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Poly(adenylic acid) Synthesis in Isolated Rat Liver Mitochondria†

Kathleen M. Rose* and Samson T. Jacob

ABSTRACT: Purified rat liver mitochondria were shown to synthesize poly(adenylic acid) (poly(A)) in vitro. Detection of the poly(A) synthesizing activity was facilitated by addition of NaF to the reaction mixture. The product of the reaction was shown to be poly(A) by its insensitivity to digestion with pancreatic RNase and RNase T_1, its degradation by venom phosphodiesterase and its retention on poly(uridylic acid) cellulose columns. The average chain length of the product was 20-23 AMP units and it was covalently attached to the endogenous RNA in the mitochondria. Poly(A) synthesis required ATP and a divalent ion and was maximally active in the pH range of 7-8. The reaction was inhibited by atractyloside, cordycepin triphosphate, Rose Bengal, rifamycin derivative AF/013, sodium pyrophosphate, and *N*-ethylmaleimide. These studies indicate that the mitochondrial poly(A) polymerase previously described in our laboratory (Jacob, S. T., Rose, K. M., and Morris, H. P. (1974), *Biochim. Biophys. Acta 361,* 312-320) is involved in the posttranscriptional addition of poly(A) sequence to mitochondrial RNA.

It is well recognized that many eukaryotic mRNAs contain a poly(A)[1] sequence at their 3′ termini (for review, see Brawerman, 1974). In addition to polysomal mRNA, mitochondrial mRNA also has been shown to contain a poly(A) tract, albeit of a shorter length (Perlman et al., 1973; Ojala and Attardi, 1974; Hirsch and Penman, 1974). Although the exact physiological function of poly(A) has not been established, there is mounting evidence to suggest that it confers stability to, and consequently enhances translational efficiency of, some mRNAs (Marbaix et al., 1975; Sheiness et al., 1975; Levy et al., 1975).

Poly(A) polymerase, the enzyme presumed to be involved in the posttranscriptional addition of poly(A) to mRNA, has been well characterized in a variety of prokaryotic and eukaryotic cells. This enzyme has been identified in the nucleus (Edmonds and Abrams, 1960; Hyatt, 1967; Niessing and Se-

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[1] Abbreviations used: poly(A), poly(adenylic acid); poly(U), poly(uridylic acid); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

keris, 1973; Jacob et al., 1976a), microsomal (Wilkie and Smellie, 1968; Rose and Jacob, 1975), and supernatant (Klemperer, 1963; Tsiapalis et al., 1973) fractions of the cell. We have characterized such an enzyme extracted from mitochondria of liver (Jacob and Schindler, 1972; Jacob et al., 1974b) and variety of rapidly growing Morris hepatomas (Jacob et al., 1972; Rose et al., 1975). These studies, however, do not unequivocally establish that this enzyme is involved in the polyadenylation of mitochondrial mRNA in vivo. One approach to resolve this problem is to investigate whether isolated mitochondria can perform this function in vitro. Use of such a system can also facilitate exploration of factor(s) controlling the polyadenylation process in mitochondria and its role in the processing of mitochondrial mRNA. The present report presents evidence to show that isolated mitochondria from rat liver can add poly(A) to the endogenous RNA and describes the characteristics of this reaction.

Materials and Methods

Buffers. Glass-distilled water was used in all buffers. Solutions were prepared immediately before use. Buffer I contained: 20 mM Tris-HCl (pH 7.4), 70 mM sucrose, 220 mM D-mannitol, 1 mM EDTA, 100 μg/ml polyvinyl sulfate, 100 μg/ml heparin, and 500 μg/ml bovine serum albumin. Buffer II consisted of buffer I without EDTA and polyvinyl sulfate and heparin was reduced to 25 μg/ml. Buffer III contained: 20 mM Tris-HCl (pH 7.4), 70 mM sucrose, and 220 mM D-mannitol.

Materials. [2,8-^{3}H]ATP (35 Ci/mmol) and scintillation cocktail 949 were obtained from New England Nuclear Corporation. Rose Bengal and *N,N'*-dimethylformamide were purchased from Eastman Kodak. All other reagents were of the highest grade obtainable.

