Transient activation of mitochondrial translation regulates the expression of the mitochondrial genome during mammalian mitochondrial differentiation

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Regulation of the expression of the nuclear-encoded β -subunit of H^+ -ATP synthase (β -F₁-ATPase) gene of oxidative phosphorylation during differentiation of liver mitochondria is mainly exerted at two post-transcriptional levels affecting both the half-life [Izquierdo, Ricart, Ostronoff, Egea and Cuezva (1995) *J. Biol. Chem.* **270**, 10342–10350] and translational efficiency [Luis, Izquierdo, Ostronoff, Salinas, Santarén and Cuezva (1993) *J. Biol. Chem.* **268**, 1868–1875] of the transcript. Herein, we have studied the expression of the mitochondrial (mt) genome during differentiation of rat liver mitochondria in an effort to elucidate the mechanisms of nucleo-mitochondrial cross-talk during biogenesis of the organelle. Estimation of the relative cellular representation of mt-DNA in liver reveals a negligible increase in mt-DNA copy number during organelle differentiation. Concur-

rently, the lack of changes in transcription rates of the mt-DNA 'in organello', as well as in steady-state levels of the mt-transcripts, suggests that organelle differentiation is not controlled by an increase in transcription of the mt-genome. However, translation rates in isolated mitochondria revealed a transient 2-fold increase immediately after birth. Interestingly, the transient activation of mitochondrial translation at this stage of liver development is dependent on the synthesis of proteins in cytoplasmic polyribosomes. These findings support the hypothesis that the expression of nuclear and mitochondrial genes during biogenesis of mammalian mitochondria is developmentally regulated by a post-transcriptional mechanism that involves concerted translational control of both genomes.

INTRODUCTION

Most mitochondrial proteins (mt-proteins) are coded for by nuclear genes [1]. However, the expression of a limited number of polypeptides encoded in the mt-genome is necessary for developing the activities responsible for electron transport and oxidative phosphorylation [1–3]. Co-ordination of the expression of the two cellular genomes is therefore required for the biogenesis of mitochondria. Very little is known about the basic regulatory mechanisms that determine the number and functional capabilities of mitochondria in cellular types of differentiated tissues, in other words, about the levels of regulation that affect the co-ordinated expression of mammalian mt-DNA with the nuclear genome. The study of mitochondrial biogenesis during liver development provides an attractive experimental system *in vivo* for the analysis of some of these mechanisms.

In the rat, biogenesis of mitochondria during liver development results in an increase in the number of mitochondria per cell, i.e. organelle proliferation [4,5], and an increase in the functional capability of pre-existing mitochondria, i.e. organelle differentiation [6–11]. Whereas proliferation of mitochondria is a continuous and subtle process that spans the whole developmental period, differentiation of mitochondria is a very rapid process occurring during the first hour of life.

As part of a project aimed at understanding the basic mechanisms that control nucleo-mitochondrial communication during biogenesis of mitochondria in mammals, we have previously

characterized the mechanisms that regulate the expression of the nuclear-encoded β -subunit of H^+ -ATP synthase (β -F₁-ATPase) gene during development of the rat liver [10–13]. Interestingly, it was found that at the stage of development in which organelle differentiation occurs (1 h post-natal), regulation of β -F₁-ATPase expression is exerted at two post-transcriptional levels of gene expression [11,12]. One level affects the relative cellular abundance of the β -F₁-ATPase mRNA [11,12] as a result of an increase in the mRNA half-life [11]. The other affects the intrinsic translational efficiency of β -F₁-ATPase mRNA, as assessed both *in vivo* [11] and *in vitro* [12], by a still unidentified mechanism. In contrast, when proliferation of the organelle occurs, control of the expression of the nuclear-encoded gene appears to shift to the transcriptional level [11]. These findings illustrate that regulation of the expression of the nuclear genome during biogenesis of mitochondria in mammalian cells could be exerted at multiple levels of gene expression. Furthermore, the findings suggest the existence of two different programmes for the regulation of organelle biogenesis that operate at different stages of liver development. The functioning of these programmes depends on whether the number (proliferation) or functional capabilities (differentiation) of mitochondria are to be changed [11].

In this work, we have studied the mechanisms involved in the expression of the mt-genome during differentiation of liver mitochondria. The results show that regulation of mitochondrial gene expression is exerted by transient activation of mito-

Abbreviations used: mt, mitochondrial-DNA, -RNA, -protein, -genome, -transcript; β -F₁-ATPase, β -subunit of H^+ -ATP synthase; SSC, 0.15 M NaCl/0.015 M sodium citrate; ND1–ND6 and ND4L, subunits of NADH dehydrogenase; COI, COII and COIII, subunits of cytochrome c oxidase; cyt b, apocytochrome b; A6 and A8, subunits of H^+ -ATP synthase; prRNA, precursor of ribosomal RNAs.

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chondrial translation, providing the first indication that the expression of nuclear [11] and mitochondrial genes (this study) in mammalian cells is regulated, in concert, by a mechanism that controls the expression of both genomes at the translational level.

