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THE GENETIC LOCALIZATION OF PRESUMPTIVE MITOCHONDRIAL MESSENGER RNAs ON RAT-LIVER MITOCHONDRIAL DNA

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Summary

High molecular weight mitochondrial (mt) RNAs were isolated from rat liver mitochondria and hybridized in the presence of excess competitor mt rRNA and/or mt tRNA to restriction fragments of mtDNA. The data reveals that there are a few areas of the mt-genome on which the complementary of these presumptive messenger RNAs is most pronounced. These areas are away from the parts of the genome which are coding for the mt rRNA or containing the D-loop.

INTRODUCTION

Mitochondria are heteronomous genetic elements present in all eukaryotic cells. They contain DNA that codes for the RNAs of the organelle ribosomes and a number of specific organelle tRNAs. It is generally believed that mtDNA also contains information for a limited number of mitochondrial proteins. In earlier studies (1) we have constructed the restriction fragment map of rat-liver mtDNA for several restriction endonucleases and localized the ribosomal RNA genes and the origin of replication on this map. In a preliminary study we have further shown that a fraction of mtRNA containing poly(A) stretches hybridizes to various isolated restriction fragments not containing the rRNA genes (2). We have now extended these studies by isolating total high-molecular weight mtRNA and the poly(A) containing fraction thereof, and by hybridizing these RNAs, after iodination in vitro, to filterstrips containing the restriction fragments of mtDNA obtained from various enzymes. Using the same method, we have also attempted to get rough information about the localization of the tRNA genes on the rat-liver mtDNA. The results of these experiments are

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reported here. Some aspects of these studies have been presented at recent meetings (3,4).

MATERIALS AND METHODS

The restriction endonuclease Eco RI was obtained from Boehringer, Mannheim, Germany; Hind III and Hha I were from New England Biolabs, Beverly, Ma. USA; Hap II was a gift of Prof. Dr. J.G.G. Schoenmakers (Nümegen, The Netherlands). Agarose was from Seakem; RNAase A was from Serva.

Mitochondria and mitochondrial DNA were isolated as described previously (5). The restriction fragmentation, the separation of the fragments on 0.7% agarose gels and the handling of the gels was performed exactly as described in ref's 1 and 5. Filterstrips (Sartorius 0.45 μm pore size) were prepared with the method of Southern (6).

RNA was isolated from mitochondria by lysis with 8M urea, and 0.4M B-mercaptoethanol; the high molecular weight RNA was isolated by centrifugation with 2M LiCl at 25000 xg for 30 min. The RNA was purified with CsCl centrifugation as described in ref. 7 and, after incubation at 60°C for 30 sec, analyzed by polyacrylamide gel electrophoresis (8). The pattern of absorbance at 260 nm was determined with the Gilford 2400-S. RNA (10 μg) was iodinated as described previously (1) and was hybridized with filterstrips put in vials for incubation at 66°C for 14 h in 4 ml of medium (2xSSC - 0.2% SDS). In some experiments the iodinated high molecular weight mitochondrial RNA was passed through an oligo(dT) cellulose column at 2°C; the bound fractions (polyA-RNA) were collected and used for hybridization. The mitochondrial tRNA was isolated as described by Buck and Nass (9), iodinated and hybridized with stripfilters as described for high molecular weight mitochondrial RNA. The rRNA and tRNA used as competitor were extracted with phenol, respectively from 55S mitochondrial ribosomes and from supernatant of centrifugation of mitochondria with 8M urea, 2M LiCl and 0.4M B-mercaptoethanol. After hybridization the filters were washed in 0.3M NaCl, 0.03M Na citrate (2xSSC) and treated with 20 μg/ml DNase-free RNase A (1); then they were dried and finally subjected to autoradiography for various times. The radioactivity associated with the different fragments was quantified by liquid scintillation counting after bachground elimination.