Isolation of Mitochondria. Mitochondria were prepared by a modification of the procedure described by Malkin (1971). Livers from male Sprague-Dawley rats (150–200 g body wt) were suspended in 0.25 M sucrose containing 0.9% (w/v) NCl. All procedures were carried out at 4 °C and as rapidly as possible. Generally 80–100 g of liver were processed at a time. Livers were minced, suspended in buffer I (2 ml/g liver), and homogenized (three strokes) in a Teflon–glass homogenizer. Heparin and polyvinyl sulfate were included in the buffers to minimize RNA degradation. After passing through cheesecloth, the filtrate was diluted with additional buffer I to 8 ml/g liver. After centrifugation at 1000*g* for 10 min, the supernatant was decanted, filtered through cheesecloth, and centrifuged again at the same speed. The nuclei-free supernatant was then centrifuged at 5000*g* for 10 min. The mitochondrial pellets were resuspended in buffer II (1 ml/g liver). Digitonin was added to a final concentration of 0.04% from a 2% (w/v) stock solution prepared just before use. After 5 min at 0 °C, the mitochondria were centrifuged at 10 000*g* for 10 min. The digitonin-treated mitochondria were washed with buffer III (1 ml/g liver) and centrifuged at 10 000*g* for 10 min. The final pellets were resuspended in 50 mM Tris-HCl (pH 8) containing 50% (v/v) glycerol (0.1 ml/g liver) and stored at −70 °C until further use.

Assay for Poly(A) Synthesis. Poly(A) synthesis was measured in a reaction mixture containing 50 mM Tris-HCl (pH 8), 30 mM KCl, 30 mM NaF, 0.5 mM $MnCl_2$, 0.5 mM $MgCl_2$, 2.5 mg/ml phosphoenolpyruvate, 5 μg/ml pyruvate kinase, 100 μM [^{3}H]ATP (15–30 cpm/pmol), and 200 to 800 μg of mitochondrial protein in a final volume of 120 μl. After 1 h at 37 °C, reaction mixtures were chilled to 0 °C and 10 μl of ATP (10 mg/ml) was added. Zero-time values were obtained by incubating the mitochondria at 37 °C for 1 h, followed by addition of 100 μg of ATP and then the [^{3}H]ATP. Aliquots of 120 μl were transferred to Whatman DE81 filters which were washed batchwise five times with 5% Na_2HPO_4. Radioactivity was determined as described previously (Rose et al., 1976b). The pmol of AMP incorporated into oligonucleotide was calculated after correcting for zero-time values (usually 100–150 cpm). All values reported are the average of triplicate determinations performed on at least two separate preparations of mitochondria.

Extraction of Mitochondrial RNA. Freshly prepared mitochondria derived from 80 g of liver were incubated using the standard conditions for poly(A) synthesis except that 200 μg/ml heparin was included in the reaction and the ATP concentration was 50 μM. After 45 min at 37 °C, the reaction mixtures were pooled and RNA was extracted by addition of 0.3 volumes of a mixture containing 3% sodium dodecyl sulfate, 3 mM EDTA, and 600 μg/ml polyvinyl sulfate. After homogenization (15 strokes) with a tight-fitting Teflon pestle, an equal volume of water-saturated phenol containing 4 μg/ml 8-hydroxyquinoline was added. The suspension was homogenized again and then vigorously shaken at 55 °C for 10 min, followed by an additional 45 min at 20 °C. The phases wereseparated by centrifugation (35 000*g* for 15 min). The phenol layer was reextracted by homogenizing with 2 volumes of 10 mM Tris (pH 9), followed by addition of 1 volume (relative to phenol) of chloroform–isoamyl alcohol (24:1). The suspension was shaken vigorously at 20 °C for 3 min and the phases were separated by centrifugation. The aqueous layers from both extractions were combined and reextracted with an equal volume of chloroform–isoamyl alcohol (24:1) as above. After centrifugation, the final aqueous layer was made 0.1 M with respect to NaCl and precipitated with 2.5 volumes of 98% (v/v) ethanol containing 2% (w/v) potassium acetate. After allowing the reaction mixture to stand for at least 12 h at −20 °C, RNA was sedimented at 40 000*g* for 20 min, dissolved in 2 ml of glass-distilled water, and reprecipitated with ethanol as before. The final RNA preparation was suspended in 2 ml of buffer containing 10 mM Tris-HCl (pH 7.5) and 0.1 M NaCl.

Chromatography on Poly(U)-Cellulose. Poly(U)-cellulose was prepared according to the procedure described by Sheldon et al. (1972). RNA samples (with a poly(A) content up to 150 μg) were suspended in buffer containing 10 mM Tris-HCl (pH 7.5) and 0.1 M NaCl and loaded onto a poly(U)-cellulose column (1.4 × 3 cm) equilibrated in the same buffer. The sample was allowed to remain in contact with the cellulose for 30 min at 4 °C. The non-poly(A)-containing RNA was removed by washing the column with 60 ml of the Tris-NaCl buffer. The column was then equilibrated for 1 h at 37 °C and the poly(A)-containing RNA eluted with 30 ml of 10 mM Tris-HCl (pH 7.5). One-milliliter fractions were collected throughout, and the elution characteristics were determined by radioactivity of an appropriate aliquot. The radioactivity detected in the high salt wash fractions was primarily due to [^{3}H]ATP, which had coprecipitated with the RNA. Either acid precipitation or processing on DE81 filters eliminated the radioactivity in these fractions. Fractions corresponding to poly(A)-containing RNA were pooled and either precipitated with ethanol or lyophilized.

