Loening (1969).

Sucrose-density-gradient centrifugation. A number of different conditions were used: (1) an isokinetic 15–33% (w/v) sucrose density gradient in 0.1 M-NaCl/0.2% sodium dodecyl sulphate, pH 7.0, at 24°C and 69000g for 16 h (Bartoov *et al.*, 1970); (2) a linear

5–20% (w/v) sucrose density gradient in 1 mM-Tris/HCl (pH 7.4)/0.25 mM-EDTA/0.2% sodium dodecyl sulphate with or without 1% formaldehyde at 24°C and 108 000g for 18 h; (3) a linear 5–20% (w/v) sucrose density gradient in 1 mM-Tris/HCl (pH 7.4)/0.25 mM-EDTA/0.1 M-LiCl/0.2% sodium dodecyl sulphate with or without 1% formaldehyde at 4°C and 108 000g for 18 h. Centrifugation was in the SW41 rotor of the Beckman L2-65B centrifuge.

Alkaline hydrolysis. RNA was precipitated with 5% (w/v) trichloroacetic acid, washed twice with 5 ml of 2% (w/v) potassium acetate in 95% ethanol, then with 5 ml each of ethanol/diethyl ether (3:1, v/v) and ether. After hydrolysis, as described previously (Fukamachi *et al.*, 1972), the resulting nucleotides were separated by descending paper chromatography on Whatman no. 1 paper with isobutyric acid/NH₃ (sp.gr. 0.88)/water (66:1:33, by vol.) as solvent. The paper was then dried cut and counted for radioactivity.

Preparation of marker rRNA. The cytosolic rRNA of 1×10^8 Chinese-hamster ovary cells, grown in 200 ml of suspension culture (Bartoov *et al.*, 1970), was labelled overnight with 1 μ Ci of [¹⁴C]uridine/ml. The cells were recovered by centrifugation, washed with phosphate-buffered saline (Bartoov *et al.*, 1970) and extracted by method no. 1 of Kirby (1965) in the presence of 1 mg of carrier rRNA.

Preparation of cytosolic poly(A). A Swiss-Webster mouse containing 7-day Ehrlich ascites cells was injected with 150 μ Ci of [³H]adenosine. After 24 h the cells were recovered and disrupted and the RNA was extracted from the 10 000g supernatant by method no. 1 of Kirby (1965). Poly(A)-containing RNA was recovered on an oligo(dT)-cellulose column and poly(A) obtained as described for mitochondrial RNA.

Measurement of radioactivity. Samples of RNA, poly(A)-containing RNA or poly(A) were dried on Whatman 3 MM filter discs, washed in two changes of ice-cold 5% trichloroacetic acid, followed by ethanol/ether (1:1, v/v) and ether. Radioactivity was measured in a toluene-based scintillation solution at 20% efficiency (Halder & Freeman, 1968). Millipore filters and paper chromatograms were also counted for radioactivity in this solvent. Fractions from sucrose density gradients were counted at 20% efficiency with 0.3 ml of water and 3.0 ml of a phase combining liquid-scintillation solvent (PCS from Amersham/Searle). Gels were cut into 1 mm slices with a Gilson Aliquotel fractionator, digested overnight in 0.15 ml of a tissue solubilizer (NCS from Amersham/Searle) diluted 2:3 (v/v) with toluene, and counted for radioactivity at 35% efficiency with 3 ml of naphthalene/dioxan scintillation solvent (Halder & Freeman, 1969). A Nuclear-Chicago Mark I liquid-scintillation spectrometer was used for radioactivity counting.

Results

RNA synthesis by isolated mitochondria

Fukamachi *et al.* (1972) showed that isolated rat liver mitochondria incorporate [³H]UTP into RNA at a linear rate for 1 h. As shown in Fig. 1, this was essentially the case for mitochondria from Ehrlich ascites cells with both [³H]AMP and [³H]UTP as precursors. In contrast, there was little increase in the incorporation of [³H]AMP into poly(A)-containing RNA (Fig. 1a) after the first 5 min. The incorporation of [³H]UTP (Fig. 1b) showed a lag at first and then was linear with time until 30 min before reaching a plateau. The difference in the first 5–10 min between the incorporation of the two precursors into poly(A)-containing RNA might reflect the synthesis of the poly(A) tail and its post-transcriptional addition.

