IDENTIFICATION OF THE HEAVY STRAND OF RAT-LIVER MITOCHONDRIAL DNA AS THE MESSENGER STRAND

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SUMMARY:

We have isolated RNA from the mitochondrial fraction of rat liver following a 6-hour pulse with ^{32}P *in vivo*. This RNA only hybridized with the Heavy strand and not with the Light strand of rat-liver mitochondrial DNA.

INTRODUCTION:

Mitochondrial DNA of vertebrate cells has unique advantages as a tool to study the regulation of transcription in animal cells: First, it consists of a homogeneous population of circular molecules that can easily be obtained free of breaks[1]. Second, its complementary strands can be separated in alkaline CsCl[2,3] and, third, the low information content of mitochondrial DNA (equivalent to about 15 000 base pairs[4]) should make it technically feasible to compare transcription products *in vivo* and *in vitro*. We have studied the transcription of mitochondrial DNA in intact rat liver, in isolated rat-liver mitochondria and with purified RNA polymerase *in vitro* and we have correlated these studies with an analysis of the binding of polyribonucleotides to the complementary strands of chick-liver mitochondrial DNA. In this paper we present our experiments with mitochondrial RNA, pulse-labeled *in vivo*.

METHODS:

DNA preparations. Rat-liver mitochondrial DNA was prepared by the procedure of Borst *et al.*[4], with small modifications (see ref. 3) to improve its stability in alkali. Annealing was carried out at a DNA concentration of about 20 µg/ml either in 1.5 M CsCl at 70°

or in 2 x SSC* at 66°. Rat-liver nuclear DNA and E.coli DNA were prepared as previously described[4], with an additional pronase treatment[5].

RNA preparations. [^{32}P]-labeled mitochondrial and "microsomal" RNA were prepared as follows: Following a 24-hour fast, 4 rats of 50 g received an intra-peritoneal injection of 1 mC carrier-free ^{32}P (Philips-Duphar). After 6 h a mitochondrial fraction was prepared from liver under sterile conditions as described previously[6]. To decrease contamination of the mitochondria with microsomal material the mitochondrial fraction was resuspended twice in 0.25 M sucrose and recentrifuged for 10 min at 24 000 x g_{max}. Mitochondrial RNA was extracted as described previously[7] with the following modifications. The mitochondria were lysed in a mixture of 2% sodium dodecyl sulphate, 4% 4-aminosalicylate and 1% tri-isopropylnaphtalene sulphonate and deproteinized with phenol containing m-cresol[8]. The RNA was further purified by an ethanol precipitation and filtration through a Sephadex G-50 column. "Microsomal" RNA was prepared in the same way from the first mitochondrial high-speed supernatant, twice centrifuged for 10 min at 24 000 x g to remove remaining mitochondria. Directly after preparation the specific activities were 190 and 150 cpm/min/µg for mitochondrial and "microsomal" RNA respectively.

[^{3}H]-ribosomal RNA of E.coli was prepared by methylating purified ribosomal RNA of E.coli with [^{3}H]-dimethylsulphate, as described by Smith et al.[9].

[^{14}C]-cRNA of mitochondrial DNA was prepared[10] with RNA polymerase[11] from E.coli, using denatured mitochondrial DNA as template and [^{14}C]-ATP and [^{14}C]-UTP of equal specific activity as labeled substrates. The purified cRNA contained 5000 cpm/µg RNA.

Hybridization. Denatured DNA in 2 x SSC was adsorbed onto membrane filters (Sartorius-Membranfilter GmbH, 0.1 µ pore size). The amount of DNA per filter was calculated from the input DNA minus DNA not adsorbed. Hybridization was carried out at 66° for 17 h in 2 ml 2 x SSC. RNA, not hybridized, was removed by a hot wash[9] or (in Expt. 2 of Table II) by ribonuclease treatment[5].

* Abbreviations: cRNA, complementary RNA made in vitro with RNA polymerase; micr., "microsomal"; mit., mitochondrial; 2 x SSC, 0.3 M NaCl + 0.03 M sodium citrate, pH 7.0.

Radioactivity of RNA on filters was determined by liquid scintillation counting.