Digestion with Nucleases. One-half of the poly(A)-containing RNA obtained after poly(U)-cellulose chromatography was incubated with 20 μg of pancreatic RNase plus 150 units of RNase T_1 in buffer containing 0.1 M NaCl and 1 mM $MgCl_2$ in a total volume of 0.35 ml. After 20 min at 37 °C,

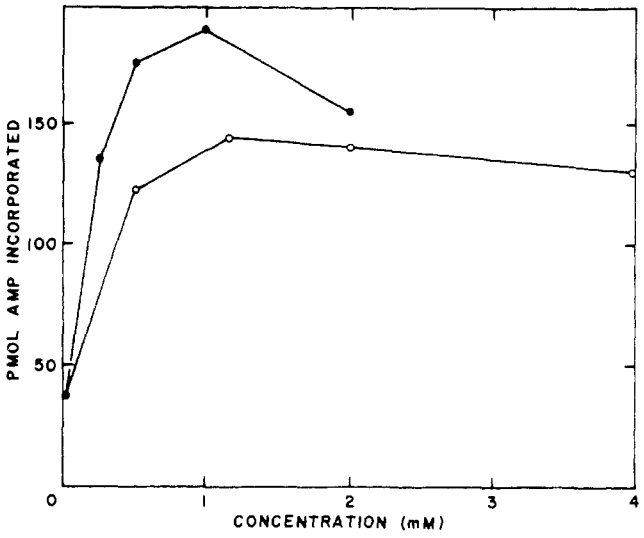

FIGURE 1: Effect of Mn^{2+} or Mg^{2+} on poly(A) synthesis by isolated mitochondria. Reaction mixtures containing 770 μg of mitochondrial protein and the divalent ion as indicated were incubated as described in Materials and Methods using standard conditions for poly(A) synthesis. (●) Mn^{2+}; (○) Mg^{2+}.

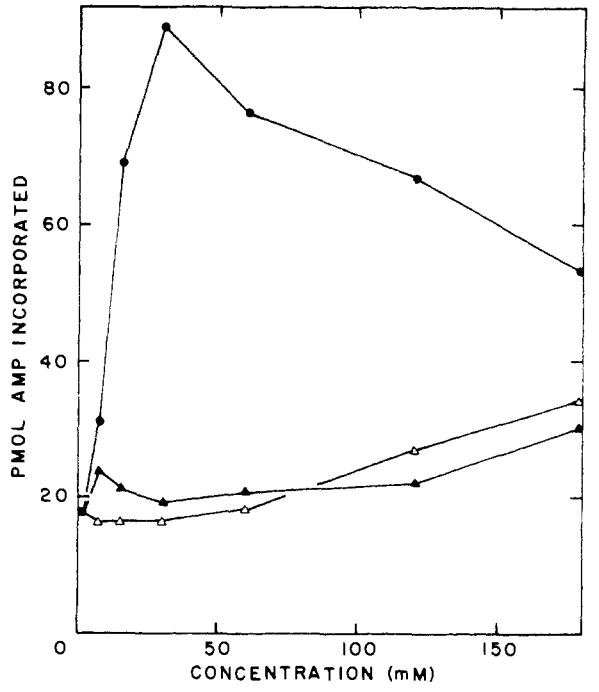

FIGURE 2: Effect of salt upon poly(A) synthesis by isolated mitochondria. Poly(A) synthesis was measured under the conditions described in Materials and Methods using 350 μg of mitochondrial protein and replacing KCl and NaF with the salt as indicated. (●) NaF; (△) NaCl; (▲) KCl.

chymotrypsin (2 μg) was added and the sample was incubated further for 4 min at 37 °C. Degradation products were removed by passing the mixture through a Sephadex G-10 column (0.9 × 14 cm). The polynucleotide fraction was then concentrated by lyophilization prior to gradient analysis.

Digestion of RNA in whole mitochondria prior to in vitro poly(A) synthesis was carried out in individual reactions of 50 μl containing 4 mM Tris-HCl (pH 7.4), 4 mM EDTA, whole mitochondria representing 200–300 μg of protein, 40 μg of pancreatic RNase, and 300 units of RNase T_1. After 20 min at 37 °C, samples were diluted to 120 μl with the appropriate cofactors, and poly(A) synthesis was determined as described above. Control samples containing buffer alone were also processed.