Previously we found that isolated rat liver mitochondria incorporate adenine nucleotides not only into RNA but also into an acid-insoluble material which on alkaline hydrolysis yielded a compound that was not 2'- or 3'-AMP (Fukamachi *et al.*, 1972). Total RNA synthesized by isolated mitochondria from Ehrlich ascites-cell mitochondria also yielded this compound after alkaline hydrolysis, but only 2'- or 3'-[³H]AMP was detected in hydrolysates of the poly(A)-containing RNA, indicating that it was RNA.

Characterization of the poly(A) tail of poly(A)-containing RNA

Poly(A)-containing RNA is usually measured by its ability to bind to oligo(dT)-cellulose, Millipore filters or poly(U)-Sephadex (Molloy *et al.*, 1974). As shown in Table 1, the first two of these methods gave essentially identical results, approx. 25% of the [³H]UTP-labelled RNA being retained either on oligo(dT)-cellulose or by Millipore filters. With [³H]AMP as the precursor about 30% was retained. Over a series of experiments this value ranged between 10% (e.g. Fig. 1a) and 35%, depending perhaps on the amount of the [³H]AMP-labelled material that was not RNA. To establish whether the RNA that was bound to oligo(dT)-cellulose contained poly(A), the bound material was hydrolysed with pancreatic ribonuclease A and T₁ ribonuclease, and the resistant material characterized by polyacrylamide-gel electrophoresis. As shown in Fig. 2 the ribonuclease-resistant material migrated with a peak of slightly less than 4S_E, with some-faster running material. In comparison cytosolic poly(A) had a peak at 7S_E. The profile of the mitochondrial poly(A) was not significantly altered by prior digestion with pancreatic deoxyribonuclease. As shown in Fig. 2, no peak was observed when [³H]UTP-labelled RNA was treated in the same way. From its resistance to the ribonucleases used and its labelling with [³H]AMP but not [³H]UTP, it was concluded that the mitochondrial 4S_E material was

Fig. 1. Incorporation of [^3H]AMP (a) and [^3H]UTP (b) into total and poly(A)-containing RNA by isolated ascites-cell mitochondria

Mitochondria were isolated and incubated with $25\text{ }\mu\text{Ci}$ of [^3H]AMP/ml or $50\text{ }\mu\text{Ci}$ of [^3H]UTP/ml in a total volume of 6 ml at 30°C . At each time-interval a 0.5 ml sample was taken, total RNA and poly(A)-containing RNA were isolated and the incorporation into each as trichloroacetic acid-precipitable radioactivity was determined. ●, Incorporation into total acid-insoluble material; ○, into poly(A)-containing RNA; □, into poly(A)-containing RNA in a separate experiment.

Table 1. Incorporation of [^3H]UTP and [^3H]AMP into ascites-cell mitochondrial total RNA, poly(A)-containing RNA and poly(A)

Mitochondria were incubated and RNA and poly(A)-containing RNA isolated from 6 ml incubations (30°C , 30 min) as described in the Materials and Methods section. The control incorporation was about 70000 c.p.m. for both [^3H]UTP (incubations with $50\text{ }\mu\text{Ci}$ /ml) and [^3H]AMP (incubations with $25\text{ }\mu\text{Ci}$ /ml) and 14000 c.p.m. for the incubation with ethidium bromide. —, No determination was made.

	Type of RNA	Method of fractionation	Incorporation (% of control)	
			[^3H]UTP	[^3H]AMP
Control				
Total			100	100
Poly(A)-containing RNA				
	Oligo(dT)-cellulose		27	28
	Millipore filter		24	31
Poly(A)				
	Oligo(dT)-cellulose		—	3
	Millipore filter		—	Negligible
+ Ethidium bromide ($3\text{ }\mu\text{g}/\text{ml}$)				
Total				100
Poly(A)-containing RNA				
	Oligo(dT)-cellulose			22
	Millipore filter			—
Poly(A)				
	Oligo(dT)-cellulose			8.4
	Millipore filter			Negligible

poly(A). The material running faster than the 4S_E peak is probably also poly(A) and not undigested oligonucleotides, as it was not labelled by [^3H]UTP and it was retained by oligo(dT)-cellulose. In comparison with the results of Hirsch & Penman (1973), the 4S_E peak corresponds to a length of 57 nucleo-

tides, but the average length of poly(A) including the smaller poly(A) would be about 50 nucleotides.

