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DETERMINATION OF SOME MITOCHONDRIAL RNAs CONCENTRATION IN ADULT RAT LIVER

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A method has been developed to accurately measure steady state concentrations of mitochondrial transcripts in adult rat liver. Total mitochondrial RNA has been hybridized with an excess of labelled mitochondrial DNA fragments coding for a single gene or a piece of it. The results obtained show that each mitochondrion contains 36 molecules of 16S rRNA, 81 of 12S rRNA and about 8 molecules of mRNAs coding for identified and unidentified reading frames. Thus, the rRNA/mRNA ratio in rat liver differs from that reported in HeLa cell mitochondria. These results are discussed in the light of mitochondrial DNA transcription regulation.

Very little information is available on the regulation of animal mtDNA expression. Data in the literature refer mainly to HeLa cell mitochondria. In these cells both DNA strands are first entirely transcribed and then processed, leading to mature gene products (1). Recently one initiation point for the L-strand transcription and two for the H-strand have been described (2). The two initiation points on the H-strand are probably involved in the rRNA and in the polycistronic mRNA synthesis, respectively. Moreover, Gelfand and Attardi (3) have reported that, in these cells, both mature rRNA and mRNA species are metabolically unstable. In conditions of inhibited transcription, however, the stability of mt mRNA species can change and this may be one of the mechanisms used by the cell to regulate the mt gene expression. The same Authors measured concentration of rRNAs and of mRNAs in HeLa cell mitochondria, by kinetic experiments.

To extend regulation of mtDNA transcription studies to more physiological systems, we decided to determinate the concentrations of rat liver mtRNAs at the steady state. This was made

Non standard abbreviations: mt: mitochondrial; bp: basepair(s); nt: nucleotite(s).

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by hybridizing total mtRNA with specific labelled mtDNA fragments.

The results obtained reveal consistent differences between mitochondrial mRNA species concentrations in HeLa cells and in rat liver.

MATERIALS AND METHODS

Liver mitochondria were isolated from an adult rat (150 g b.w.) through differential centrifugations (4). Care was taken to reduce nuclear contamination by centrifuging the postnuclear supernatant several times at low speed. After lysis in 120 mM NaCl, 10 mM TRIS-HCl pH 7.4, 1 mM EDTA, 1.2% SDS, the nucleic acids were isolated through extraction with phenol-chloroform-isoamylic alcohol (50/49/1) and repeated ethanol precipitations.

The DNA fragments used as probe in the hybridization experiments were isolated by cutting recombinant plasmids containing Eco RI fragments A, B, C, D and E of rat liver mtDNA with the appropriate restriction enzymes. After separation on polyacrylamide gel, the fragments were eluted and ethanol precipitated as previously described (5). Probe labelling was carried out by nick translation (6) for 2 h at 15°C in presence of 75 μCi/mmole of α (^{32}P)dCTP. At the end of the reaction the labelled DNA was isolated by chromatography on a G-50 Sephadex column eluted with 10 mM TRIS-HCl pH 8.0, 1 mM EDTA. The DNA size, determined by urea-polyacrylamide gel electrophoresis, ranged from 200 to 400 bp and its specific activity was around 1.10^7 cpm/μg.

The solution hybridization experiments were done in a volume of 40 μl using the conditions described by Casey and Davidson (7). After incubation (20 h at 48°C) the samples were digested with 100 U/ml of S_1 nuclease (Sigma) for 30 min at 45°C. The amount of hybrid formed was determined by trichloroacetic acid precipitation.

The DNA and RNA concentration was determined by colorimetric methods as diphenylamine and orcinol, or (in case of DNA) using the fluorimetric assay developed by Labarca and Paigen (8). Protein concentration was determined through the Waddel method (9).

RESULTS

The strategy used to accurately measure the steady state concentrations of mitochondrial transcripts was based on the determination of the amount of DNA-RNA hybrids formed by hybridization of a known quantity of total mtRNA with an excess of a radioactive mtDNA fragment coding for a single gene or a piece of it. This could be done, since gene organization of rat liver mtDNA is known (10) and cells of E.coli harbouring the chimeric plasmid pSF2124, containing the Eco RI A, B, C, D and E fragments of rat liver mtDNA were available. Total DNA was extracted from these cells, E.coli and plasmid DNAs were separated on a CsCl gradient and finally probes were obtained by cutting the plasmids with several restriction enzymes. The probes used contained the genes for the two mt rRNA 16S and 12S and for five

mRNA (CoI, CoIII, ATPase-6, URF1 and URF4) so to obtain a representative pattern of all mt transcripts.