For nuclease digestion of the product synthesized in vitro, several reaction mixtures were pooled, heated at 100 °C for 2 min to terminate the reaction and quickly cooled to 0 °C. The solution was clarified by centrifugation at 5000g for 15 min. One hundred percent of the reaction product was located in the supernatant. Digestion by viper venom phosphodiesterase was carried out by addition of 6 units of enzyme/ml and $MgCl_2$ to a final concentration of 5 mM. Degradation after 90 min at 37 °C was measured by spotting 200-μl aliquots onto DE81 filters which were processed as described for poly(A) synthesis. The decrease in radioactivity retained on the filters after incubation relative to zero-time aliquots was taken as an indication of product degradation. Sensitivity of the in vitro product to pancreatic RNase and RNase T_1 was conducted in a similar manner except that the degradation reaction contained 0.1 M NaCl, 3 mM Tris-HCl (pH 7.4), 3 mM EDTA, 20 μg of pancreatic RNase/ml, 150 units of RNase T_1/ml and was incubated for 10 min at 37 °C.

Estimation of Protein. Protein was estimated by the modification of the Lowry procedure as described by Bennett (1967) using purified bovine serum albumin as a standard.

Results

Purity of Mitochondria

In order to eliminate contamination of cytoplasmic components, the mitochondria used for the present studies were prepared by treatment with digitonin. This technique has been reported to remove most of the outer mitochondrial membrane and yet leave the mitochondria functionally intact (Malkin, 1971). In our hands, 1 g of liver yielded 1.2–1.6 mg of mitochondrial protein. The sucrose density gradient centrifugation of RNA extracted from the isolated mitochondria showed distinct species of RNA sedimenting at 13 and 16 S with minimal levels of the cytoplasmic 18S species.

Reaction Characteristics

Dependence on Mn^{2+} and Mg^{2+}. AMP incorporation into oligonucleotides by isolated mitochondria was stimulated by addition of either Mn^{2+} or Mg^{2+}. As shown in Figure 1, product formation was almost completely dependent upon addition of exogenous divalent ion. The presence of Mn^{2+} stimulated AMP incorporation fivefold at the optimal concentration of 1.0 mM. Mg^{2+} could replace Mn^{2+} and, at its optimal concentration of 1 mM, stimulated product formation more than threefold relative to control. The activity in the presence of both Mn^{2+} and Mg^{2+} (0.5 mM each) was 0.15 nmol of AMP incorporated, a value intermediate between that observed for either Mn^{2+} or Mg^{2+} alone (0.17 and 0.12 nmol of AMP incorporated, respectively).

Effect of Salt and pH. Increasing concentrations of NaF, NaCl, and KCl were added to isolated mitochondria and product formation was measured. Figure 2 shows that NaF significantly increased detection of oligonucleotide synthesis. A fivefold stimulation was observed at 30 mM. NaCl and KCl could also stimulate product recovery, although the major effect of these two salts was observed at higher concentrations. When increasing concentrations of KCl were added to reaction mixtures containing 30 mM NaF, only a slight stimulation was observed. Since we have previously shown nuclease activity to be present in mitochondrial extracts (Jacob and Schindler, 1972) and a poly(A) degrading nuclease from mitochondria

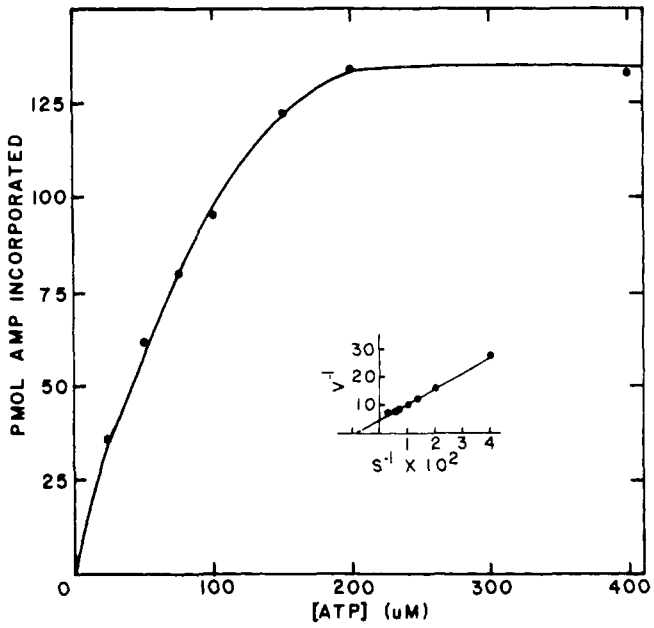

FIGURE 3: Effect of ATP concentration on poly(A) synthesis by isolated mitochondria. Poly(A) synthesis was measured using standard conditions as described in Materials and Methods. Individual reaction mixtures contained 430 μg of mitochondrial protein and the ATP concentration as indicated. The specific activity of [^{3}H]ATP was from 8000 to 500 cpm/nmol.