EXPERIMENTAL

Animals

Timed pregnant albino Wistar rats weighing 200 g were fed standard laboratory chow and water *ad libitum*. Pregnancy was confirmed on day 19 of gestation. The fetuses (0 h old) (5.2 ± 0.1 g) were delivered by rapid hysterectomy from cervically dislocated pregnant rats on day 22 of gestation. The newborns were maintained without feeding as previously described [7] and killed by decapitation at the time intervals indicated in the Figures. In those experiments in which cytosolic protein synthesis was inhibited, newborns were injected intraperitoneally with 10 mg of cycloheximide/kg of body weight at the time of delivery [7].

Isolation of mitochondria

Fetal, newborn and adult rat liver mitochondria were prepared from decapitated animals, under sterile conditions. The dissected livers were homogenized in 4 vol. of ice-cold isolation medium (0.32 M sucrose/1 mM K-EDTA/10 mM Tris/HCl, pH 7.4) and mitochondria were isolated as reported previously [7]. Protein concentrations were determined with the Bio-Rad protein assay using crystalline BSA as standard.

Quantitative analysis of mt-DNA

Total cellular DNA was extracted from 1 g of liver after digestion with RNase and proteinase K, using standard protocols. Cellular DNA samples were digested with *Bam*HI. mt-DNA was prepared from 1 mg of rat liver mitochondria. Mitochondria were resuspended in a medium containing 10 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA and 2% SDS and were incubated at 37 °C for 10 min in the presence of 50 µg/ml proteinase K [14]. Nucleic acids were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), once with ethyl ether and afterwards were precipitated with ethanol. The nucleic acids were resuspended in 10 mM Tris/HCl/1 mM EDTA, pH 8, and incubated with RNase A (50 µg/ml) at 37 °C for 15 min. The mt-DNA extracted as above was precipitated with ethanol, resuspended in H₂O and further digested with *Bam*HI. The DNA fragments were resolved on 0.8% agarose gels and transferred to nylon membranes (Gene-Screen®, Du Pont–NEN) [11]. Membranes were incubated with [³²P]dCTP-labelled DNA probes. The rat liver DNA probes used in this study were β -F₁-ATPase cDNA, for a nuclear-encoded oxidative phosphorylation gene transcript [15] (provided by Dr. P. L. Pedersen, The Johns Hopkins University, Baltimore, MD, U.S.A.), and specific DNA probes for the mitochondrial genes 12 S rRNA, cytochrome *b*, ATP synthase subunits 6 and 8 (A6 and A8) and cytochrome *c* oxidase subunit III (COIII), NADH dehydrogenase subunit 1 (ND1) and cytochrome *c* oxidase subunit I (COI) [16] (provided by Dr. P. Cantatore, University of Bari, Italy). DNA probes were labelled by nick translation at 15 °C for 1 h using 50 µCi of [α -³²P]dCTP (3000 Ci/mmol). Hybridization was carried out at 42 °C for 72 h. After hybridization, membranes were washed according to the following protocol: (1) 6 × SSC, at room temperature for 10 min (where SSC is 0.15 M NaCl/0.015 M sodium citrate); (2) 2 × SSC containing 0.1% SDS, at 65 °C for 10 min; and (3) 0.1 × SSC containing 0.1% SDS, at 65 °C for

10 min. Hybridization signals of the samples were always within the linear range of the film. For stripping labelled DNA probes, membranes were incubated in sterile water at 90–100 °C for 20 min. Membranes were exposed to X-ray films and analysed by laser densitometric scanning.

RNA hybridization analysis

Total liver RNA was extracted from 1 g of tissue as previously described in detail [10,12]. mt-RNA was extracted from lysed mitochondria with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) [14]. RNA samples were separated by electrophoresis in formaldehyde/1.4% agarose gels. Denatured RNAs were transferred to Gene-Screen® membranes (Du Pont–NEN) by vacuum transfer. mt-DNA probes [16] were labelled by nick translation. Hybridization conditions, membrane washing, stripping of DNA probes and quantification of hybridization signals were as detailed above.

Transcription and isolation of labelled mitochondrial nucleic acids 'in organello'

Incorporation of [α -³²P]UTP by isolated mitochondria was carried out as described earlier [17]. Briefly, liver mitochondria (~2 mg/ml) were resuspended in 0.5 ml of 10 mM Tris/HCl, pH 7.4, containing 25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K₂HPO₄, 0.05 mM EDTA, 5 mM MgCl₂, 1 mM ATP, 10 mM glutamate, 2.5 mM malate, 1 mg/ml BSA (incubation buffer) and 20 µCi of [α -³²P]UTP (400–600 Ci/mmol) in microfuge tubes. Incubation was carried out at 37 °C for 60 min in a rotatory shaker. After incubation, mitochondria were pelleted at 12000 *g* for 1 min and washed twice with 10% glycerol/10 mM Tris/HCl (pH 6.8)/0.15 mM MgCl₂ [18]. Lysis of mitochondria and nucleic acid extraction were carried out as described previously [14]. The total mitochondrial nucleic acid fraction was analysed by electrophoresis through 1.4% agarose slab gels in the presence of de-ionized CH₃HgOH [19]. After the run, the gels were stained with ethidium bromide, dried and exposed for autoradiography. Quantification of the autoradiograms was carried out by laser densitometry. Transcription rates were linear over the times measured and were expressed relative to both the amount of mt-protein and mt-DNA in each assay.