RESULTS:

Table I shows that rat-liver pulse-labeled RNA specifically hybridized with rat-liver mitochondrial DNA. Up to 19% of input mitochondrial RNA was bound at high DNA/RNA ratio's, but no plateau was reached. A higher percentage hybridization could hardly

Table I

Hybridization of pulse-labeled mitochondrial RNA with mitochondrial DNA; specificity of the hybridization and the effect of RNA and DNA concentration.

DNA on filter	RNA added	Radioactivity on filter	
		cpm	% of input cpm bound
2 µg mit.	13 µg mit.[^{32}P]-RNA	73	4
4 µg mit.	13 µg mit.[^{32}P]-RNA	130	7
3 µg mit.	7 µg mit.[^{32}P]-RNA	33	5
5 µg mit.	7 µg mit.[^{32}P]-RNA	50	8
12 µg mit.	7 µg mit.[^{32}P]-RNA	88	14
26 µg mit.	7 µg mit.[^{32}P]-RNA	125	19
4 µg mit.	27 µg micr.[^{32}P]-RNA	2	0.1
4 µg mit.	10 µg E.coli[^{3}H]-RNA	0	0
40 µg nuclear	13 µg mit.[^{32}P]-RNA	0	0
50 µg E.coli	13 µg mit.[^{32}P]-RNA	0	0
107 µg E.coli	10 µg E.coli[^{3}H]-RNA	309	2.7

All values are corrected for RNA bound to filters not containing DNA. The blank values (including background) were about 20 cpm for the [^{32}P]-RNA's, about 55 cpm for the [^{3}H]-RNA and about 60 cpm for the [^{14}C]-RNA (Table II).

be expected, however, since earlier work[7] has shown that most of the RNA present in rat-liver mitochondrial preparations is due to microsomal contamination.

To decide which of the complementary strands of mitochondrial DNA acts as messenger strand in vivo, we have separated the two strands in alkaline CsCl, as shown in Fig. 1. Annealing experiments and other studies[12] have shown that the hatched peaks labeled L and H represent the complementary strands of mitochondrial DNA: After self-annealing for 3 h, the equilibrium density in an analytical neutral CsCl gradient[4] was 1.715 g/cm^3 for Light (L)-strand DNA and 1.725 for Heavy (H)-strand DNA. After annea-

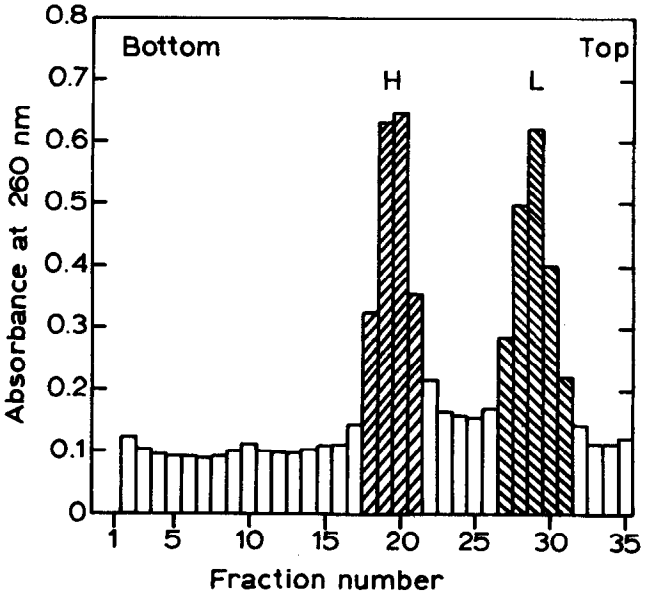

Figure 1. Preparative separation of the complementary strands of rat-liver mitochondrial DNA in an alkaline CsCl equilibrium gradient. 4.0 ml of a solution containing 200 µg DNA in 0.0625 M Na_3PO_4 was mixed with 5.35 g CsCl and the pH was adjusted to 12.3 with NaOH using a Methrohm pH meter model E 350 B with a UX electrode. The mixture was centrifuged in a polyallomer tube of the 50 (angle) rotor at 43 000 rev./min for 42 h in a Beckman-Spinco L II 50 ultracentrifuge. Following the run the tube was punctured and 40-drop fractions were collected. After dilution with 0.6 ml 25 mM sodium phosphate, containing 0.1 mM sodium ethylenediamine tetraacetate, pH 6.0, the A_{260} was read on a Zeiss PMQ II spectrophotometer (1 cm light path).

ling a 1:1 mixture of H- and L-strand DNA one very sharp band at 1.705 g/cm^3 was obtained.