TABLE I: Effect of Various Compounds on Poly(A) Synthesis by Isolated Mitochondria.[a]

Addition	Concn	Act. (%)
		100
Cordycepin 5′-triphosphate	60 μM	71
	120 μM	66
	240 μM	59
	480 μM	45
Atractyloside	100 μM	64
N-Ethylmaleimide	0.5 mM	21
	10 mM	11
Sodium pyrophosphate	1 mM	21
Sodium phosphate	1 mM	100
Minus phosphoenolpyruvate and pyruvate kinase		32
α-Amanitin	150 μg/ml	88
Rose Bengal	30 μg/ml	61
	300 μg/ml	0
Rifamycin	500 μg/ml	93
AF/013	500 μg/ml	59

[a] Poly(A) synthesis was carried out using the standard assay conditions as described in Materials and Methods with the additions (or deletions) as indicated. One hundred percent activity corresponded to 150 pmol of AMP incorporated/h. Separate control reactions were run for colored compounds (Rose Bengal, rifamycin, AF/013) with addition of the inhibitors at the end of the reaction to account for reduced efficiency of scintillation counting due to color retention on the filters. Rifamycin and AF/013 were dissolved in dimethylformamide and used in 10-μl aliquots. Dimethylformamide itself did not inhibit the reaction.

has been characterized (See and Fitt, 1972), the degradation of radioactively labeled RNA and poly(A) was examined in the presence and absence of NaF, NaCl, and KCl. These results indicated that mitochondria can actively degrade both poly(A) and RNA in the absence of salt. Addition of NaF (30 mM) significantly reduced the degradation of poly(A) with little effect on the RNA. However, the addition of a high concentration of KCl or NaCl was more effective in inhibiting the RNA degradation than the poly(A) degradation (data not shown).

Product formation in isolated mitochondria was maximum in the pH range 7–8. Increase in pH beyond 8 inhibited the reaction substantially.

Reaction Kinetics. Poly(A) synthesis could be detected with varying mitochondrial concentrations ranging from 250 to 1300 μg of protein. At intermediate levels of protein, oligonucleotide synthesis was linear to 60 min and no decline was observed up to 120 min (not shown).

The effect of increasing ATP concentrations upon the reaction velocity is shown in Figure 3. AMP incorporation increased with increasing ATP up to a concentration of 180 μM. The K_m(app) for ATP was 125 μM (Figure 3, insert).

Effect of Inhibitors. Table I shows the result of addition of various compounds upon AMP incorporation in isolated mitochondria. Cordycepin 5′-triphosphate (3′-deoxy-ATP) was an effective inhibitor of product synthesis. Fifty-five percent inhibition was observed at 480 μM. Atractyloside, a compound which inhibits the transport of adenine nucleotides across the inner mitochondrial membrane (Winkler et al., 1968), also produced inhibition of product formation. A concentration of 100 μM was sufficient to produce 36% inhibition, using standard conditions of oligonucleotide synthesis. Reduction of the ATP concentration in the reaction mixture rendered product formation more sensitive to inhibition by atractyloside. The sulfhydryl binding agent *N*-ethylmaleimide inhibited AMP incorporation by 79% at 0.5 mM with virtually complete inhibition being observed at 10 mM. Addition of sodium pyrophosphate (1 mM) almost completely eliminated product formation. Sodium phosphate (1 mM) did not alter product formation. However, some inhibition was seen at higher concentrations of phosphate. Deletion of phosphoenolpyruvate and pyruvate kinase resulted in a 68% decrease in activity.

The mushroom toxin, α-amanitin, which inhibits nuclear RNA polymerases II and III (Jacob et al. 1970a,b; Kedinger et al., 1970; Lindell et al., 1970; Weinman and Roeder, 1974), showed only a slight inhibition of product formation even at relatively high concentrations. Rose Bengal (tetrachlorotetraiodofluorescein), an inhibitor of bacterial RNA polymerase (Wu and Wu, 1973), was also an effective inhibitor of AMP incorporation by isolated mitochondria. Although the protein concentration in the reaction was fairly high (310 μg/ml), this dyestuff inhibited product formation partially at 30 μg/ml and completely at 300 μg/ml. Rifamycin, the inhibitor of bacterial RNA polymerase (Hartman et al., 1967), produced little effect on AMP incorporation by isolated mitochondria. Rifamycin derivatives have been previously shown to inhibit partially purified poly(A) polymerase from liver mitochondria (Jacob and Rose, 1974; Jacob et al., 1974a). The rifamycin derivative AF/013 (3-formylrifamycin SV: *O*-*n*-octyloxime) could also inhibit product formation in isolated mitochondria (41% reduction at 500 μg/ml). It should be noted that the interpretation of the effect of various compounds to a complex system such as isolated mitochondria may be extremely difficult since such compounds may interact at sites distinct from poly(A) synthesis.