Mitochondrial protein synthesis and analysis of mitochondrial translation products 'in organello'

Incorporation of [³⁵S]methionine by isolated mitochondria was carried out in a sterile medium similar to that used for determining rates of RNA synthesis. Briefly, liver mitochondria were resuspended in incubation buffer (1 mg/ml) containing 0.1 mM complete amino acid mixture (minus methionine), 100 µg/ml cycloheximide and 60 µCi/ml [³⁵S]methionine (> 1000 Ci/mmol). In some experiments, chloramphenicol (100 µg/mg of mt-protein) was added to the basic medium to inhibit mt-protein synthesis. All incubations were carried out at 30 °C in a rotatory shaker. After incubation, 5 µl aliquots of the incubation reaction were precipitated with 5% (v/v) ice-cold trichloroacetic acid [20] to determine the radioactivity incorporated into mt-proteins. The rest of the incubation reaction was precipitated with 5 vol. of acetone for 10 min at –20 °C. After centrifuging for 10 min at 12000 *g*, the pellets were dried at room temperature and resuspended in a sample buffer containing 8 M urea, 62.5 mM Tris/HCl (pH 6.8), 2% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 1 mM EDTA and 0.01% (w/v) Bromophenol Blue [21]. Mitochondrial translation products were fractionated in

SDS/15%-PAGE in the presence of 4 M urea. The gels were further processed for fluorography [22]. The intensity of the bands in the resulting autoradiograms was determined by laser densitometric scanning. Exposure times were chosen to fall within the linear response range of the films.

Other methods

Determination of the relative amount of the β -subunit of the F_1 -ATPase complex in mitochondria was carried out as previously described in detail [9,10,12]. Briefly, 50 μ g of mt-proteins were fractionated on SDS/12%-PAGE, transferred to poly(vinylidene difluoride) membranes (Millipore) and blotted with affinity-purified antibodies recognizing the β -subunit of the mitochondrial F_1 -ATPase complex [11,12]. The intensity of the immunoreactive band was determined by laser densitometric scanning and expressed as a percentage of that present in mitochondria from 0 h-old samples. Cytochrome *c* oxidase activity was assayed in isolated mitochondria by standard enzymic procedures as described previously [7,9].

RESULTS

Changes in the relative content of mt-DNA during liver development

It has been proposed that genome dosage is one of the major determinants of the expression of mt-DNA [23,24]. Furthermore, the content of mt-DNA has been shown to increase in rat liver

Figure 1 Changes in mt-DNA copy number during liver development

Cellular DNA was isolated from 1 g of liver. Digested DNA (10 μ g) were resolved on 0.8% agarose gels, transferred to nylon membranes and hybridized subsequently with the mitochondrial (mt-12 S) and the nuclear (β -F1) DNA probes for the 12 S rRNA and β -subunit F_1 -ATPase genes from rat liver respectively. A representative blot with two different samples for each experimental condition, fetal (0 h), 1 h- and 6 h-old neonatal and adult is shown. Quantification of the data from various experiments is presented in Table 1.

during development [25]. Therefore, we determined the relative cellular content of mt-DNA in liver at the stage of development in which mitochondrial differentiation occurs (1 h post-natal) (Figure 1 and Table 1). mt-DNA content was assessed in Southern blots from the 12 S hybridization signal. The content of cellular DNA was assessed from the signal of the nuclear encoded β -F₁-ATPase gene of oxidative phosphorylation. The results obtained illustrate no significant increase in mt-DNA content of the liver immediately after birth, although a significant increase was noted at 6 h post-natal (Figure 1 and Table 1). The adult liver contained ~6-fold higher amounts of mt-DNA than the term fetal liver (Figure 1 and Table 1), a finding that is in agreement with similar estimates [25] and with the reported differences in the number of mitochondria per hepatocyte [4,5] and in the amount of β -F₁-ATPase protein/g of liver [11] between both stages of liver development. These findings therefore suggest that mt-DNA replication is activated after birth, although no significant differences in mt-DNA content could be observed in the liver at the stage of organelle differentiation.

Steady-state amounts of mt-RNAs during organelle differentiation

In order to find out whether differentiation of mitochondria is accompanied by changes in mitochondrial transcription rates, the steady-state levels of several mt-transcripts were determined in the liver at these two stages of development (Figure 2 and Table 1). The results obtained revealed two different trends. Whereas 12 S rRNA and ND1 and A6 + A8 + COIII mRNAs (COIII is a subunit of cytochrome *c* oxidase) showed no changes between the fetal and 1 h-neonatal liver, apocytochrome *b* (cyt *b*) and COI mRNAs showed a 2-fold increase in steady-state amounts during this short time-interval (Figure 2; Table 1). Similar findings were obtained when (1) steady-state mt-mRNA levels were expressed relative to the 12 S hybridization signal and (2) steady-state transcript levels were determined relative to the amount of mt-RNAs extracted per mg of mt-protein (results not shown). It should be mentioned that the yield of isolated mt-RNA/mg of mt-protein was roughly the same in both fetal and 1 h-neonatal samples (7.3 ± 1.2 and 6.9 ± 0.1 μ g of RNA/mg of mt-protein respectively). Since these mt-mRNAs are transcribed as a single polycistronic molecule from the same transcription unit of the heavy chain of mt-DNA [26], the changes observed in the steady-state content of COI and cyt *b* mRNAs suggest a specific regulation of the stability of these mt-transcripts at this stage of liver development (see below).

