POLY(A)-CONTAINING RIBONUCLEIC ACID IN MITOCHONDRIA FROM RAT LIVER AND KREBS II ASCITIC CARCINOMA CELLS

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Received 27 July 1973
Original figures received 28 August 1973

1. Introduction

There is no unequivocal data concerning the transcriptional origin of mitochondrial messenger RNA [1, 2]. Our previous studies showed that a certain part of mitochondrial rapidly-labelled RNA was complementary to nuclear DNA in hybridization experiments while the other part was of 'autonomous' origin [3, 4]. However, in standard annealing conditions there is a significant contribution of highly reiterated sequences into hybrid formation, which hampered greatly the precise interpretation of results and quantitative determination of the fraction size of mitochondrial mRNA transcribed from nuclear genes [5].

The existence of untranslatable 3′-terminal sequence rich in adenylic acid is one of the characteristic features of different messenger RNA's in eukaryotic cell [6–9]. On the other hand such sequences were not found in bacterial mRNA [10]. Since marked structural and functional similarities exist between bacterial and mitochondrial genomes [11, 12] it could be suggested that transcripts of mitochondrial DNA did not contain A-rich sequences. In this respect the detection of poly(A) in mitochondrial RNA would be one of the proofs of the nuclear origin of this RNA fraction and poly(A) could serve as a 'marker' for isolation of this minor RNA fraction from mitochondria.

The results described in this paper showed that a certain fraction of mitochondrial RNA from animal cells actually contained adenylate-rich sequences.

2. Materials and methods

The experiments were performed with mitochondria isolated from regenerating rat liver and Krebs II ascitic carcinoma cells. Regenerating liver was taken 30 hr after partial hepatectomy. Ascites cells were used 7–8 days after intraperitoneal inoculation.

Rat liver mitochondrial RNA was isolated 2 hr after intraperitoneal injection of carrier-free $H_3{}^{32}PO_4$ (5 mCi per rat) with or without [^{3}H]adenine (1 mCi per rat). Krebs II cells were incubated aerobically at 30°C for 3 hr in minimal phosphate-free Earle medium containing 100 μCi/ml $H_3{}^{32}PO_4$.

Mitochondria were isolated from postnuclear supernatants of cell homogenates by isopicnic centrifugation (d = 1.612 g/ml) in linear sucrose density gradients. The mild digitonin treatment was used as an additional purification step removing microsomal contaminants [13].

Mitochondrial RNA was isolated using phenol-chloroform mixture according to Perry et al. [14].

The resolution of poly(A)-containing RNA and poly(A)-free RNA was achieved by: i) Millipore retention technique [15] or ii) hydroxyapatite chromatography after annealing with polyuridylic acid [9]. The proportion of adenylate-rich sequences in RNA fractions was detected as ribonuclease-resistant radioactivity after hydrolysis (pancreatic RNAase, 50 μg per 1 ml; T_1 RNAase, 200 units per ml, 37°C, 60 min). The sizes of RNAase-resistant fragments were determined by sucrose concentration gradient centrifugation using yeast transfer RNA as a marker.

RNA radioactivity was measured after precipitation

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Table 1
Poly(A)-containing RNA in mitochondria from regenerating rat liver and Krebs II ascitic carcinoma cells.

[^{32}P]RNA fractions	Source of mitochondria	
	Regenerating liver	Krebs II cells
Total RNA	100	100
Millipore-retained fraction	51.9	79
RNAase-resistant fraction	15.0	35

Labelling conditions, RNA preparation and fractionation – see 'Methods'. Radioactivity was expressed as a proportion of total RNA radioactivity taken for 100%.

with cold 5% trichloroacetic acid onto nitrocellulose filters. ^{3}H- and ^{32}P-radioactivity was counted in toluene scintillation mixture PPO–POPOP in a 'Nuclear Chicago' liquid scintillation counter.

3. Results

RNAase treatment of mitochondrial RNA revealed the existence of RNAase-resistant fraction constituting about 15% of the initial [^{32}P]RNA radioactivity (table 1).