Characterization of the Product. The product synthesized by isolated mitochondria in the presence of [^{3}H]ATP was shown to be poly(A) by its resistance to digestion by pancreatic RNase combined with RNase T_1, and by its complete retention on poly(U)-cellulose. AMP incorporated into poly(A) was completely sensitive to digestion by snake venom phosphodi-

TABLE II: Characteristics of Product Synthesized by Isolated Mitochondria in the Presence of ATP.[a]

Treatment	Act. (% of control)
Before reaction	
Ethidium bromide (50 μg/ml)	104
Actinomycin D (20 μg/ml)	94
DNase I (5 μg/ml)	95
After reaction	
Pancreatic RNase + RNase T_1	92
Viper venom phosphodiesterase	0
Poly(U)-cellulose chromatography	
High salt wash	1
Low salt wash	99

[a] Additions to poly(A) synthesizing reaction were carried out by preincubating isolated mitochondria with the additions as indicated for 20 min at 0 °C followed by addition of cofactors and determination of pmol of AMP incorporated into oligonucleotide as described in Materials and Methods. One hundred percent activity equals 150 pmol of AMP incorporated. Digestion of the product with nucleases was carried out on the reaction product from several standard reaction mixtures by heating, quickly cooling, and centrifugation as described in Materials and Methods. One hundred percent activity corresponded to 100 pmol of AMP incorporated into oligonucleotide. Poly(U)-cellulose chromatography was carried out on RNA extracted with sodium dodecyl sulfate and phenol from mitochondria after the poly(A) synthesizing reaction in vitro as described in Materials and Methods. Activity refers to cpm in trichloroacetic acid precipitable fraction. From 120 mg of mitochondria, 100% activity corresponded to 20 000 cpm.

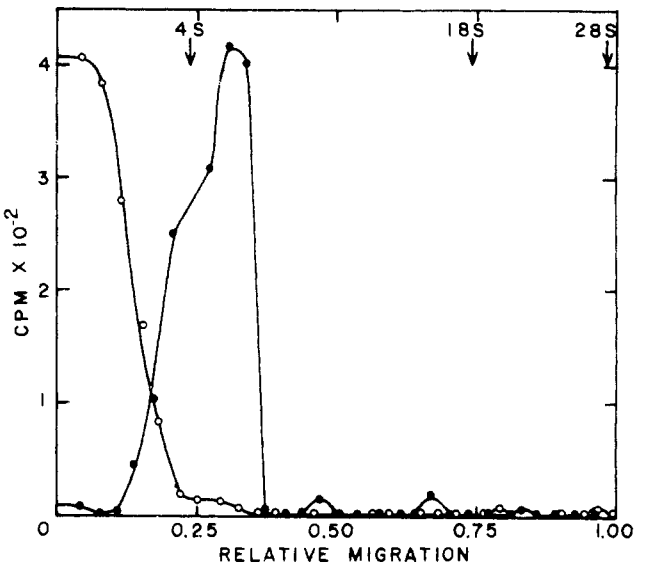

FIGURE 4: Sedimentation analysis of poly(A) containing RNA labeled in vitro by isolated mitochondria. Poly(A) containing RNA was obtained after polyadenylation in vitro and chromatography on poly(U)-cellulose as described in Materials and Methods. In order to prevent aggregation, the fraction was heated at 70 °C for 5 min and quickly cooled (Ojala and Attardi, 1974). One-half of the sample was treated with pancreatic RNase and RNase T_1, followed by chymotrypsin as described in Materials and Methods. The remaining 50% was applied to the gradient without further treatment. Centrifugation was carried out on 5–20% sucrose gradients in buffer containing 0.1 M NaCl, 20 mM NaOAc (pH 5.1), and 1 mM EDTA for 18 h at 38 000 rpm in the SW 40.1 rotor. Fractions (0.5 ml) were collected, scintillation cocktail (Bray's solution) was added, and radioactivity was determined in a liquid scintillation counter. Reaction product before (●) and after (○) nuclease digestion. Arrows indicate the position of cytoplasmic RNA markers run in parallel gradients.

esterase as would be expected for poly(A). Furthermore, resistance to inhibition by actinomycin D, ethidium bromide, or DNase indicated that the polyadenylation reaction was template independent. The above data are summarized in Table II.

