POLY ADENYLIC ACID SEQUENCES IN MITOCHONDRIAL RNA.

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SUMMARY

RNA isolated from a mammalian mitochondria was investigated for Poly-A sequences. 34-39% of mitochondrial RNA was found to contain Poly-A as tested by two different techniques. The Poly-A sequences are 150-180 nucleotides (4-5S) long. The possibility of poly-A containing RNA being a cytoplasmic contaminant was excluded by treating mitochondria with RNAse and digitonin.

INTRODUCTION

RNA biosynthesis in mitochondria has been one of the most active fields of research during the last five years. Data reported in various systems clearly show that mitochondrial DNA codes for mitochondrial rRNA's and many of the tRNA species (1). Nevertheless, it is not certain if there is a transport of mRNA from the nucleus to the mitochondria. Almost all mammalian mRNA's (excepting the histone mRNA's) are known to contain poly adenylic acid sequences (Poly-A) covalently linked with the coding regions of the RNA molecules (2-7). As one possible approach to the problem of nuclear mRNA in mitochondria, it was decided to look for Poly-A in mitochondrial RNA.

In this report we show that about 40% of the RNA isolated from the purified mitochondria of Lettrë Ehrlich hypotetraploid ascites cells contain Poly-A sequences, probably, on their 3' terminals.

MATERIALS AND METHODS

Seven day old Lettrë Ehrlich ascites tumor cells grown in the peritoneal cavity of Swiss colony mice (8) were used as the source of mitochondria.

^{32}P Labelling: Cell suspension from 30-40 mice (30-40ml packed cells and 90-120ml ascites fluid) were diluted 3 fold with Krebs Ringer medium containing

no phosphate, and incubated at 37°C for 4 1/2 hrs. At zero time 4 mCi ^{32}P orthophosphate, carrier free (Schwartz and Mann Laboratories, N.Y.) was added. At the intervals of 1, 2 and 3 hrs. 2 mCi each of ^{32}P was added.

Isolation of Mitochondria: Cells were packed at 1,000 x g for 5 minutes, washed twice with ice-cold normal saline and finally suspended in two volumes of 0.25 M sucrose containing 2mM EDTA. Homogenization was carried out using a motor-driven glass homogenizer at 1,200 RPM for 3-4 minutes. Two preliminary centrifugations were carried out at 1,100 x g (4-5°C), one for 5 minutes and the second for 10 minutes in a refrigerated centrifuge (Beta-Fuge" Model A-2, Lourdes Instrument Corp.). Supernatant from these runs was centrifuged at 7,500 x g for 15 minutes. The crude mitochondrial pellet thus obtained was suspended in 3 times its volume of 0.25M sucrose containing 5 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.5), and 40 mM KCl, and incubated with 2ug/ml DNAse (Worthington Biochemical Corp.) for 30 minutes on ice. Mitochondria were pelleted at 7,500 x g for 15 minutes and washed 3 times with 0.25 M sucrose containing 2 mMEDTA.

RNA Isolation: RNA was extracted by a procedure modified from Warren et al(9). Mitochondrial pellet was suspended in 6 volumes of RNA-extraction buffer (0.01 M acetate buffer pH 6.0; 0.5% Naphthalene Disulfonic Acid, disodium salt; 0.1% Macaloid; 0.04% Poly Vinyl Sulfuric Acid; and 0.2% Sodium Dodecyl Sulfate.). Two volumes of Phenol-Chloroform (1:1) containing 0.1% 8-hydroxyquinoline (Sigma) was added and the mixture was shaken at 55°C for 10 minutes. The aqueous phase was separated from the phenolic phase by centrifugation at 10,000 x g for 10 minutes, and was removed by aspiration. The interphase was re-extracted with RNA-extraction buffer and Phenol:Chloroform as above and the aqueous phase recovered. Two aqueous phases from the first and the second extraction steps were pooled and treated with Phenol:Chloroform two times. RNA from the aqueous phase was precipitated with 2 volumes of 70% Ethanol containing 3.0% Potassium Acetate at -20°C for 12-18 hrs. The RNA precipitate was washed three times with 70% Ethanol and the resultant pellet was dissolved either in the phosphate buffer (10 mM Sodium Phosphate, pH 6.8 and 1 M NaCl) at a concentration of