Table 1 mt-DNA copy number, steady-state mt-RNA amounts and transcription rates 'in organello' of mt-DNA during liver development

For specific details see legends to Figures 1, 2 and 3 respectively. The results shown are means $\pm$ S.E.M. The numbers in parentheses represent the number of values used in calculating the means. The relative cellular content of mt-DNA during liver development is expressed as the ratio of the hybridization signals of 12 S rRNA to β -F₁-ATPase. For quantification of mitochondrial transcription rates, only the bands of newly synthesized transcripts shown in Table 2 were considered: ^a $P < 0.05$, ^b $P < 0.025$, ^c $P < 0.0025$ and ^d $P < 0.0005$ when compared with 0 h samples by Student's *t*-test; ^e $P < 0.05$ when compared with 1 h samples by Student's *t*-test. Abbreviation: a.u., arbitrary unit.

Developmental stage	Cellular content of mt-DNA (relative units)	mt-RNA (a.u./ μ g cellular RNA)					Mitochondrial transcription (a.u./mg of mt-protein)
		12 S	ND1	A6 + A8 + COIII	cyt <i>b</i>	COI	
0 h-Fetal	1.00 ± 0.06 (6)	2.2 ± 0.3 (6)	3.3 ± 0.4 (6)	1.4 ± 0.2 (6)	3.7 ± 0.5 (6)	9.3 ± 0.9 (6)	1.5 ± 0.3 (4)
1 h-Neonatal	1.24 ± 0.12 (6)	1.9 ± 0.1 (6)	4.3 ± 1.1 (6)	1.5 ± 0.1 (6)	5.8 ± 0.8 (6) ^a	16.0 ± 1.4 (6) ^c	1.7 ± 0.3 (4)
6 h-Neonatal	1.67 ± 0.36 (2) ^b	—	—	—	—	—	—
Adult	5.83 ± 0.66 (8) ^d	—	—	—	—	—	0.5 ± 0.3 (3) ^e

Figure 2 Steady-state mt-DNA transcription products in fetal and neonatal rat liver

Total cellular RNA was prepared from livers of fetal (0 h) and neonatal (1 h) rats. After fractionation by electrophoresis of 30 μ g of total RNA, the membrane was hybridized with 32 P-labelled DNA probes for rat liver mt-DNA transcription products (12 S rRNA and ND1, A6 + A8 + COIII, cyt *b* and COI mRNAs). Six different samples assayed in the same Northern blot are shown for each experimental condition. Quantification of the data is presented in Table 1.

Transcription rates of mt-DNA during liver development

We have recently reported that steady-state transcript levels in the liver of fetal and neonatal rats are much higher than in the adult liver for both the nuclear and mitochondrially encoded mRNAs of the ATP synthase [11]. This is a surprising finding considering the much lower cellular representation of mt-DNA (Figure 1 and Table 1) at this stage of liver development [25]. In order to verify that transcription rates of mt-DNA do not change during organelle differentiation (Figure 2; Table 1), and to find out whether the higher availability of mt-transcripts during organelle differentiation [11] results from developmental changes

Figure 3 mt-DNA transcription rates in isolated liver mitochondria

Mitochondria were isolated from fetal (0H), 1 h-old (1H) neonatal and adult rat liver. Transcription assays were carried out 'in organello' in the presence of [32 P]UTP. Transcription products were resolved on agarose-CH₃HgOH slab gels as described in the Experimental section. (A) A representative autoradiogram of the labelled transcription products obtained from two different samples of each experimental condition assayed in the same gel. Note the higher intensity of most of the transcription products in the autoradiogram of fetal and neonatal samples. The nomenclature of mitochondrial transcription products is as follows: 4, 4a, prRNAs; 5, mRNA of ND5; 6, the mRNA precursor of COI (pCOI); 7, 9, the mRNAs for the mature COI and subunits ND4 and ND4L; 16S, the mature 16 S rRNA; 11, the mRNA of cytochrome *b*; 12, the mRNA of ND2; 13, 12S, the mRNA of ND1 and the mature 12 S rRNA respectively; 14, 15, subunits A6, A8 and COIII; 16, COII. Quantification of data from various experiments is presented in Table 1. (B) Southern blot illustrating the higher representation of mt-DNA/mg of mt-protein in fetal and neonatal rat liver mitochondria when compared with adult. mt-DNA was isolated from 1 mg of mt-protein. After fractionation and transfer of the digested mt-DNA, the membranes were hybridized with 32 P-labelled DNA probes for the 12 S rRNA and COI mitochondrial genes. It should be noted that the higher intensity of most of the transcription products in the autoradiogram of fetal and neonatal samples is due to the different content of mt-DNA/mg of mt-protein between samples (see B). In fact, when transcription rates were normalized by the amount of mt-DNA loaded in each experiment, the transcription rates of mt-DNA were the same throughout development (0.9 ± 0.1 relative units/mt-DNA).

in mt-DNA transcription, transcription rates were determined using a recently developed 'in organello' system that transcribes mt-DNA *in vitro* with high fidelity [17,18]. Figure 3(A) illustrates the profile of synthesized mt-transcripts in isolated mitochondria from fetal, 1 h-neonatal and adult liver. The profile of synthesized transcripts is similar to that reported previously for mitochondria from rat liver and brain [17,18] and from human HeLa cells [27].

