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Synthesis and turnover rates of four rat liver mitochondrial RNA species

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The synthesis and turnover rates of the two 12 S and 16 S mt rRNAs and of the mt mRNAs for subunits I and III of cytochrome oxidase have been determined by measuring the kinetics of incorporation of [^{3}H]uridine in the mtRNA of rat hepatocytes. All the RNA species examined have approximately the same turnover ($t_{\frac{1}{2}} \sim 100$ min) and therefore the rate of synthesis, which is about 10-times higher for the rRNAs, seems to be the factor responsible for the different mt rRNA and mRNA steady-state levels.

mtRNA synthesis rate; Turnover rate; Transcription regulation; (Rat hepatocyte)

1. INTRODUCTION

Rat liver mtDNA codes for 2 rRNAs, 11 mRNAs specifying 13 respiratory complex polypeptides and 22 tRNAs (reviews [1,2]). The steady-state concentration of several of these transcripts has been measured in our laboratory by hybridizing total mtRNA to specific DNA probes [3–5]. The results obtained showed that the rRNAs are about 10-times more abundant with respect to the mRNAs. By considering that the mtRNA species decay with first-order kinetics [6,7] their steady-state level is given by the ratio between the rate of synthesis K_s, and that of degradation, K_d. Knowledge of these parameters and comparison with those of other mammals may throw light on the factors controlling the relative abundance of mRNA and rRNA species in the same and different cell types. Here, by measuring the kinetics of incorporation of [^{3}H]uridine in the mtRNA of primary hepatocyte cultures, we have determined the synthesis rates and the metabolic half-lives of four mtRNA species. The data reported show that the different abundance of rat liver ribosomal and messenger mRNAs is mainly regulated at level of the rate of synthesis.

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Abbreviations: mt, mitochondrial; bp, base pair(s); COI, COIII, subunit I and III of cytochrome oxidase

2. MATERIALS AND METHODS

2.1. *Isolation of mtRNA from labelled rat hepatocytes*

In a typical experiment rat hepatocytes were prepared from two 200–300 g male Wistar rats, by the collagenase method as in [8]. Isolated cells (usually $1–2 \times 10^8$/liver) were at least 85% viable as determined by trypan blue exclusion. They were suspended at $0.5–1 \times 10^6$ cells/ml in Ham F12 medium (Flow), supplemented by 15% (v/v) bovine foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin and transferred to 2 l glass flasks capped by rubber corks. The cells were incubated at 37°C and 90 rpm in a rounding incubator and after 30 min exposed to

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[5,6-^{3}H]uridine (25 μCi/ml). During incubation the cells maintained completely their viability up to 7 h. At the indicated times aliquots of the cells were harvested and washed twice with cold NKM (130 mM NaCl, 5 mM KCl, 7.5 mM $MgCl_2$, 1 mM Tris-HCl, pH 7.0). They were then suspended in 15–20 ml of 2 mM NaCl, 0.5 mM $MgCl_2$, 3.5 mM Tris-HCl pH 7.8, incubated for 5–10 min on ice and homogenized with a motor-driven teflon pestle. Mitochondria, prepared as in [9], were divided in two fractions that were used for nucleic acid extraction and determination of the specific activity of the UTP pool, respectively. Total mtRNA prepared as reported by Cantatore et al. [3] was suspended in water and used for hybridization experiments.