RESULTS

Mapping of high-molecular weight mtRNAs

For the isolation of high-molecular weight mtRNA we used a high-salt precipitation (8M urea - 2M LiCl) in the presence of mercaptoethanol to inhibit nucleases. This RNA was purified by CsCl gradient centrifugation. The resulting RNA preparation was undegraded as can be inferred from the electrophoretic mobility pattern shown in Fig. 1. It can be seen that there is no low molecular weight RNA present in the preparation: moreover the two mt-rRNA (16S and 12S) are present in an absorbance ratio of about 2:1, whereas the bulk of the RNA has a

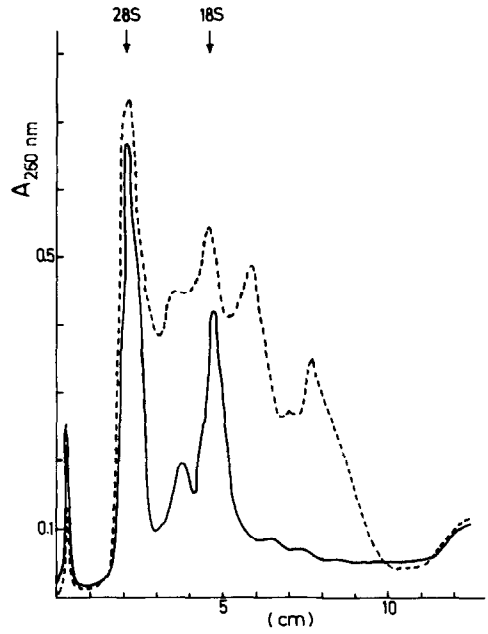

Fig. 1 - Polyacrylamide-gel electrophoresis of high molecular weight mtRNA.
The mtRNA extracted as described on the text was layered on a 2.7% polyacrylamide gel. The cytoplasmic rRNAs were used as markers.
————cytoplasmic rRNAs
------high molecular weight mtRNA

molecular weight higher than the 16S rRNA. Some contamination with cytoplasmic ribosomal RNA seems likely. After alcohol precipitation the RNA was iodinated as described in Materials and Methods. The poly(A)-containing fraction of iodinated RNA was separated by an oligo(dT)-cellulose column at 2°C. At this temperature RNA molecules with poly(A) tails as short as 16 adenylate residues are bound to the column. The total high molecular weight mtRNA and the poly(A)-containing RNA fraction obtained at 2°C were hybridized with restriction fragments of various enzymes, immobilized on millipore filterstrips. Examples of the autoradiograms obtained after exposures of the strips to Rö-film are shown in Fig. 2 together with a photograph of the agarose gel, from which the filters were prepared. It can be seen that the fragments containing the rRNA genes (Eco A, Eco D) are most strongly labelled also in the experiment with poly(A)-RNA. For this reason further experiments were