The poly(A) tail of the mitochondrial poly(A)-containing RNA could be bound to oligo(dT)-cellulose but not to Millipore filters, as shown in Table 1. It had about 10% of the radioactivity of the

poly(A)-containing RNA. Assuming an average length of poly(A) of 50 nucleotides and that the content of A residues in mitochondrial RNA is 35% (Freeman *et al.*, 1973) then the average length of the poly(A)-containing RNA is about 1300 nucleotides.

Fig. 2. Polyacrylamide-gel electrophoresis of the poly(A) tail of mitochondrial and cytosolic poly(A)-containing RNA

Mitochondria were isolated and incubated with 25 μ Ci of [3 H]AMP/ml or 50 μ Ci of [3 H]UTP/ml in a total volume of 6 ml at 30°C for 30 min. The RNA and poly(A)-containing RNA were isolated and the poly(A)-containing RNA was digested with T₁ and pancreatic ribonucleases, as described in the Materials and Methods section. The poly(A) was recovered, re-adsorbed and eluted from oligo(dT)-cellulose and characterized on 14% polyacrylamide gels. The cytosolic poly(A) was obtained as described in the Materials and Methods section. Electrophoresis was for 3.5 h at 8 V/cm. The gels were sliced and counted for radioactivity as described in the Materials and Methods section. The marker was *E. coli* tRNA run in a separate gel and localized by scanning the gel at 260 nm on a Gilford spectrophotometer. ●, Cytosolic poly(A); ○, mitochondrial poly(A) labelled with [3 H]AMP; Δ, mitochondrial poly(A) labelled with [3 H]UTP.

Mitochondrial origin of poly(A)-containing RNA

Although the size of the poly(A) tail was different from that of cytosol poly(A)-containing RNA, it was still possible that the poly(A)-containing RNA was synthesized in nuclei or nuclear fragments present in the mitochondrial fraction or in some other cellular organelle. To prevent possible synthesis by nuclei, [3 H]AMP was used, as isolated nuclei only incorporate nucleotides from nucleoside triphosphates, whereas the nucleoside monophosphate would be phosphorylated in mitochondria. The site of synthesis was examined further with selective inhibitors. As shown in Table 2, atractyloside, an inhibitor of adenine nucleotide translocation into mitochondria (Winkler & Lehninger, 1968; Vignais & Vignais, 1970), and ethidium bromide, an inhibitor of labelling of RNA in mitochondria (Zylber *et al.*, 1969; Fukamachi *et al.*, 1970; Hirsch & Penman, 1974a,b) both inhibited [3 H]AMP incorporation. Further, as shown in Table 2, immobilized pancreatic ribonuclease digested only 10% of the [3 H]AMP-labelled poly(A)-containing RNA in the mitochondrial fraction, indicating that this RNA was protected within a membrane. It should be noted that under these conditions of digestion in low salt concentrations, poly(A) is hydrolysed (Beers, 1960).

Characterization of the poly(A)-containing RNA

The poly(A)-containing RNA was characterized by sucrose-density-gradient centrifugation and polyacrylamide-gel electrophoresis. As shown in Fig. 3, both [3 H]AMP- and [3 H]UTP-labelled poly(A)-containing RNA gave similar patterns on 15–33% sucrose density gradients under non-denaturing conditions. There was a broad peak of material at about 18S and also much larger material. The high *s* values could reflect the true size of the RNA, its conformation or its aggregation. This was examined

Table 2. Effect of inhibitors on [3 H]AMP incorporation

The experiment was performed as for Table 1. In one experiment the mitochondria were washed after the incubation with 0.3M-sucrose/2 mM-EDTA/2 mM-Tris/HCl, pH 7.4, and then incubated in this medium for 15 min at 30°C in the presence of 1 unit of immobilized ribonuclease/ml.

