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RESEARCH ARTICLE

Mitochondrial gene expression is regulated at multiple levels and differentially in the heart and liver by thyroid hormones

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Abstract Biogenesis of the oxidative phosphorylation system (OXPHOS) requires the coordinated expression of the nuclear and the mitochondrial genomes. Thyroid hormones play an important role in cell growth and differentiation and are one of the main effectors in mitochondrial biogenesis. To determine how mtDNA expression is regulated, we have investigated the response of two different tissues, the heart and liver, to the thyroid hormone status in vivo and in vitro. We show here that mtDNA expression is a tightly regulated process and that several levels of control can take place simultaneously. In addition, we show that the mechanisms operating in the control of mtDNA expression and their relevance differ between the two tissues, being gene dosage important only in heart while transcription rate and translation efficiency have more weight in liver cells. Another interesting difference is the lack of a direct effect of thyroid hormones on heart mitochondrial transcription.

Keywords mtDNA · Transcription · Translation · RNA stability · *In organello* · Thyroid hormones

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Introduction

Each cell type develops and maintains a specific oxidative phosphorylation (OXPHOS) capacity to satisfy its metabolic and energetic demands (Benard et al. 2006; Goffart and Wiesner 2003; Moyes and Hood 2003). For that, the coordinated expression of genes located in the nuclear and mitochondrial compartments is required. Different programs of regulation and levels of action are possible and, at present, there is no clear picture as to how this complex process is executed (Kelly and Scarpulla 2004; Moyes and Hood 2003; Enriquez et al. 1999b).

Thyroid hormones play an important role in cell growth and differentiation and have been known for a long time to regulate the respiratory capacity of cells (Gustafson et al. 1965), being one of the main effectors in mitochondrial biogenesis. In most tissues one of the more prominent effects of increased thyroid hormone levels is the stimulation of mitochondrial respiratory rate (Lanni et al. 2001; Pillar and Seitz 1997). This effect is brought about through non-genomic effects like changes in the lipid composition of the inner mitochondrial membrane, in intracellular Ca^{2+} concentration or in some enzyme activities (Davis and Davis 1996; Franco et al. 2006; Lanni et al. 2001; Pillar and Seitz 1997). In addition, thyroid hormones modify the expression of respiratory genes (Lanni et al. 2001; Mracek et al. 2005; Pillar and Seitz 1997) and other genes involved in ATP producing and consuming functions (Jansen et al. 2000; Klein and Ojamaa 2001; Portman et al. 2000). Thus, in liver cells, they modify transcription of some key nuclear genes and of all mitochondrial DNA-encoded genes (Enriquez et al. 1999a; Gadaleta et al. 1990; Mutvei et al. 1989; Van Itallie 1990). In the heart, the effect of thyroid hormones on mitochondrial protein synthesis (Cote and Boulet 1985; Leung and McKee 1990) and on other steps of mitochondrial gene expression has been

addressed (Goldenthal et al. 2004; Sheehan et al. 2004; Wiesner et al. 1994). From these studies it is well established that thyroid hormones influence mitochondrial biogenesis in the heart and liver, although contradictory reports on the nature and extent of the effects are frequent (Bahi et al. 2005; Das and Harris 1991; Goldenthal et al. 2005; Paradies et al. 1993; Sheehan et al. 2004; Wiesner et al. 1994) and a complete understanding on the genomic effects of these hormones, particularly on mtDNA expression, is still lacking.

Mammalian mtDNA encodes genes for 13 polypeptides that are essential subunits of the OXPHOS complexes I, III, IV and V and also for RNA components of the mitochondrial translation apparatus (2 rRNAs: 12S and 16S; and 22 tRNAs). The regulation of mitochondrial gene expression can take place at several steps, from gene dosage, transcription and translation, to assembly and turnover of the complexes. Most studies have focused on transcription regulation and although other regulation levels have been analyzed, their relative importance and hierarchy in each cell type and in each particular situation have not yet been defined (Enriquez et al. 1999a; Garstka et al. 1994; Goglia et al. 1999; Leung and McKee 1990; Polosa and Attardi 1991; Wiesner et al. 1994).

To get a deeper insight into the regulatory mechanisms operating in OXPHOS biogenesis and further analyze how thyroid hormone controls mtDNA gene expression, we have investigated the response of several mammalian tissues to thyroid hormone status in vivo and in vitro. Here, we report an integrated analysis of the thyroid hormones influence on heart and liver mitochondrial gene expression at different levels. First of all, we have confirmed the validity of our experimental approach by determining the changes in cytochrome c oxidase (COX) activity in the different thyroid states. Then, to characterize the possible regulatory steps in mitochondrial gene expression in response to thyroid hormones, we searched for changes in mtDNA and mtRNA concentrations in vivo, and in mitochondrial RNA and protein synthesis using an *in organello* system. Thus, we describe the existence of several control levels in mtDNA expression that overlap in the regulation and adaptation of the respiratory capacity to physiological situations. Furthermore, we show that heart and liver cells respond differently to thyroid hormones, supporting the idea that mtDNA expression is differentially regulated, in a tissue-specific manner, in its contribution to mitochondrial biogenesis.