2.2. *Preparation of DNA filters and hybridization*

DNA fragments coding for the four mitochondrial transcripts analyzed (see [3] for reference) were cloned in pUC8 and loaded on 25 mm Schleicher and Schüll BA 85 nitrocellulose filters as in [10]. Filters were prehybridized in 2 ml of 50% (v/v) deionized formamide, 5 × Denhardt's, 0.1% (v/v) SDS, 5 × SSPE, 50 μg/ml *E. coli* tRNA [1 × Denhardt's: 0.02% (w/v) bovine serum albumin, 0.02% (w/v) polyvinylpyrrolidone 40, 0.02% (w/v) ficoll 400; 1 × SSPE: 180 mM NaCl, 10 mM sodium phosphate (pH 7.7)] for 4 h at 42°C. Hybridization of heat-denatured [^{3}H]RNA to plasmid DNA was carried out in 2 ml of 50% (v/v) deionized formamide, 1 × Denhardt's, 0.1% (v/v) SDS, 5 × SSPE, 50 μg/ml *E. coli* tRNA, at 42°C with gentle shaking for 20 h. The filters were washed twice at 68°C for 30 min with 2 ml of 2 × SSPE, 0.1% (v/v) SDS and twice at 68°C for 30 min with 2 ml of 0.2 × SSPE, 0.1% (v/v) SDS. Then they were dried and the radioactivity bound to each filter counted using a toluene-based scintillation cocktail. Hybridization was carried out by using a 20–50-fold molar excess of DNA over the specific RNA, as determined by hybridizing increasing amounts of RNA to a set of identical filters. Generally we hybridized 10–25 μg total mtRNA with 4–20 μg specific DNA fragments. To determine the efficiency of hybridization, a rat mtDNA 715 bp fragment, obtained from the digestion of the *Eco*RI A fragment with *Hpa*II [11], was cloned in the plasmid pSP65 and transcribed with SP6 RNA polymerase as reported by Melton et al. [12] by using [α-^{32}P]UTP or [^{3}H]UTP as radioactive precursor. The synthesized RNA was reacted with a filter-bound DNA clone carrying its gene. The reaction was set up to mimic the sequence concentration of the RNAs labelled in vivo included in this study. The hybridization of [^{32}P]- or [^{3}H]RNA proceeded to 50% completion, as determined by comparing the amount of input radioactive RNA with that bound to the filters at each time point. Hybridization data were analyzed by using a non-linear least-squares program, MESSAGE [13], kindly provided by Dr E.H. Davidson (Cal. Tech.). In this program the mitochondrial decay and entry rates are varied assuming the form

$$R(t) = K_s \int_0^t S(t')\exp[-(t' - t)k_d]dt'$$

where R is the amount of radioactive (^{3}H) UMP incorporated in the specific transcript at each time point (dpm/cell), S is the specific activity of the precursor ([^{3}H]UTP) pool at each time pont (dpm/pg UMP), K_s is the apparent entry rate of the transcripts in the mitochondrial compartment (molecules/min per cell), K_d is the decay rate of the transcripts (min^{-1}) and t' is the variable of the integration.

2.3. *Determination of the specific activity of the UTP pool*

The mitochondrial fraction to be used for UTP pool specific activity was further purified by repeated centrifugations at 7500 × g for 10 min and then treated with 20% trichloroacetic acid (0.3 ml of 20% trichloroacetic acid per 10^7 cells). The suspension was incubated for 20 min at 2–4°C, centrifuged for 5 min at 2500 × g and the residue extracted again with 0.5 vol. of 20% trichloroacetic acid. The combined supernatants, neutralized by repeated diethyl ether extractions, were lyophilized and dissolved in 200–400 μl water. UTP was separated from the rest of the acid-soluble fraction by high-performance liquid chromatography on a CI8 Spherisorb ODS 2 column (Violet), by using a Waters liquid chromatograph, equipped with a numeric integrator. The column was eluted with a linear gradient of 0.01 M $NH_4H_2PO_4$ (pH 6.4)-0.6 M $NH_4H_2PO_4$ (pH 6.4), 20% methanol at a flow rate of 1.5 ml/min. Fractions of 1.5 ml were collected and their radioactivity determined in a liquid scin-

tillation counter by adding 10 ml Triton X-100/xylene scintillation mixture.

3. RESULTS AND DISCUSSION

Knowledge of the metabolic properties of the mtRNA species represents an important piece of information in understanding the regulation mechanism of mitochondrial gene expression in mammalian cells. Here, we have used primary rat hepatocyte cultures to analyze the kinetics of the in vivo incorporation of nucleotide precursors in four mtRNA species. Rat hepatocytes, prepared by liver perfusion and collagenase treatment [7], were labelled up to 5 h with [^{3}H]uridine. At fixed times samples of the cells were removed and used for measurement of the specific activity of the cellular and mitochondrial pool and for the preparation of total mtRNA. Fig.1 shows that the kinetics of labelling of cytoplasmic and mitochondrial UTP pool are similar: they reach saturation at around 30 min and decline over the next 60 min. The decline is likely due to the entering in the UTP pool of unlabelled UMP, produced by the degradation of RNA synthesized from unlabelled precursors. The absolute values of the UTP pool specific activity are however different: the cytoplasmic pool has a specific activity which is 10-times that of the