Inhibitor	Fraction	Incorporation (% of control)	
		Total	Poly(A)-containing RNA
Control	...	100	100*
+Atractyloside (210 μ M)		22	20
+Ethidium bromide (3 μ g/ml)		20	20
+Immobilized ribonuclease		81	90

* Incorporation into poly(A)-containing RNA was 10% of total incorporation.

further by characterization of denatured RNA. When the [^3H]AMP-labelled poly(A)-containing RNA was denatured by heating, as described in the Materials and Methods section, and centrifuged in a 5–20% sucrose density gradient in 1 mM-Tris/HCl, pH 7.4, at 25°C, a peak of material at 12S was obtained as shown in Fig. 4. When denatured [^3H]AMP-

Fig. 3. Characterization on sucrose density gradients of mitochondrial poly(A)-containing RNA synthesized in the presence or the absence of ethidium bromide

Mitochondrial poly(A)-containing RNA, obtained as described in Table 1, was centrifuged for 16 h at 69000g at 24°C in a 15–33% (w/v) sucrose density gradient in 0.1 M-NaCl/0.2% sodium dodecyl sulphate, pH 7.0. The markers were [^{14}C]uridine-labelled cytosolic rRNA from Chinese-hamster ovary cells run in a parallel tube. The fraction size was 0.22 ml. ●, [^3H]AMP-labelled poly(A)-containing RNA; △, [^3H]UTP-labelled poly(A)-containing RNA; ○, [^3H]AMP-labelled poly(A)-containing RNA synthesized in the presence of 3 µg of ethidium bromide/ml.

Fig. 4. Characterization of denatured [^3H]AMP-labelled poly(A)-containing RNA on a sucrose density gradient

Total [^3H]AMP-labelled mitochondrial RNA was obtained as described in Table 1. It was denatured at 70°C for 5 min and the poly(A)-containing RNA was adsorbed on and eluted from oligo(dT)-cellulose and denatured again, as described in the Materials and Methods section. Centrifugation was on a 5–20% (w/v) sucrose density gradient in 1 mM-Tris/HCl (pH 7.4)/0.25 mM-EDTA/0.2% sodium dodecyl sulphate at 24°C and 108000g for 18 h. The markers are from denatured [^{14}C]uridine-labelled cytosolic rRNA from Chinese-hamster ovary cells.

or [^3H]UTP-labelled poly(A)-containing RNA was centrifuged in a 5–20% sucrose density gradient in 0.1 M-LiCl at 4°C, two major peaks of material at 16S and 12S were observed (Fig. 5). If the [^3H]AMP-labelled poly(A)-containing RNA was denatured in the presence of formaldehyde and centrifuged at 25°C in the presence of formaldehyde, two major peaks of material at 16S and 12S were observed as well as some RNA with a lower rate of sedimentation (results not shown). The same result was observed if this RNA was centrifuged at 4°C in the presence of formaldehyde. On 2.5% polyacrylamide gels the denatured poly(A)-containing RNA ran between 12S_E and 16S_E (results not shown).

Characterization of poly(A)-containing RNA synthesized in the presence of ethidium bromide

Although ethidium bromide inhibited the incorporation of [^3H]AMP into poly(A)-containing RNA, some labelling was still observed, as shown in Table 1. This reflects the synthesis of both the poly(A) tail and the rest of the poly(A)-containing RNA, but the poly(A) tail contained 35% of the radioactivity. This higher percentage might be expected if the ethidium bromide inhibits transcription. This was examined further by characterizing the poly(A)-containing RNA and its poly(A) tail. As shown in Fig. 3, the poly(A)-containing RNA sedimented at about 5–10S in a 15–33% sucrose density gradient in 0.1 M-NaCl. In 5–20% gradients, this peak separated into components of 6S, 12S and shoulder of a material

Fig. 5. Characterization of denatured poly(A)-containing RNA on a sucrose density gradient

Denatured poly(A)-containing RNA, labelled with either [^3H]AMP or [^3H]UTP, was obtained as described in Fig. 4. Centrifugation was on a 5–20% (w/v) sucrose density gradient in 0.1 M-LiCl/1 mM-Tris/HCl (pH 7.4)/0.25 mM-EDTA/0.2% sodium dodecyl sulphate at 4°C and 108000g for 18 h. ●, [^3H]AMP-labelled poly(A)-containing RNA; ○, [^3H]UTP-labelled poly(A)-containing RNA.

above 12S. On 14% polyacrylamide gels the poly(A) tail of this RNA was shorter than the major poly(A) peak of poly(A)-containing RNA synthesized in the absence of ethidium bromide (Fig. 2).