As shown in Fig.1, the fragment containing the gene for the 16S rRNA was the 1304 bp long one, deriving from the digestion of pSF2124 (D) with HhaI and Eco RI; the 12S rRNA gene

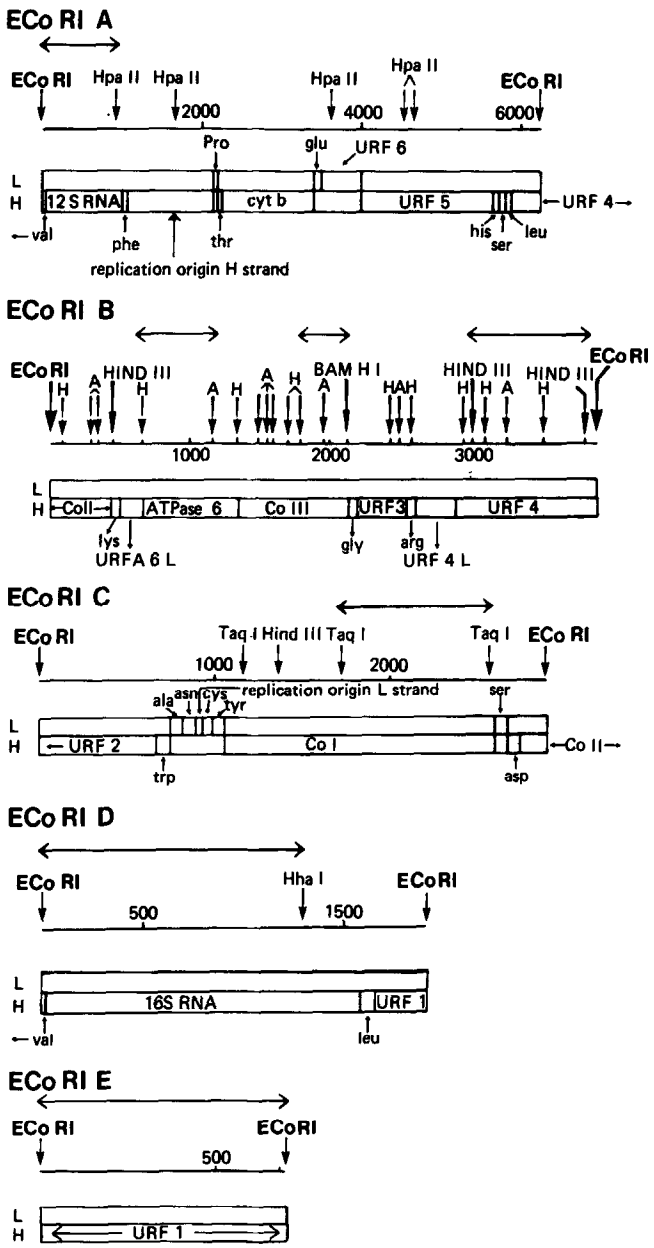

Figure 1. Physical and transcription map of rat liver mtDNA fragments A, B, C, D and E. Only the restriction sites used for probe preparation are shown. A two headed arrow indicates the fragments that were labelled by nick translation. A and H are the Hae III and Hinf I sites.

was contained in the fragment of 934 bp, originated by the digestion of Eco A with HpaII. The CoI gene resulted from the digestion of Eco RI C with Taq I, whereas the URF1 gene was contained in the fragment Eco RI E. The probes for CoIII, ATPase-6 and URF4 have been prepared from the Eco RI B fragment. The URF4 probe is contained in two Hind III sites, whereas the other two, were prepared digesting Eco RI B, first with Hind III and Bam HI and then with Hae III and Hinf I. To determine fragment concentrations, E.coli cells containing plasmids were grown in the presence of ^{3}H-thymidine (0.5 µCi/ml) and the plasmid DNA specific activity (about 1000 cpm/µg) was used to calculate the concentrations of the DNA fragment to be hybridized.

The hybridization experiments were carried out by using total mitochondrial RNA and increasing concentrations of DNA fragments labelled by nick translation, in conditions maximizing the RNA-DNA hybrid formation (7).

The mtRNA preparation used for hybridization also contained the DNA coextracted from the mitochondria with the RNA. It was not eliminated since its amount was used as internal standard in the calculation of the concentration of mtRNA species. Therefore an equivalent quantity of cold DNA was added in the blanks and corrections were made for the specific activity of the radioactive probes. Hybridization reached saturation levels with all the probes used. The pg of RNA hybridized at saturation are reported in Tab.1. These quantities were used for concentration determination of each mtRNA species, by relating them to the amount of mtDNA coextracted from the mitochondrial fraction with the RNA and assuming a mean number of 5 DNA molecules per mitochondrion (11). The results, reported in Tab.1, show that there are 36 molecules of 16S rRNA and 81 molecules of 12S rRNA per mitochondrion. The mRNAs are present at lower levels, ranging from 6 molecules of URF4 mRNA to 16 molecules of CoI mRNA.