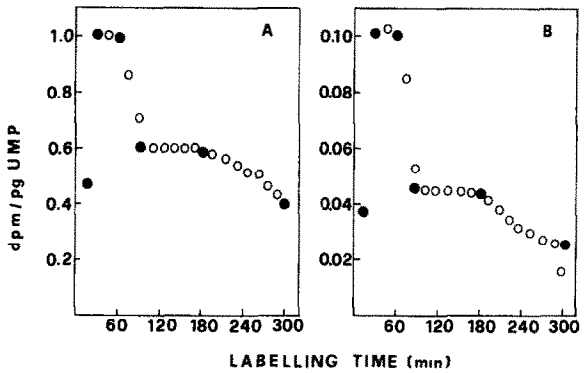

Fig.1. Variation of mitochondrial and cytoplasmic UTP precursor pools' specific activity during the labelling period. Specific activity values (dpm/pg UMP) obtained from the experimental data were normalized for counting efficiency of the scintillation system used (0.3), divided by 4 and supplied to the MESSAGE program. (●) Measured pool specific activities; (○) interpolated form of the time function $S(t)$ used for the synthesis rate calculation.

mitochondria. This may depend on slow mitochondrial UTP uptake, as it is also suggested by different UTP levels in the cytoplasm (1.3 mM) and mitochondria (0.1 mM) [14,15]. To measure the kinetics of incorporation of [^{3}H]uridine in the mtRNA species, mtRNA from each time point was reacted with filters containing an excess of specific DNA probes. The RNA species analyzed were the two 12 S and 16 S rRNAs and the mRNAs for subunits I and III of cytochrome oxidase (COI and COIII). The hybridized cpm were normalized for counting and hybridization efficiency. Then they were reduced as dpm/cell by measuring as reported by Bestwick et al. [16] the amount of mtDNA coextracted with the mtRNA and assuming a mean content of 0.1 μg mtDNA per 10^6 liver cells [17]. The hybridization data were analyzed by using a non-linear least-squares method [13], which gives the best solutions for the rate of appearance in mitochondria of the individual RNA species (k_s) and for their rate of decay (k_d). Fig.2 shows the labelling and turnover kinetics of the four transcripts and table 1 lists the values of k_s, k_d and $t_{1/2}$. Whereas the rate of appearance of the mt rRNAs is about one order of magnitude higher than that of the mRNAs, the k_d values of all the RNA species analyzed are similar. The calculated half-lives are about 100 min for both rRNAs and mRNAs. The ratios k_s/k_d, which give estimates of the steady-state amount of the individual RNAs, are also reported in table 1. They are in good agreement with the molar amount per cell previously calculated by us, titrating rat mtRNA with labelled DNA probes [3,4]. These results support the conclusion that the 10-times higher content of rat mt rRNAs with respect to the mRNAs is mainly due to a higher rate of synthesis of the rRNAs. This is in agreement with data indicating the existence of two different transcription units for rRNAs and mRNAs, respectively [1,2].

Data obtained in several experimental systems [18,19] raised the possibility that, as for some cytoplasmic mRNA species (the mRNAs for casein, globin and histones [20–22]), at least one control of mitochondrial gene expression may take place at the level of RNA stability [6]. According to this hypothesis, it could have been expected that the about 10-times higher level of rat mt mRNAs with respect to the corresponding HeLa cells species might be mainly due to a stabilization of rat