The average chain length of the poly(A) synthesized in vitro was shown to be 23 nucleotides. The chain length was determined by incubating standard reaction mixtures with [^{14}C]-ATP for 45 min. After acid precipitation, the product was hydrolyzed for 18 h with 0.4 N KOH and subjected to thin-layer chromatography on poly(ethylenimine)cellulose (Randerath and Randerath, 1967). The sample was eluted in the first direction with glass-distilled water to separate the adenosine from the charged species, followed by chromatography in the second direction with 2.0 M LiCl. Adenosine, now separate from AMP, migrated with a R_f of 0.6 and had radioactivity corresponding to 80 cpm above background. AMP had an R_f of 0.7 and 1773 cpm. Less than 1% of the total cpm comigrated with the tetraphosphate (R_f 0.5), indicating little or no unprimed synthesis. No radioactivity was detected anywhere else on the chromatogram. The results of another experiment yielded an average chain length of 20 with 250 cpm with adenosine and 4700 cpm with AMP.

Covalent Attachment of Poly(A) to Preexisting RNA. We have previously demonstrated that poly(A) polymerase extracted and partially purified from liver mitochondria requires a polynucleotide primer for its activity (Jacob et al., 1974b). To test whether poly(A) synthesis in isolated mitochondria was dependent upon RNA already present in the organelle, mitochondria were incubated with pancreatic RNase plus RNase T_1, prior to addition of cofactors for poly(A) synthesis. Pretreatment of the mitochondria with the nucleases resulted in a 66% reduction in polyadenylation relative to control (5 and 15 pmol AMP, respectively). Under the conditions employed, neither exogenous [^{3}H]poly(A) nor the product of the reaction was degraded. Since some RNA is already polyadenylated prior to isolation of mitochondria, the poly(A) synthesis observed after nuclease treatment could be due to chain extension of preexisting poly(A) fragments.

The covalent attachment of the newly synthesized poly(A) to RNase-sensitive endogenous RNA was investigated as follows: Total RNA was extracted from mitochondria following incubation with [^{3}H]ATP and the other cofactors for poly(A) synthesis. The [^{3}H]poly(A)-containing RNA was separated from the bulk of the extracted RNA by poly(U)-cellulose chromatography, as described in Materials and Methods. One-half of this RNA was digested with pancreatic RNase and RNase T_1 (see Materials and Methods). The nuclease-treated and untreated fractions were then subjected to sucrose density gradient centrifugation. As shown in Figure 4, the untreated [^{3}H]poly(A)-RNA sedimented from 4 to 7 S. After nuclease treatment, the sedimentation rate was significantly reduced (3 S and less). This finding is consistent with the contention that the majority of the poly(A) synthesized in vitro is of short length and is covalently attached to preexisting RNA.

Discussion

The present studies demonstrate that isolated mitochondria are capable of adding a poly(A) sequence to the endogenous RNA. The product of the reaction was shown to be poly(A) by its insensitivity to combined treatment with pancreatic RNase and RNase T_1, its degradation by venom phosphodiesterase, and by its complete retention on poly(U)-cellulose.

Detection of poly(A)-synthesizing activity in the isolated mitochondria was facilitated by inclusion of NaF in the reaction mixture. Previously, NaF has been shown to inhibit poly(A)-degrading nuclease activity present in the nuclei (Lazarus and Sporn, 1967). Our studies suggest that NaF is also an effective inhibitor of poly(A)-degrading nuclease known to be present in the mitochondria (Jacob and Schindler, 1972; See and Fitt, 1972).

Several factors indicate that the observed polyadenylation reaction is truly of mitochondrial origin. (1) The mitochondria were treated with digitonin to remove the outer membrane and the bulk of the cytoplasmic contaminants. Thus, mitochondrial preparations were only minimally contaminated with cytoplasmic RNAs and sedimentation profiles were comparable to the data given for mitochondria prepared by similar methods (Malkin, 1971). (2) Even though small amounts of cytoplasmic RNA were detected in the mitochondrial fraction, it is unlikely that even the presence of small quantities of cytoplasmic ribosomes could lead to contamination by cytoplasmic poly(A) polymerase since we have been unable to detect any poly(A) polymerase activity associated with rat liver ribosomes. Extranuclear enzyme activity appears to be present essentially in the postmicrosomal supernatant (Rose et al., 1976a) and in the membrane portion of the microsomal fraction (Rose and Jacob, 1975). Digitonin treatment can release the membrane-associated poly(A) polymerase (but not the mitochondrial enzyme), thus virtually eliminating contamination of the mitochondrial preparation by cytoplasmic poly(A) polymerase. (3) The polyadenylation reaction was at least partially inhibited by atractyloside which prevents transport of adenine nucleotides across the inner mitochondrial membrane (Winkler et al., 1968).

Synthesis of the poly(A) tract appeared to be dependent on the preexisting RNA in the mitochondria and was covalently attached to it, as shown by a significant decrease in the polyadenylation following pretreatment of the mitochondria with the nucleases and by a reduction in the size of the product after the combined treatment with pancreatic RNase and RNase T_1. It is not yet known whether the poly(A) added in vitro is attached to RNA which has not been polyadenylated or whether the poly(A) tract is added onto RNA which already contains a very short poly(A) segment. Since both de novo synthesis of nuclear poly(A) and additional cytoplasmic polyadenylation have been reported (Brawerman and Diez, 1975), it is possible that one or both of these processes are occurring in isolated mitochondria.