This approach to calculate mtRNA concentration is the more direct assuming that the DNA and RNA are coextracted with the same yield from mitochondria and that the DNA present in mtRNA preparation is of mitochondrial origin only. The first assumption was verified by determining the DNA/RNA ratio during nucleic acids extraction from the mitochondria. Furthermore, the DNA extracted from the mitochondria was 1% of that extracted

TABLE I
Determination of the concentration of several mitochondrial RNA species by hybridization

RNA species	RNA ng	DNA hybridized pg	RNA molecules per mitochondrion
12S	10	102 ± 2	81.0 ± 3
	20	225 ± 7	
16S	10	72 ± 4	36.0 ± 4
	13	82 ± 3	
	25	145 ± 8	
URF 1	100	82 ± 4	8.5 ± 0.5
CoI	100	185 ± 17	16.0 ± 2
ATPase-6	100	49 ± 7	7.1 ± 1.2
CoIII	100	38 ± 8	7.3 ± 1.6
URF 4	100	67 ± 2	6.4 ± 0.4
	150	71 ± 2	

The values reported in the third column are those obtained at saturation. They are the mean of at least three experiments far each RNA concentration, run in triplicate. The background was determined by measuring the S_1 resistance of samples incubated without mtRNA. The number of RNA molecules per mitochondrion has been calculated as described in the text.

from the homogenate of the same preparation as reported in literature (11). Finally, the nuclear marker enzyme, RNA polymerase II, was absent in the mitochondrial preparation. Estimates of mtRNA concentration were also made by referring the pmoles of hybridized RNA either to the mitochondrial proteins or to the total cellular DNA. The values obtained, normalized for the losses of mitochondria and RNA occurring during the extraction, were of the same order of magnitude as those reported in Tab.1.

DISCUSSION

In this paper the results obtained by measuring the concentration of the two rRNAs and of five mRNAs in rat liver mitochondria are reported. They show that the concentration of 12S RNA is double than 16S RNA. This difference could be due to the

gene order in rat liver mtDNA: the 12S RNA is in fact immediately adjacent to the transcription initiation point. The concentration of the mRNAs is about one order lower than that of rRNAs. It is about the same for all mRNA measured except for CoI mRNA and it is independent of gene order confirming the polycistronic transcription pattern of mitochondrial mRNA genes.

The only data existing in the literature on the steady state concentration of mitochondrial transcripts have been reported by Gelfand and Attardi (3). These Authors measured the concentration of 12S rRNA and of mRNAs in HeLa cell mitochondria. From the comparison of the results obtained in rat liver and in HeLa cells it comes out that the concentration of 12S rRNA is similar in the two systems whereas the concentration of mRNAs is 10 to 100 times lower in HeLa cells. The concentration of CoI and CoIII mRNAs in the two systems correlate well with their respective cytochrome aa_3 content (12,13); it seems clear therefore that the higher concentration of mRNAs in rat liver is related to the higher respiratory rate of rat liver cells with respect to the HeLa.

Some data suggest that the mtRNA synthesis proceeds in a constitutive fashion. Studies carried out in sea urchin have demonstrated that in spite of the absence of mtDNA replication and mt protein synthesis even synthesis of mtRNA takes place during early development (14-16). In the cerebellum, an organ which in rats develops almost completely after birth, mtDNA and mt protein synthesis peak up at 10 days of age, whereas RNA synthesis remains quite constant along the first three weeks of postnatal life (17,18). By assuming that RNA decays with a kinetic of the first order, its concentration at the steady state is given by the ratio between the rate of synthesis and that of degradation (19). Several Authors have indicated that when mtRNA synthesis is inhibited (20,21) the stabilities of mt mRNA species are increased, suggesting that their steady state concentrations are regulated at decay level. The data on mRNAs reported in this paper might also be explained by this mechanism. In conditions of elevated respiratory rate, as in rat liver, the increased concentration of mitochondrial mRNA could depend on an increase of their stability. A regulation operating at promoter mtDNA level cannot, however, be excluded and only experiments directly addressed to measurement of mtRNA stability may settle

the problem. This model does not apply, however, to the rRNAs since the similarity of rat liver and HeLa cells 12S level suggests that their concentration is independent of cell metabolism. Data from other systems will eventually confirm this possibility. The approach used here could be useful in studying mitochondrial gene expression during development or in different physiological conditions. Concentration measurements of specific mitochondrial transcripts could help in finding out if the increased oxidative phosphorilation observed in rat liver at critical stages of development (22) is due either to the rise of the number of mitochondria per cell or to that of RNA concentration per mitochondrion. The same considerations could apply to the study of thyroid hormone action on mitochondrial biogenesis (23).

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