Discussion

Two major approaches have been used to determine the site of synthesis of mitochondrial proteins and RNA (Borst, 1972; Schatz & Mason, 1974). In the first, the macromolecules are labelled in whole cells, and inhibitors that act selectively are used to stop synthesis of macromolecules either in or outside the mitochondria. In the second, mitochondria are isolated and the macromolecules that they synthesize are determined directly. With the latter approach it must be shown that mitochondria are the site of the observed synthesis. In the present experiments, the inhibition by ethidium bromide and atractyloside and lack of digestion by immobilized ribonuclease indicate that mitochondria were the site of synthesis of poly(A)-containing RNA. It is concluded that Ehrlich-ascites-cell mitochondria synthesize a poly(A)-containing RNA. Further, since isolated nuclei do not synthesize poly(A) at low concentrations of ATP (Jelinek, 1974), with [^3H]AMP as the precursor, it would not be expected that nuclei or nuclear fragment would account for the synthesis of poly(A) by the mitochondrial fraction.

We have shown that isolated rat liver mitochondria synthesize mitochondrial rRNA (Fukamachi *et al.*, 1970, 1972). The poly(A)-containing RNA also appeared as discrete species if it was denatured before centrifugation. Two broad peaks of material centred at 16S and 12S were observed on sucrose density gradients centrifuged at 4°C (Fig. 5) or at 25°C in the presence of formaldehyde. This result is very similar to that observed by Ojala & Attardi (1974c). We have not observed the 7S component that they did. The non-denatured poly(A)-containing RNA sedimented more rapidly. The higher *s* value could reflect aggregation of the RNA when it was isolated, as suggested by Ojala & Attardi (1974a,b,c). On denaturation the aggregates are dispersed. The alternative possibility, that the RNA is hydrolysed on heating or contains hidden nicks, cannot be entirely discounted.

Assuming that poly(A)-containing RNA when denatured was not partly hydrolysed, then its mol.wt. would be about 400000 for the average of the two major components. This is similar to the value of 390000 calculated on the basis of the size of the poly(A) tail and the percentage of the poly(A) tail in the poly(A)-containing RNA. It is not known at present if this RNA is mRNA, but if it is, then this size is sufficient to code for the proteins synthesized in mammalian mitochondria (Coote & Work, 1971; Lederman & Attardi, 1973; Burke & Beattie, 1974; Wallace *et al.*, 1975). In the presence of ethidium

bromide the poly(A)-containing RNA synthesized was smaller both in its poly(A) tail and in the rest of the molecule. No free poly(A) was observed. This is similar to the results of Hirsch & Penman (1974a), but differs from that of Ojala & Attardi (1974b), who observed the synthesis of free poly(A) in HeLa-cell mitochondria in the presence of ethidium bromide.

The poly(A) tail of the poly(A)-containing RNA was smaller than that of cytosolic poly(A)-containing RNA. This was also found in HeLa cells (Hirsch & Penman, 1973; Ojala & Attardi, 1974) and in Ehrlich ascites cells (Avadhani *et al.*, 1974); however, the poly(A) tail synthesized in isolated mitochondria is more heterogeneous. The major peak of material had a similar electrophoretic mobility to that observed by Hirsch & Penman (1973), who calculated a length of 57 nucleotides for it.

As discussed above, our results are very similar to those of Hirsch & Penman (1973) and Ojala & Attardi (1974a,b,c). Mitochondria do appear to synthesize a poly(A)-containing RNA. Avadhani *et al.* (1973, 1974) were unable to show that isolated mitochondria and digitonin particles synthesize a poly(A)-containing RNA. Although there are some apparently minor differences between their approach and ours, the reason for the difference in results is not known at present.

This work was supported by the Medical Research Council of Canada, grant MT-1940.

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