The results show that mitochondrial transcription, when it is expressed relative to the amount of mt-DNA, is the same at all

Table 2 Relative synthesis of mitochondrial transcription products in isolated liver mitochondria

For details see legend to Figure 3. Dried gels were exposed to X-ray films at -70°C with intensifying screens. The autoradiograms were quantified by laser densitometric scanning and the percentage of each transcription product of mt-DNA calculated assuming as 100% the sum of those presented below. The nomenclature of mt-transcripts as given in Figure 3 is given in parentheses. The results shown are the means $\pm$ S.E.M. of 3–4 different experiments: ^a $P < 0.0005$ and ^b $P < 0.005$ when compared with adult samples by Student's *t*-test; ^c $P < 0.01$ when compared with 1 h-neonatal samples by Student's *t*-test.

Developmental stage	prRNA (4, 4a)	ND5 (5)	ND4, ND4L, COI (7, 9)	16 S	cyt <i>b</i> (11)	ND2, ND1, 12 S (12, 13, 12S)
0 h-Fetal	10 ± 2^a	8 ± 1	19 ± 3	$12 \pm 1^{b,c}$	14 ± 1	30 ± 5
1 h-Neonatal	8 ± 4^b	8 ± 3	20 ± 0.3	23 ± 1^a	16 ± 1	25 ± 3
Adult	29 ± 1	8 ± 1	18 ± 3	5 ± 1	15 ± 1	27 ± 2

Figure 4 Protein synthesis in isolated liver mitochondria

Mitochondria were isolated from fetal (lanes 1 and 2), 30 min-old (lanes 3 and 4), 1 h-old (lanes 5 and 6), 6 h-old (lanes 7 and 8) neonatal and adult (lanes 9 and 10) rat liver. Assays for synthesis of mt-proteins *in vitro* were carried out with ~ 1 mg/ml of mt-protein in the presence of [^{35}S]methionine and 100 $\mu\text{g/ml}$ cycloheximide (lanes 1–10) plus (lanes 1, 3, 5, 7 and 9) or minus (lanes 2, 4, 6, 8 and 10) 100 $\mu\text{g/ml}$ chloramphenicol. (A) Electrophoretic pattern of the translation products *in vitro* of rat liver mitochondria at various stages of development. Equivalent mitochondrial samples were electrophoresed after treatment with acetone, as described in the Experimental section. The inhibitory effect of chloramphenicol on mitochondrial translation is shown in the odd numbered lanes for each time-point. The unidentified chloramphenicol- and cycloheximide-resistant band (see Results section) is marked with an asterisk. The ND5 translation product is not shown because it overlaps the band identified with an asterisk. (B) Quantification of the relative rates of mt-protein synthesis was calculated after laser densitometric scanning of the bands resulting in X-ray films. The values shown are means $\pm$ S.E.M. of 3–7 different mitochondrial preparations, expressed as a percentage of the synthesized proteins (arbitrary units/mg of mt-protein) found in 0 h-old samples assayed in the same gel. $P < 0.0005$ when results for 1 h-old samples are compared with 0 h-old, 6 h-old and adult samples by Student's *t*-test.

stages of liver development (0.9 ± 0.1 relative units/mt-DNA). In fact, a significant linear correlation was obtained between transcription rates and the amount of mt-DNA in each assay ($m = 2.036$; $b = 0.14$; $r = 0.9882$; $P < 0.01$). However, transcription rates in adult mitochondria were ~ 2 – 3 -fold lower than those determined in fetal mitochondria when expressed relative to the amount of mt-protein (Figure 3A and Table 1). This later finding is in agreement with the lower representation of mt-DNA/mg of mt-protein found in the adult mitochondria (Figure 3B). Furthermore, the rates of mt-transcription/mg of mt-protein in fetal and 1 h-old neonatal mitochondria were not significantly different (Figure 3A and Table 1), which coincides with the lack of significant changes in mt-DNA content (Figure 1, Figure 3B and Table 1) and in the steady-state amounts of liver 12 S rRNA and

ND1 and A6+A8+COIII mRNAs (Figure 2 and Table 1) between these two stages of liver development. These results therefore suggest that differentiation of the organelle is not coupled to a transcriptional burst of the mt-genome. Furthermore, the finding that transcription rates of cyt *b* (11 in Figure 3A) and COI (9 in Figure 3A) remained unchanged between fetal and 1 h-old samples (Figure 3A and Table 2) strongly suggests that an additional specific mechanism of regulation, that affects the stability of these mt-transcripts, is operating at this stage of development in the mammalian liver.