Millipore filtration experiments demonstrated rather a high proportion of mitochondrial RNA retained on filters (70% and 52% for mitochondrial [^{32}P]RNA from Krebs II cells and from rat liver respectively). Thus a significant fraction of mitochondrial rapidly-labelled RNA contained adenylate-rich sequences.

These sequences were identified in further experiments with poly(U) hybridization followed by hydroxyapatite chromatography. It can be seen (fig. 1) that untreated RNA eluted from columns with low

Table 2
^{3}H/^{32}P ratio in ^{32}P-orthophosphate- and ^{3}H-adenine-labeled mitochondrial RNA from regenerating rat liver.

RNA fraction	^{3}H/^{32}P ratio
Total RNA	2.0
Millipore-retained RNA	4.0
Millipore-filtered RNA	1.6
RNAase-resistant RNA	8.5

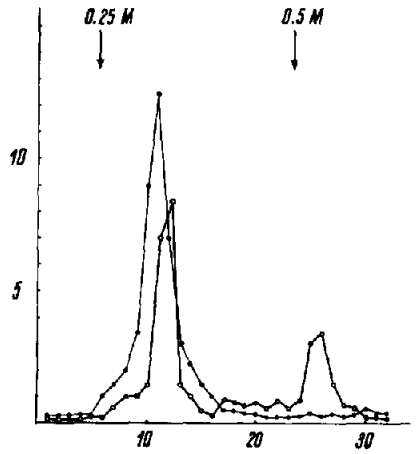

Fig. 1. Isolation of poly(A)-containing RNA of rat liver mitochondria on hydroxyapatite. Mitochondrial [^{32}P]RNA (200 000 cpm) was annealed with 100 μg/ml poly(U) in 1 M sodium chloride–0.01 M sodium phosphate buffer, pH 6.8–0.1% sacrosile for 60 min at 23°C. Incubation mixture was then diluted 10-fold with solution containing 0.2 M sodium chloride, 0.01 M phosphate buffer and 0.1% sarcosile and applied onto a hydroxyapatite-cellulose column prepared according to Greenberg and Perry [9]. Fractions (2.0 ml each) were obtained by stepwise elution with phosphate buffer of increasing concentration (incubated by arrows). Abscissa' fraction numbers; Ordinate: acid-insoluble ^{32}P-radioactivity (cpm, 10^{-4}), (○—○—○) [^{32}P]RNA annealed with poly(U); (●—●—●) [^{32}P]RNA annealed without poly(U).

Table 3
The effect of actinomycin D and ethidium bromide on the synthesis of mitochondrial poly(A)-containing RNA in Krebs II cells.

Incubation conditions	Radioactivity of mitochondrial RNA fractions		
	Total	Millipore-retained	RNAase-resistant
Control	100	79	35
Actinomycin D, 5 μg/ml	100	58	28
Ethidium bromide 1 μg/ml	100	32	21

Cells were pre-incubated with inhibitors for 3 hr and then incubated with $H_3{}^{32}PO_4$ for three more hours. All the samples (including control) contained 0.04 μg/ml actinomycin D to suppress rRNA synthesis. The values are expressed as a proportion of the radioactivity in the RNA fraction (the total radioactivity of RNA from a given sample being taken for 100%).

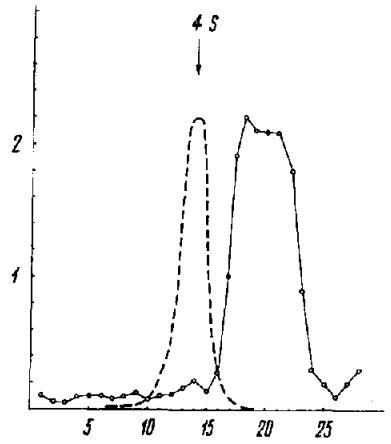

Fig. 2. Sedimentation profile of RNAase-resistant fragments of [^{32}P]RNA from Krebs II cell mitochondria. [^{32}P]RNA after ribonuclease treatment (see 'Methods') was deproteinized with phenol–chloroform mixture at pH 6.0 and precipitated from aqueous phase with 2 vol of ethanol and yeast tRNA as a carrier. RNA precipitate was dissolved in 0.3 M sodium chloride–0.03 M sodium citrate (2 × SSC) and layered upon linear sucrose density gradient (5–20%, W/W) made on 2 × SSC. The gradients were centrifuged in Spinco L2 ultracentrifuge, rotor SW50, at 49 000 rpm for 5 hr at 4°C. Abscissa: fraction numbers (from bottom); Ordinate: 2-acid-insoluble radioactivity (desintegrations per minute $\times 10^{-2}$); The position of marker (4 S RNA) is indicated by arrow.