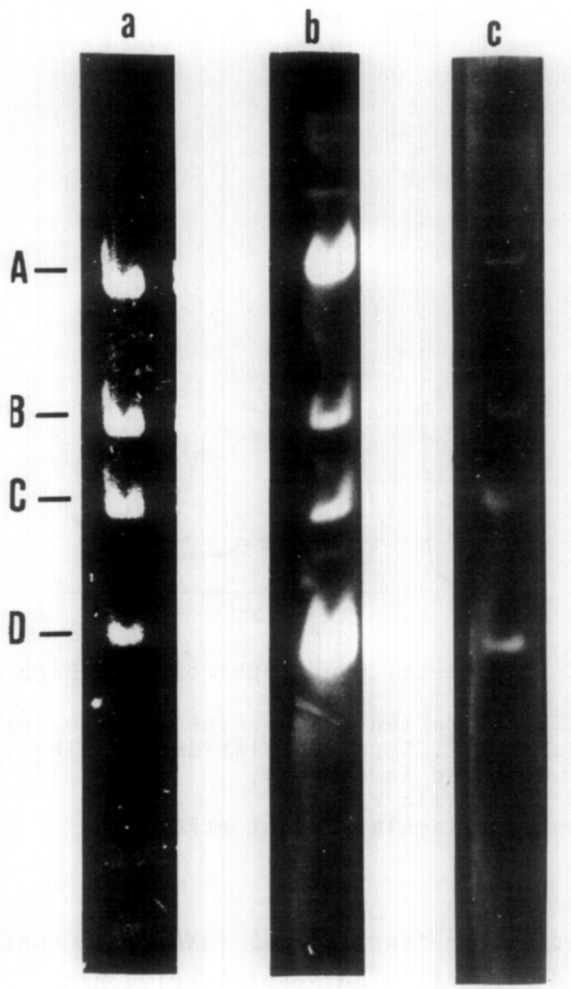

Fig. 2 - Photograph of a tipical electrophoretic pattern on 0.7% agarose slab gel of the rat liver mtDNA digested by Eco RI: fragments visualized by U.V. lamp after ethidium bromide staining (a). Detection by autoradiography of hybridization with Eco RI fragments of high molecular weight RNA (b) and poly(A)-RNA species (c), extracted and iodinated as described in Materials and Methods.

done in the presence of excess unlabelled mt-rRNA. To exclude a contribution of minor contamination with tRNA and to compete with tRNA sequences possibly present in larger precursor molecules, unlabelled mt-tRNA was added in some experiments as well. After autoradiography the filters were cut into pieces and counting was performed as described (1). The results of this approach are given in Table I. In order to provide

Table I - Hybridization of mitochondrial high molecular weight RNA species and poly(A) RNA with various restriction fragments of rat liver mitochondrial DNA.

Fragments		% of total counts bound per 1000 bp's lenght total mRNA	poly(A) RNA
Eco RI	A	0.5	2.36
	B	16.3	7.04
	C	14	11.69
	D	0.7	17.22
Hap II	A	5	2.8
	B	9.12	1
	C	-	-
	D		
	E	27.6	25
	F	9.8	6
Hind III	A	3.8	
	B	3.4	
	C	13	
	D	14.7	
	E	6.2	

The high molecular weight RNA was extracted from mitochondria and iodinated as described in Materials and Methods. 21×10^6 cpm of ^{125}I-RNA were passed through an oligo(dT)-cellulose column. The bound poly(A)-containing fraction (1.2×10^5 cpm) was collected and used in stripfilters hybridization experiments. The hybridization of total mitochondrial RNA with various fragments was carried out in presence of rRNA (80 µg) as competitor. Total counts bound: Eco RI; 8150 cpm, total mRNA; 2534 cpm poly(A)RNA Hap II:8120 cpm total mRNA; 792 cpm poly(A) RNA; Hind III: 10840 cpm total mRNA.

an index of the degree of complementarity the results are expressed as radioactivity of the various fragments per unit length.

Mapping of mitochondrial transfer RNAs

Total mt-tRNAs were isolated and iodinated as described under methods, using the same approach as described above for the high molecular weight mtRNAs. In these experiments unlabelled mt-rRNAs were added as competitors in order to avoid a contribution of contaminating rRNA fragments. The quantitative results are shown in Table II. The tRNA data are expressed as the number of genes per fragment, assuming that the minimal

Table II - Number of tRNA genes located on different restriction fragments.

Fragments		Nr of tRNA genes	Fragments		Nr of tRNA genes
Eco RI	A	7	Hap II	A	5
	B	6		B	5
	C	5		C	3
	D	1		D	2
	E	1		E	1
	F	1		F	2
		Σ 21		G	1
				H	1
					Σ 20
Hind III	A	8	Hha	A	12
	B	7		B	4
	C	4		C	3
	D	1		D	1
		Σ20			Σ20

Stripfilter hybridization of ^{125}I tRNA (s.a. 10-50 $\cdot 10^6$ cpm/µg) in the presence of large excess of competitor rRNA was performed as described elsewhere (1). The calculation of the number of the genes are based on the assumption that minimal percentage of counts bound to any fragment (varying from 4-6%) represents one tRNA gene.

radioactivity bound to any fragment represents the contribution of one tRNA. This amounts to 4-6% in the various experiments; 5% was used as the average value. The total number of tRNA genes obtained in this way is therefore 20, a figure well in accordance with data from others.