In Table II our hybridization experiments using these L- and H-strand preparations and various RNA fractions are summarized. Expt. 1 shows that mitochondrial RNA hybridizes efficiently

Table II

Hybridization of mitochondrial RNA with purified H- and L-strand mitochondrial DNA.

Mit.DNA on filter	Period of DNA self-annealing (h)	RNA added	Radioactivity on filter	
			cpm	% of input cpm bound
Experiment 1				
3 µg H-strand	3	13 µg mit.[^{32}P]-RNA	279	16
3 µg L-strand	3	13 µg mit.[^{32}P]-RNA	15	0.9
2 µg H-strand	3	27 µg micr.[^{32}P]-RNA	2	0.1
2 µg L-strand	3	27 µg micr.[^{32}H]-RNA	2	0.1
2 µg H-strand	3	3 µg E.coli[^{3}H]-RNA	11	0.4
2 µg L-strand	3	3 µg E.coli[^{3}H]-RNA	7	0.2
2 µg H-strand	3	0.7 µg mit.[^{14}C]-cRNA	552	16
1 µg L-strand	3	0.7 µg mit.[^{14}C]-cRNA	863	25
Experiment 2				
3 µg H-strand	3	13 µg mit.[^{32}P]-RNA	101	7.7
3 µg L-strand	3	13 µg mit.[^{32}P]-RNA	7	0.5
3 µg H-strand	20	133 µg mit.[^{32}P]-RNA	145	2.3
2 µg L-strand	20	133 µg mit.[^{32}P]-RNA	0	0

Corrections as in Table I. The RNA hybridized was determined in Experiment 1 by the hot wash procedure and in Experiment 2 by ribonuclease resistance (see METHODS).

with H-strand DNA, whereas less than 1% of the input counts are bound to L-strand DNA. Appropriate controls demonstrate that this result is not due to artefacts. Expt. 2 of Table II shows that the small amount of mitochondrial RNA bound to L-strand DNA could not be removed by treatment with ribonuclease, whereas it was completely eliminated by self-annealing the L-strand for 20 h instead of 3 h prior to hybridization. Control experiments (not shown) proved that the L-strand was not degraded by the long annealing period, because it still hybridized with mitochondrial cRNA.

DISCUSSION:

The results presented in this paper show that rat-liver mitochondrial RNA is transcribed *in vivo* and that the H-strand acts as the exclusive messenger-strand under the conditions chosen for our experiments, *i.e.* a 6-hour [^{32}P]-pulse in rats one month old. Since at this age the liver is vigorously growing[13] and since there is a continuous turnover of mitochondria in liver cells[14], it seems likely that the 6-hour pulse will label all RNA species made in rat liver with mitochondrial DNA as template. We, therefore, conclude that transcription of mitochondrial DNA is totally asymmetric and that the H-strand acts as the exclusive messenger strand. This conclusion is subject to an obvious reservation: In the liver of a one-month old rat part of the mitochondrial genes could be completely repressed or transcribed at such a low rate that the transcription products would not show up in pulse-labeled RNA. We cannot exclude that such hypothetical repressed cistrons are transcribed from the L-strand. In view of the very low information content[4] of mitochondrial DNA, however, it seems rather unlikely that actively multiplying mitochondria could afford the luxury of repressed genes. We are testing this unlikely possibility by studying the competition in hybridization experiments of mitochondrial RNA with mitochondrial cRNA.

The finding that only the H-strand of mitochondrial DNA is transcribed *in vivo* is of interest in connection with other studies on mitochondrial transcription in this laboratory. In collaboration with C.Saccone and M.N.Gadaleta of the University of Bari, Italy, we have shown[10] that the asymmetry of transcription of rat-liver mitochondrial DNA *in vivo*, is preserved in isolated mitochondria. In collaboration with Ruttenberg, we have shown[12] that both poly Uridylate and a 1:1 copolymer of Inosinate and

Guanylate strongly interact with the L-strand of chick-liver mitochondrial DNA and hardly at all with the H-strand. This result is compatible with the concept[15] that pyrimidine-rich clusters are also involved in the regulation of transcription in vertebrate cells, dT-rich clusters being the ones involved in the case of mitochondrial DNA.

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