ionic strength phosphate buffer while after poly(U) hybridization a significant RNA fraction (36%) was firmly adsorbed onto columns and eluted only with high ionic strength buffer.

In some experiments mitochondrial RNA was labelled in vivo with $H_3{}^{32}PO_4$ and [^{3}H]adenine and ^{3}H/^{32}P ratio was measured in total RNA preparations, in Millipore-retained and Millipore-filtered fractions and in the RNAase-resistant fraction. The data presented in table 2 show that the ^{3}H/^{32}P ratio for Millipore-retained fraction was twice as high as for total RNA while this ratio was somewhat lower for Millipore-filtered fraction. Four-fold increase of ^{3}H/^{32}P ratio was found for RNAase-resistant fragments in comparison to total mitochondrial [^{3}H,^{32}P]RNA. Thus Millipore-retained RNA and especially RNAase-resistant acid-insoluble material are markedly enriched in adenylic acid content (see [16]).

The sedimentation profile of ribonuclease-resistant fragments of mitochondrial RNA from Krebs II cells appeared to be rather heterogeneous; the bulk of acid-insoluble radioactive material being somewhat lighter than 4 S-RNA (fig. 2).

A preliminary attempt was undertaken to study the origin of poly(A) in mitochondrial RNA with the aid of specific inhibitors. As can be seen from table 2, both actinomycin D in low doses (unable to penetrate into mitochondria within a cell) and ethidium bromide depressed significantly the synthesis of mitochondrial poly(A)-containing RNA in Krebs II cells.

4. Discussion

The data obtained may be considered as evidence for the presence of poly(A)-containing RNA fraction in mitochondria from regenerating rat liver and from Krebs II ascitic carcinoma cells. This RNA fraction could be identified according to the following criteria: i) the retention on Millipore filters; ii) the annealing with polyuridylic acid revealed by hydroxyapatite chromatography; iii) the increase of ^{3}H/^{32}P ratio in RNAase-resistant and Millipore-retained fractions of mitochondrial RNA which was labelled in vivo with $H_3{}^{32}PO_4$ and [^{3}H]adeninine and iv) the size distribution of RNAase-resistant fragments (< 4 S).

The sensitivity of mitochondrial poly(A)-containing RNA synthesis to low doses of actinomycin D seemed to be an evidence for the nuclear origin of this RNA fraction, which is in agreement with our previous results [4, 5, 17]. Ethidium bromide in our experiments also inhibited partially the synthesis of mitochondrial poly(A)-containing RNA. In this respect our results differ somewhat from those of Perlman et al. [18] which described total ethidium bromide sensitivity. The sensitivity of mitochondrial poly(A)-containing RNA synthesis to ethidium bromide could be considered as evidence for the 'autonomous' origin of this RNA fraction. However, the following circumstances must be taken into account: i) ethidium bromide in low concentrations (1 μg/1 ml) depressed the synthesis of poly(A)-containing mRNA's in cell nuclei [19] and in the cores of vaccinia virus [20]; ii) ethidium bromide-sensitive transcription of the mitochondrial genome could be well a prerequisite for the transport of nuclear poly(A)-containing mRNA into mitochondria.

Thus the transcriptional origin of mitochondrial poly(A)-containing RNA remains to be clarified in further experiments. In particular, preparative scale

isolation of this RNA fraction and its careful hybridization study would be of value.

When our manuscript was in preparation, work appeared [21], in which poly(A)-containing mitochondrial RNA was described. These results are in good agreement with our data.

Acknowledgements

The valuable technical assistance of Miss V.V. Denezkina and Miss O.N. Shalaeva is appreciated.

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