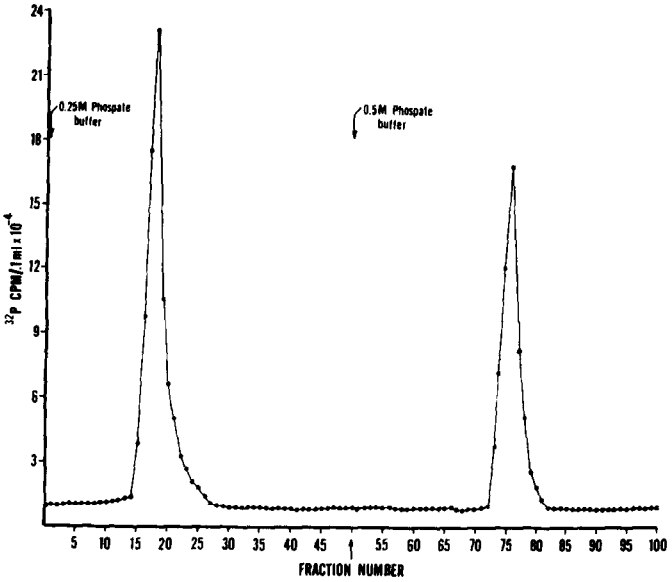

Fig. 1 Separation of Poly-A containing RNA from the bulk RNA by hybridization with Poly-U. About 400 µg of ^{32}P labeled mitochondrial RNA was hybridized with 200 µg of Poly-U (Miles Research Laboratories) at room temperature as described by Greenberg and Perry (11). The ribonucleic acid was adsorbed on 2 gm of hydroxylapatite (Bio Rad, HTP) and mixed with 2.0 gm of cellulose powder (course grade; W & R Balston, Ltd.) also as described (11). The mixture was transferred into a column and was washed with 100 ml each of 0.25 M and 0.5 M phosphate buffer (sodium phosphate, pH 6.8) containing 0.1 M NaCl and 0.1% Sarkosyl (K & K Laboratories) as indicated on the graph. 100 fractions of 2.0 ml each were collected and 0.1 ml of each fraction was tested for radioactivity. Other details were as described by Greenberg and Perry (11).

0.25 mg/ml or in the Tris buffer (25 mM Tris-Cl, pH 7.5, 40 mM KCl and 1 mM $MgCl_2$) at a concentration of 2.0 mg/ml. RNA thus obtained from ^{32}P labelled cells had a specific activity of 70-90,000 CPM/ug.

RESULTS AND DISCUSSION

Poly-A containing RNA's are known to bind to nitrocellulose filters at high salt concentrations (4, 5, 10). Also, a portion of RNA which binds to the filters has been known to be resistant to ribonuclease digestion (4). The ribonuclease resistant portion is now known to be Poly-A (4-7, 10). When ^{32}P labelled mitochondrial RNA is brought in contact with the Millipore filters at 0.5M NaCl as described by Lee et al (4), about 30-34% of acid-insoluble counts were retained by the filter. About 14% of the filter-bindable counts are

TABLE -1

Detection of Poly-A by Millipore-binding technique

RNA treated with	^{32}P counts bound to the filter (%)
None	100
RNAse- A + T1	14
Venom Diesterase	1.2
RNAse-A + T1 and Venom Diesterase	0.9

RNA samples in the Tris buffer as described in the Materials and Methods were incubated at 37°C for 30 min. with or without added enzymes, diluted 30 fold with a buffer containing 25 mM Tris-Cl (pH 7.5), 0.5M NaCl and 1 mM $MgCl_2$, and filtered through Millipore filters as described (6). RNAse treatment was as described in Fig. 2. Reaction with Venom phosphodiesterase (Worthington Biochemical Corporation) was according to Keller (16).

resistant to RNAse (Table 1). Recently, a highly quantitative and unequivocal method for detecting Poly-A has been reported by Greenberg and Perry (11). In this method, the Poly-A portion of the RNA is hybridized with Poly-U and the resultant hybrid is separated from the single-stranded structures using hydroxylapatite chromatography (11). When this technique is used with the mitochondrial RNA, approximately 61% of the ^{32}P counts are eluted out as single stranded RNA and the remaining 39% of the counts eluted out as hybrids (Fig. 1). When checked for RNAse-effect, over 98% of the counts eluted with 0.25 M phosphate buffer (Fig. 1), were sensitive, while 14-16% of the counts eluted with 0.5 M phosphate buffer were resistant, indicating a quantitative separation of the Poly-A containing RNA.