It should be stressed that the percentage of mature synthesized mt-mRNA transcripts was the same at the three stages of rat liver development studied (Table 2). However, adult mitochondria showed a remarkably lower relative synthesis of mature

Figure 5 Inhibition of cytosolic protein synthesis prevents transient activation of mitochondrial translation

Cytosolic protein synthesis was inhibited in newborn rats at the time of delivery (0 h) by the intraperitoneal administration of 10 mg/kg body weight of cycloheximide (CHX). Mitochondria were isolated 1 h after the treatment (1 h) and the amount of β -F₁-ATPase protein (Western blot in **A**), cytochrome *c* oxidase activity (Table 3) and protein synthesis *in vitro* (autoradiogram in **B**) determined. Quantification of data from various experiments is presented in Table 3. (**A**) mt-Proteins (50 μ g) were fractionated by SDS/PAGE and probed with affinity-purified anti- β -F₁-ATPase antibodies. A representative blot is presented. (**B**) A representative autoradiogram illustrating the electrophoretic pattern of the products translated *in vitro*.

ribosomal 16 S rRNA (16 S in Figure 3A) than fetal and 1 h-neonatal mitochondria (see Table 2). Interestingly, the lower rate of synthesis of the mature rRNA in adult mitochondria resulted from a lesser maturation of the precursor for rRNAs (prRNA) (see 4 and 4a in Figure 3A and Table 2), a finding that could not be observed for the mature 12 S rRNA because it overlapped with other mt-transcripts (Figure 3A).

Translation of mt-mRNAs during liver development

Altogether, the findings described thus far suggest the possibility that the expression of the mt-genome during organelle differentiation could be exerted by modulation of the translation rates of the available transcripts. Consequently, we determined the rates of mt-protein synthesis following an '*in organello*' approach similar to those previously described by others [2,28–30].

Basically, the procedure to determine mt-protein synthesis consisted in measuring the incorporation of [³⁵S]methionine into

proteins in the presence of an inhibitor of cytosolic ribosomes (cycloheximide). The '*in organello*' system revealed a linear incorporation of the tracer with respect to time and mt-protein concentration (results not shown), resulting in optimal quantification of fractional incorporation rates. Protein synthesis determined in the presence of chloramphenicol, an inhibitor of mt-protein synthesis, revealed negligible and non-time-dependent incorporation of labelled methionine into proteins (results not shown; see Figure 4A for a comparison of mt-protein synthesis with and without chloramphenicol).

Figure 4(A) illustrates the relative rates of synthesis of mt-proteins at various stages of liver development. The profile of translation products synthesized in liver mitochondria is basically the same as that reported previously for human HeLa cells [2], rat fibroblast [2] and rat liver [30], muscle [2] and brain [29] mitochondria. In agreement with the findings of Polosa and Attardi [29], a pronounced labelled band moving somewhat more slowly than the COI subunit of cytochrome *c* oxidase (~69 kDa) (marked with an asterisk in Figure 4A) was observed in the protein-labelling patterns from rat liver mitochondria. The nature of this cycloheximide- and chloramphenicol-resistant band is not known [29]. Since this band has a similar electrophoretic mobility to the ND5 subunit of NADH dehydrogenase, it prevented reliable visualization and quantification of this translation product.

Quantification of the relative contribution of each translation product to the total synthesized protein revealed that cyt *b* (30%), ND1 (7%), COIII (8%), COII (19%), A6 (11%) and ND6 (14%) accounted for most of the translation products in rat liver mitochondria. Furthermore, the relative contribution of synthesized products did not show appreciable differences with respect to the stage of development studied (Figure 4A), a surprising finding when compared with the changes observed in steady-state levels for cyt *b* and COI mRNAs in the liver of fetal (0 h) and 1 h-old neonatal rats (Figure 2; Table 1).

However, the overall rates of mitochondrial translation did reveal a significant and transient increase at 1 h-post-natal (Figure 4A). On a quantitative basis, the rate of labelling of the mitochondrial translation products/mg of mt-protein at 1 h post-natal was 2-fold higher than the rates observed in fetal and adult mitochondria (Figure 4B). Interestingly, this increase in mt-protein synthesis is transient, since rates of protein labelling in mitochondria from 30 min-old and 6 h-old neonates were basically the same as in the adult (Figures 4A and 4B).

Cytosolic protein synthesis controls the transient increase in mitochondrial translation during organelle differentiation

The unexpected transient nature of the activation of mitochondrial translation (Figure 4) raised questions regarding the mechanism involved in this rapid and reversible response of the mitochondrial translation machinery. Therefore, we studied whether cytosolic protein synthesis could be involved in mediating such a transient response. Newborn rats were injected at the time of delivery with cycloheximide to inhibit cytosolic protein synthesis, or with NaCl, as controls. One hour after the treatment, mitochondria were isolated and mitochondrial translation rates were determined. Treatment of the neonates with cycloheximide has previously been shown to affect the rates of cytosolic protein synthesis [7] and to impair post-natal development of mitochondrial functions in newborn rats [7] as a result of the arrest of mitochondrial differentiation [7,8]. In agreement with previous findings [7], mitochondria from cycloheximide-treated neonates showed a significant reduction in the amount of β -F₁-ATPase protein (Figure 5A and Table 3) and cytochrome *c* oxidase

Table 3 Inhibition of cytosolic protein synthesis prevents transient activation of mitochondrial translation