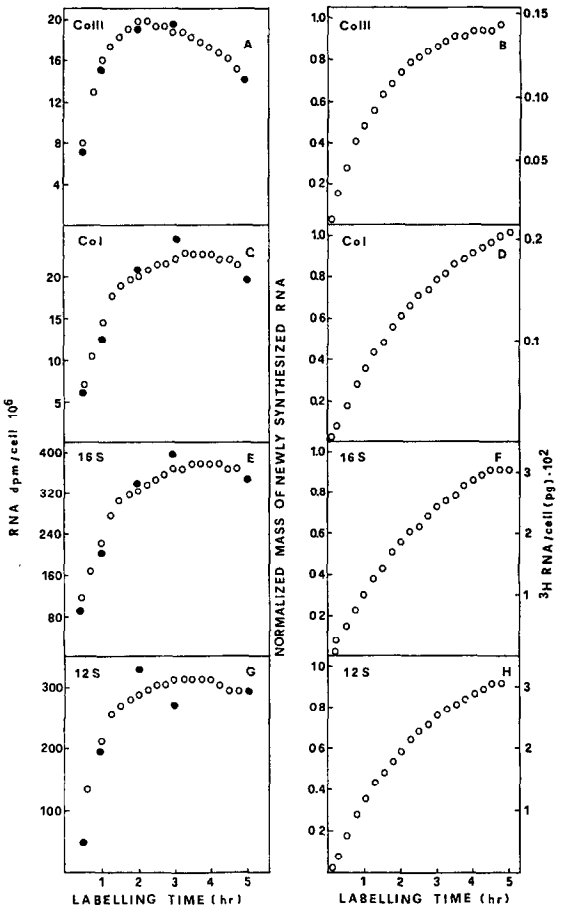

Fig.2. Labelling and turnover kinetics of mitochondrial transcripts. (A,C,E,G) Accumulation of [^{3}H]UMP in the specific mitochondrial transcripts on a per cell basis. Data (●) are fitted with curves (○) generated by application of the equation reported in section 2 to give the least-squares solution for the values of K_s and K_d. (B,D,F,H) Time functions for relative mass accumulation M, for each of the four clones, generated from the solutions for K_s and K_d according to the expression $M = (K_s/K_d)(1 - e^{-k_d t})$. Each curve was measured several times with 5–6 time points for the experiment, using mtRNA from 0.5×10^7–1×10^8 cells per point.

mRNAs. Comparison of the kinetic parameters of HeLa cells [6,7] and rat (this paper) mt mRNAs seems to exclude this model: both HeLa cells and rat mt mRNAs have a comparable stability, so that the higher content of rat mt mRNAs is mainly due to an overall higher rate of synthesis. The low stability of the mtRNA species, perhaps a characteristic of the mammalian mitochondrial system, may allow us to argue that mitochondrial transcription is a process which seems to be regulated in a not particularly tight fashion. This might explain the fact that in sea urchins, in spite of the absence of mtDNA replication and mt protein synthesis, even synthesis of mtRNA takes place during development [23]. Moreover, in the cerebellum, an organ which in rats develops almost

Table 1

Gene	Probe length (bp)	Rate of synthesis[a] K_s (no. of molecules/min per cell)	Rate of degradation K_d (min^{-1})	Half-life[c] (min)	Steady-state content (no. of molecules/cell)	
					K_s/K_d	Direct[c] determination
12 S	954	402	6.7×10^{-3}	115, 95, 118, 80 av. 102	59600	80000
16 S	1304	276	5.3×10^{-3}	147, 120, 142, 115 av. 131	51000	80000
COI	855	17	6.0×10^{-3}	105, 120, 100, 135 av. 115	2900	8000
COIII	394	41	1.1×10^{-2}	48, 85, 55, 70 av. 63	3760	8000

[a] Represents the rate of appearance of the RNA species

[b] Half-life is $\ln 2/K_d$, where K_d (see also legend to fig.2) is the decay rate obtained in the least-squares solution to the labelling and pool specific activity data

[c] Data calculated from [3] assuming a mean content of 1000 mitochondria per rat liver cell

completely after birth, mtDNA and mt protein synthesis peak at 10 days of age whereas RNA synthesis remains quite constant during the first 3 weeks of postnatal life [24]. The relative underregulation of mitochondrial transcription is further confirmed by the estimate of the translation efficiency of the mt mRNAs. The ratio of the number of synthesized cytochrome oxidase subunit I molecules/mRNA molecule per min, to the maximal activity of the mRNA, calculated as reported by Kim and Warner [10], gives a translation efficiency that in both rat liver and HeLa cells is less than 10%. The inefficiency or under-utilization of the mt mRNAs could be related to their lack of features as the cap structures at the 5′-end, and the Shine-Dalgarno sequence, both thought to be important elements for stabilization of the translation initiation complex [1,2]. Moreover, since the mitochondrially coded polypeptides assemble with the nuclear coded subunits to form inner membrane respiratory complexes, it is likely that the extramitochondrial environment also participates in the modulation of mammalian mt mRNA translation in mammalian cells.

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