When a ribonuclease-digest of ^{32}P labelled mitochondrial RNA was electrophoresed on poly acrylamide gels (6,15), the main peak of radioactivity was slightly behind the 4S peak (see Fig. 2). These results clearly show that about 39% of the RNA populations from purified mitochondria contain Poly-A

Table -2

Effect of various treatments on the Poly-A content of mitochondrial RNA.

Mitochondria treated with	^{32}P counts retained on the filter (%)
None	34.3
RNAse	29.8
Digitonin	31.4

Mitochondrial pellet was suspended in 4 times its volume of 0.25 M sucrose containing 2 mM EDTA and mixed with RNAse A and RNAse T1 at final concentrations of 10 and 5 µg/ml respectively. Incubation was carried out at room temperature for 20 min. After treatment with RNAse, mitochondria were washed twice with 0.25 M sucrose containing 2 mM EDTA. Treatment of mitochondria with digitonin (Sigma) was carried out as described by Schnaitman and Greenwalt (13). Other details were as described in the Materials and Methods and in Table 1.

sequences of 150-180 nucleotides. Further, the Poly-A is probably situated at the 3' terminals of the RNA molecules, since both the Poly-A containing RNA's and the Poly-A sequences obtained after RNAse digestion of the RNA's are completely sensitive to Venom diesterase (Table 1). This is possible only if the Poly-A is on the 3' end.

It is unlikely that the Poly-A containing RNA isolated from the mitochondrial preparations results from contamination with bytoplasmic polysomes since the Poly-A content is unaltered after the mitochondria were subjected to treatments with RNAse, and digitonin (Table 2). RNAse should have served to degrade any cytoplasmic polysomal RNA contaminating mitochondria (12). Treatments with digitonin drastically reduce (12, 13) the contaminating endoplasmic reticulum in mitochondrial preparations.

It is not yet, however, known if Poly-A containing RNA's from mitochondria are of nuclear or mitochondrial origin. Since it is established that prokaryotes do not generally contain Poly-A in their RNA (14), it is very important to investigate the origin of the Poly-A containing RNA in mitochondria. This might also throw light on the origin of these organelles.

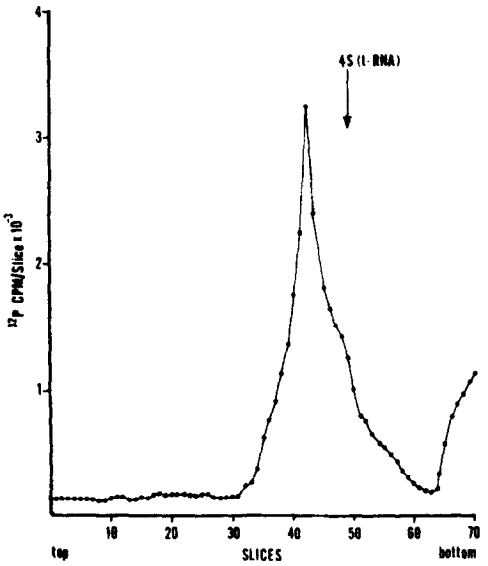

Fig. 2 Electrophoretic pattern of the RNAse-resistant portion of Mitochondrial RNA. ^{32}P labeled mitochondrial RNA was treated with 2 µg/ml RNAse A and 1 µg/ml RNAse T1 (both purchased from Worthington Biochemicals Corp.) in a medium containing 25 mM Tris-Cl (pH 7.5), 40 mM KCl, 1 mM $MgCl_2$. After 30 min. incubation at 37°C, the mixture was dialysed against distilled water for 12-18 hours. About 36,000 RNAse resistant counts were layered over 6.0% polyacrylamide gels and electrophoresed according to Bishop, Claybrook and Spiegelman (15). The gels were cut into 1mm thick slices and counted.

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