Cytosolic protein synthesis was inhibited in newborn rats at the time of delivery by the intraperitoneal administration of 10 mg/kg body weight of cycloheximide (CHX). Age-matched neonates were administered NaCl. Mitochondria were isolated from neonatal livers at 0 h (0 h-fetal) and 1 h (1h + NaCl and 1h + CHX) after the treatment. For details see legend to Figure 5. The results shown are means $\pm$ S.E.M. The numbers in parentheses represent the number of values used in calculating the means. Quantification of the relative mitochondrial amount of β -F₁-ATPase is expressed as a percentage of the amount of β -F₁-ATPase (arbitrary units/ μ g of protein) found in 0 h samples assayed in the same blot. Cytochrome *c* oxidase activity is expressed as units/mg of mitochondrial protein. Total mitochondrial protein synthesis is expressed as a percentage of that at 0 h: ^a*P* < 0.005 and ^b*P* < 0.0005 when compared with 0 h by Student's *t*-test; ^c*P* < 0.05 and ^d*P* < 0.005 when compared with 1 h-old NaCl-treated age-matched neonates.

Developmental stage	Cytochrome <i>c</i> oxidase (units/mg of mt-protein)	β -F ₁ -ATPase protein (% at 0 h)	Mitochondrial translation (% 0 h)
0 h-Fetal	0.40 $\pm$ 0.03 (5)	100 $\pm$ 2 (3)	100 $\pm$ 3 (3)
1 h + NaCl	0.76 $\pm$ 0.04 (3) ^b	168 $\pm$ 3 (3) ^b	200 $\pm$ 16 (3) ^a
1 h + CHX	0.52 $\pm$ 0.02 (5) ^d	126 $\pm$ 7 (5) ^d	130 $\pm$ 16 (5) ^c

activity (Table 3), when compared with mitochondria from age-matched NaCl-treated neonates. Likewise, mitochondria from cycloheximide-treated neonates showed a significant reduction in mitochondrial translation rates at 1 h post-natal (Figure 5B and Table 3). These findings suggest that the transient activation of the mitochondrial translation machinery that occurs during organelle differentiation depends on proteins synthesized *de novo* on cytoplasmic ribosomes.

DISCUSSION

Very little is known about the physiological and developmental regulation of mitochondrial gene expression in mammals [1,2]. Even less is known about the basic mechanisms that exert a co-ordinated response in the expression of both genomes during organelle biogenesis. The results described herein illustrate that the expression of the mt-genome during differentiation of mitochondria, a critical process required for adaptation of mammals to extra-uterine life [8,9], is regulated at the translational level. Interestingly, a translational regulatory mechanism also controls the expression of a nuclear-encoded oxidative phosphorylation gene at this stage of mammalian development [11,12].

Replication and transcription of mt-DNA during liver development

In rabbit [23,24] and mouse [31] muscle, parallel changes in mt-DNA copy number and mt-mRNA amounts have been observed, leading to the suggestion that genome dosage is one of the major determinants of the expression of mt-DNA. These findings are in contrast with the results described in the liver during development, where a ~6 fold increase in the relative content of mt-DNA (Figure 1 and Table 1) is paralleled by a concurrent ~6-fold reduction in the relative content of H⁺-ATPase A6+A8 mRNA (see Figure 4 in ref. [11]). However, it should be stressed that steady-state amounts of mt-transcripts in developing liver are regulated by additional post-transcriptional levels of gene expression [11,32]. Therefore, the apparent discrepancy between mt-DNA copy number and mt-mRNA availability is just restricted to the stage of liver development when organelle differentiation occurs [7,10–12,33]. This finding further supports the idea of the existence of two different biological programmes (differen-

tiation versus proliferation) for exerting the expression of the two genomes that affect organelle biogenesis during liver development [11]. In fact, a constitutive transcription of mt-DNA during liver development (Figure 3 and Table 1 and see Results section) suggests that the replication of mt-DNA (Figure 1 and Table 1) is essential for maintaining steady-state levels of mt-transcripts compatible with mitochondrial proliferation [4,5,11,33]. In this regard, it should be stressed that the relative changes in the content of mt-DNA in the liver (Figure 1 and Table 1 and see ref. [25]) closely parallel the developmental changes in the transcription rates of the nuclear-encoded β -F₁-ATPase gene [11]. This parallelism strongly suggests that co-ordination in the expression of oxidative phosphorylation genes during proliferation of liver mitochondria is exerted at the transcriptional level on the nuclear genome and at the level of replication on the mt-genome, i.e. that mt-DNA is one of the major determinants of the expression of the mt-genome during proliferation of liver mitochondria. Regulatory links for the concerted expression of the two genomes at these levels have been discussed previously [1,34–41].

It has been suggested that mt-DNA transcription, both in cultured cells [27,42,43] and in rat liver [44], is not rate-limiting for mitochondrial biogenesis because its capacity exceeds actual cellular needs. Our findings do not support this hypothesis, at least when certain stages of development of the liver are considered, because the higher availability of mt-mRNAs [11] during differentiation of mitochondria appears to result from a profound increase in mt-mRNA half-lives [32]. Interestingly, the half-life of the nuclear-encoded β -F₁-ATPase mRNA is also much higher (4-fold) at earlier stages of development [11], strongly suggesting the existence of an additional mechanism for exerting the concerted expression of both genomes during organelle differentiation that involves the regulation of mRNA turnover [32].