The presence of a polyadenylating system in rat liver mitochondria is not an unexpected finding since we have previously demonstrated poly(A) polymerase activity extracted from liver mitochondria (Jacob and Schindler, 1972; Jacob et al., 1974b). The endogenous primer for the enzyme is at least partially retained in these extracts but is dissociated from the enzyme by chromatography on phosphocellulose (Jacob et al., 1974b). Furthermore, the existence of poly(A) tracts attached to mRNA from HeLa cell mitochondria has been demonstrated in two laboratories (Perlman et al., 1973; Ojala and Attardi, 1974; Hirsch and Penman, 1974). The size of the poly(A)-containing RNA observed after in vitro polyadenylation (4–7 S, see Figure 4) agrees well with the previously reported value for HeLa cells (~7 S). The slightly lower sedimentation value we obtained may be due to anticipated degradation during incubation at 37 °C.

Studies on RNA and protein synthesis in isolated mitochondria indicate that this organelle retains many of its in vivo properties when studied in vitro (Coote and Work, 1971; Lederman and Attardi, 1973; Ibrahim et al., 1973; Poyton and Groot, 1975; Aaij et al., 1970). The present investigation has demonstrated that isolated mitochondria are also capable of posttranscriptional modification of RNA (presumably messenger RNA) by adding a poly(A) tract to it. These data complement our studies using another organelle system, rat liver nuclei, which were shown to be capable of adding a poly(A) tract to the endogenous RNA in vitro (Jacob et al., 1976a).

These studies have established a basis for future investigations on the characterization of the mitochondrial polyadenylation process in response to physiological stimuli or in neoplastic tissues. We have recently demonstrated that the levels of partially purified mitochondrial poly(A) polymerase from the rat liver mitochondria are dramatically altered in response to amino acid supply (Jacob et al., 1976b). It would be interesting to investigate whether such effects can also be observed in isolated mitochondria containing both poly(A) polymerase and the native RNA primer.

Note Added in Proof

Following submission of this manuscript, polyadenylation of RNA in isolated mitochondria from Ehrlich ascites cells has been reported (Aujame and Freeman, 1976).

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Alkylation of Ribose in RNA Reacted with Ethylnitrosourea at Neutrality†

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ABSTRACT: Ribose oxygens in TMV-RNA are ethylated by the carcinogen ethylnitrosourea in neutral aqueous solution (pH 6.1–7.3). 2′-*O*-Ethyladenosine, 2′-*O*-ethylguanosine, 2′-*O*-ethyluridine, and 2′-*O*-ethylcytidine have been identified as reaction products. The four 2′-*O*-ethyl nucleosides are found in approximately equal amounts and the total extent of ribose alkylation is about 15% of the total ethylation. This finding, in conjunction with earlier results showing that all ring and phosphate oxygens can be ethylated, signifies that every oxygen in RNA or polyribonucleotides can react with ethylnitrosourea. The possible biological significance of ribose alkylation, resulting from chemical rather than enzymatic reaction, is discussed. The preparation of the new derivative 2′(3′)-*O*-ethylguanosine is described.

N-Nitroso compounds, particularly ethylating nitroso compounds, have been shown to alkylate preferentially the oxygens of nucleosides rather than the nitrogens. We have reported that, in RNA and DNA treated with ethylnitrosourea in vitro and in vivo, the phosphodiesters are the major site of ethylation and, of the nucleophilic sites of the bases, the O^6 of guanine is modified to a high extent (Singer and Fraenkel-Conrat, 1975; Sun and Singer, 1975).

Cytidine in neutral aqueous solution is alkylated on the O^2, with the ethylating agents being more effective than the analogous methylating ones (Singer, 1976). All nucleophilic centers of the uracil ring in poly(U)[1] (O^2, N-3, and O^4) are found reactive when treated with carcinogenic alkylating agents at neutrality; the ratio of O/N alkylation is about 300 when ethylnitrosourea is used (Kuśmierek and Singer, 1976b). In addition to the ring alkylation, phosphodiesters and ribose are alkylated in poly(U) (Kuśmierek and Singer, 1976b).

The finding that the ribose-OH group is also susceptible to

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[1] Abbreviations used are: Et, ethyl; Ado, Guo, Cyd, and Urd are the four ribonucleosides; O′ indicates ribose substituted on the 2′(3′) position; poly(U), poly(uridylic acid); uv, ultraviolet; EDTA, ethylenediaminetetraacetic acid.