DISCUSSION

In view of the known order and mutual relations of the various restriction fragments the data obtained permit the localization of the various genes. The results of our deductions and considerations are show in Fig. 3. It appears as expected (HeLa, Xenopus) that the tRNA genes are scattered around the genome. There is one tRNA gene in the region between the two rRNA genes, a phenomenon well known for bacteria, and for mitochondrial DNA from other animals and Neurospora crassa. There are only few area without tRNAs that are long

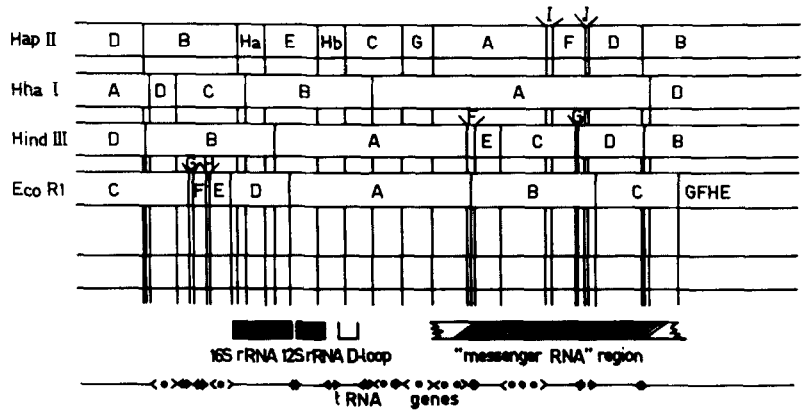

Fig. 3 - The restriction fragment map of rat-liver mitochondrial DNA and the localization of the rRNA and tRNA genes, the origin of replication (D-loop) and "messenger RNA" region.
From top to bottom are shown: the maps for the restriction endonucleases Hap II, Hha I, Hind III and Eco RI, the map with the positions of the 30 restriction sites for these enzymes and the localization of the various genetic markers. The map is largely constructed from the data presented previously (1-4). The fragments Hap II I and Hind G were not mapped in previous publication. The data on which these localizations are based will be published elsewhere. For further details see text.

enough to accomodate information for messenger RNAs. Among these are the Eco RI C, Hap II D region, the Hind III E region and the Hap II F region. Of course the presence of spliced genes cannot be excluded. Furthermore, our method does not distinguish between the two strands of mtDNA. It is feasable that the same region of mtDNA codes for one RNA product in the one strand and for another RNA in the other strand.

The hybridization experiments performed with the high molecular weight RNA in the presence of excess rRNA as competitor clearly indicate that the presumptive mitochondrial messenger RNA species are not scattered all over the mt genome but mainly localized in the region which does not contain either rRNA genes or the origin of the replication. From the overlapping of the fragments obtained with the various restriction endonuclease enzymes it appears that in particular the region of mitochondrial genome showing complementarity to messenger RNA species is the Hind III E -C -D area (Fig.3).

The hybridization of poly(A)-RNA fraction to mtDNA fragments clearly shows that the fraction bound to oligo(dT) cellulose column retains rRNA sequences. This could be explained either assuming that mt rRNA is able to be bound to the oligo(dT) cellulose and therefore is simply present as contaminant or that the poly(A) fraction contains polyadenylated ribosomal precursors. The quantitative hybridization data furthermore show no preferential hybridization of poly (A)-RNA to any particular region of mt genome.

The probable messenger nature of the high molecular weight non ribosomal RNAs extracted from mitochondria is suggested by their ability to stimulate protein synthesis in a E. coli cell-free extract (results not shown). Messenger activity was found either in the RNA fraction which was eluted from the oligo(dT) cellulose column or in the fraction bound at 2°C, whereas very little stimulation was obtained with the RNA fraction bound at room temperature and thus probably containing the majority of contaminating messenger RNAs of cytoplasmic origin with longer poly(A) sequences.

It is generally accepted that mitochondria from higher animal contain poly(A)-rich RNA species whereas mitochondria from lower eukariotes do not. In a previous paper we have also demonstrated that isolated mitochondria are able to synthesize poly(A) containing RNA (10) probably by a specific mitochondrial poly(A) polymerase enzyme. However from the data reported in this paper it could be argued that the mt messenger RNA species in rat liver may also be either free from adenylate sequences or contain a short poly(A) tail. Further investigations are necessary to clarify this point.

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