Besides, the increase in liver content of COI and cyt *b* mRNAs observed during the first hour post-natal (Figure 2 and Table 1) suggests the existence of specific stabilization of these transcripts during organelle differentiation. In the specific case of COI and cyt *b* mRNAs, it seems reasonable to suggest that the mammalian homologue of the protein product of the yeast nuclear gene NAM1 [45,46] could perform such a task. However, although cyt *b* and COI mRNAs significantly increase in the neonatal liver (Figure 2 and Table 1), the relative amount of their translated products does not change in parallel (Figure 4), which might suggest that stabilization of these transcripts affects their translation (see below).

It is conceivable that the changes in mitochondrial mRNA/rRNA ratio observed during liver development (see Figure 4 in ref. [11]) could also result from a developmental change in the control of transcription initiation at the two promoter sites of the H-strand [27,47] or in the termination event at the end of the 16 S rRNA [48–50]. In fact, the prRNA is found to be one of the major synthesized transcription products in adult mitochondria, which is not the case in fetal and neonatal mitochondria (Figure 3 and Table 2). However, it is also possible that differences in the processing of the prRNA during development, more active in the fetal and neonatal than in the adult mitochondria (Table 2), could cause the difference in prRNA synthesis.

Translational regulation of mitochondrial gene expression during organelle differentiation and liver development

The maintenance of a constant level of 12 S rRNA during liver development [11], in spite of a concurrent increase in the number of mitochondria/hepatocytes [4,5,11], suggests that mitochondria

at early stages of development have a higher relative abundance of the translational machinery than at later stages. Likewise, the situation is even more favourable when the availability of mt-mRNAs/mitochondria is considered during development [11]. Therefore, if mitochondrial translation rates during liver development are expressed relative to these parameters, adult mitochondria have a higher efficiency of translation than fetal or neonatal mitochondria. However, the finding of a constant rate of mitochondrial translation during liver development (Figure 4), with the exception of the 1 h-old time-point (Figure 4), is consistent with the suggestion that only a fraction of mt-mRNAs is marked for translation [1,44], and with the finding that there is no change in the intrinsic capacity of mitochondrial translation during hepatic mitochondrial proliferation [30].

In yeast, a set of mutants with altered translation of specific mitochondrially encoded mRNAs has been reported [1,51,52]. However, in mammalian mitochondria it appears that translational activation at 1 h-post-natal affects the whole set of mitochondrial translation products equally (Figure 4). Thus, it is reasonable to suggest that the translational response at this stage of development has more to do with a general regulation of the translational machinery than with factors affecting translation of specific mRNAs [53]. In this regard, it has been suggested that the availability of cytoplasmically synthesized subunits of inner membrane complexes could define the rates of translation of mt-mRNAs [1,2].

Interestingly, mitochondrial differentiation is also coupled with a specific increase in the translational efficiency of the mRNA encoding mitochondrial β -F₁-ATPase on cytosolic poly-ribosomes [12]. Although the *cis*- and *trans*-acting factors affecting the translational efficiency of β -F₁-ATPase mRNA are still to be defined, it is tempting to suggest that regulation of mitochondrial translation during organelle differentiation is dependent on the previous activation of the translation of the nuclear-encoded transcript. The inhibition of the activation of mitochondrial translation observed in cycloheximide-treated neonates (Figure 5B and Table 3) supports this suggestion. This situation provides a mechanism for the co-ordination of the response of both genomes required for appropriate assembly of the inner membrane complexes, and thus for efficient mitochondrial biogenesis. In fact, the activation of the intrinsic activity of mitochondrial translation occurs at the stage of development in which the synthesis of nuclear-encoded subunits of the ATP synthase shows a profound increase [11,12] and both the respiratory and oxidative phosphorylation complexes of the mitochondria increase roughly 2-fold (Figure 5A and Table 3), as assessed both in the isolated organelle [7,8] and in the liver *in situ* [11]. In other words, the activation of mitochondrial translation takes place only at the stage of liver development in which mitochondrial structure and function has experienced a profound change [7,8,11].

In conclusion, differentiation of mitochondria in rat liver [7] provides the first example in which the expression of the two cellular genomes that affect biogenesis of mitochondria is exerted in concert at the translational level. The expression of nuclear [11,12] and mitochondrial genes (this study) is the result of transient activation of translation of oxidative phosphorylation transcripts. The operation of a programme of organelle biogenesis (differentiation) controlled at post-transcriptional levels of gene expression certainly provides a mechanism of regulation of nucleo-mitochondrial cross-talk that will allow cells to respond to environmental changes more quickly than a programme of biogenesis controlled at the level of transcription (proliferation). On the other hand, the operation of this programme assures the adaptation of the mammalian newborn to extra-uterine life,

because the onset of mitochondrial function is a pre-requisite for survival of the organism. It is obvious that future investigations aimed at identifying the *trans*-acting factors responsible for determining the translational efficiency of the transcripts will help our understanding of mammalian mitochondrial biogenesis.

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