Materials and methods

Animals and treatments

Hypothyroidism was induced in male Wistar rats weighing 150–200 g by administration of 0.05% (w/v) propylthiouracyl in drinking water for 5–8 weeks. A subgroup of hypothyroid rats (treated animals) was injected intraperitoneally once a day with 20 μg and 3 μg/100 g body weight, of T_4 (3,3′,5,5′-tetraiode-L-thyronine) and T_3 (3,3′,5-triiode-L-thyronine), respectively, for 2 days and the animals were killed 15 h after the second treatment. Control hypothyroid and euthyroid rats (referred to hereafter as hypothyroid and control animals, respectively) were injected for the same time period with the same volume of vehicle (0.9% NaCl/propylenglycol). These treatments produce clear and significant differences in the blood hormone levels among the three animal groups (Enriquez et al. 1999a).

The organs were removed from animals, placed on ice-cold medium A (0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4), weighed, washed and homogenized as described earlier for the liver (Enriquez et al. 1999a) and heart (Asín-Cayuela et al. 2001; Fernández-Vizarra et al. 2006).

COX and CS activity measurement

COX and CS activities were measured on heart and liver total homogenates by spectrophotometric standard methods (Acin-Perez et al. 2003; Fernández-Vizarra et al. 2006).

Protein quantification

Total sample protein concentration was measured using Bio-Rad's protein assay on the basis of the Bradford dye-binding procedure.

Nucleic acids extraction and quantification

DNA. Total DNA was extracted from liver and heart total homogenates by proteinase K digestion and phenol extraction followed by ethanol precipitation. Total DNA was transferred to nylon membranes using a Schleicher and Schuell Minifold® II Slot–Blot manifold. The slot–blot membranes were hybridized with specific radioactive probes for the rat 12S mitochondrial rDNA and 18S nuclear rDNA, generated by PCR and labeled using Roche's "High Prime" kit. Membranes were prehybridized for 4 h, at 68°C, in 6× SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 0.1 mg/ml salmon sperm ssDNA and 5× Denhardt's solution, and hybridized overnight under the same conditions but adding the ^{32}P-labeled probe.

RNA. Total RNA was extracted from rat liver and heart using Promega's "RNAgents® Total RNA Isolation System". Quantification of mitochondrial transcripts was performed by Northern blot; 5 μg of total RNA was separated through methylmercury hydroxide/agarose gels (see below) and then blotted into nylon membranes, using a vacuum blotting system (Pharmacia Biotech). The RNA

membranes were prehybridized for 4 h, at 42°C, in 40% formamide, 6× SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 0.1 mg/ml salmon sperm ssDNA and 5× Denhardt's solution, and hybridized overnight under the same conditions but adding the ^{32}P-labeled probe.

Following hybridization, the membranes were washed and then exposed for autoradiography at −70ºC with a DuPont screen intensifier. Quantification of the relative amount of DNA or RNA was carried out in the autoradiograms, after selecting the appropriate exposures, using an LKB Ultroscan XL laser densitometer and a GelScan XL software.

In organello RNA synthesis and pulse–chase experiments

Mitochondria were isolated from the rat heart as previously described (Fernández-Vizarra et al. 2006) and suspended at a final concentration of 2 mg/ml of mitochondrial protein in 0.5 ml of incubation buffer [25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM K_2HPO_4, 0.05 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 10 mM glutamate, 2.5 mM malate, 10 mM Tris-HCl, pH 7.4 and 1 mg/ml of bovine serum albumin (BSA)], in 1.5 ml Eppendorf tubes. For *in organello* transcription analysis, 20 μCi of [α-^{32}P]-UTP (400–600 Ci/mmol) was added to the medium and incubation was performed at 37°C for 60 min in a rotary shaker (12 rpm). For pulse–chase experiments, isolated mitochondria were prelabeled with [α-^{32}P]-UTP for 2 h, then the mitochondrial samples were pelleted at 13,000 g_{av} for 1 min and the supernatant with the non-incorporated [α-^{32}P]-UTP was removed. Then, the mitochondria were resuspended in fresh incubation medium in the presence of a 200-fold excess of cold UTP and incubated for several periods of time (chase) before harvesting. Mitochondrial nucleic acids were extracted and analyzed by methylmercury hydroxide/agarose gels as described earlier (Enriquez et al. 1996; Enriquez et al. 1999a). After running, the gels were first stained with ethidium bromide and photographed under UV light, and then dried and exposed for autoradiography either at −70ºC with a DuPont screen intensifier, or at room temperature.

In organello protein synthesis

An *in organello* system optimized to maintain the translation capacity of the isolated organelles in a way that could closely reproduce the in vivo situation was developed for this study. We assayed different incubation conditions, finding that the inclusion in the incubation buffer of inhibitors of cytoplasmic protein synthesis and of all 20 amino acids yielded the best results. Under these conditions, heart and liver mitochondria were able to maintain a linear translation activity for about 1 h (not shown) and produced a pattern of labeled polypeptides very similar to that obtained with intact cells. Isolated mitochondria from the rat liver and heart were suspended at a final protein concentration of 2 mg/ml in 0.5 ml of incubation buffer, in 1.5 ml Eppendorf tubes. For the *in organello* protein synthesis assays >75 μCi of Amersham's PRO-MIX^{TM} L-[^{35}S] in vitro Cell Labelling Mix (>1,000 Ci/mmol, labeled methionine plus cysteine), as well as 0.1 mg/ml Emetine, 0.1 mg/ml Cycloheximide and 10 μM of the 20 L-amino acids (including methionine and cysteine), were added to the medium and incubation was performed at 37°C for 30 min in a rotary shaker (12 rpm). The mitochondrial samples were then pelleted at 13,000 g for 1 min, washed twice with 1 ml of 0.32 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and lysed with 5× sample buffer. The labeled mitochondrial proteins were electrophoresed as described earlier (Chomyn 1996), gels were dried and exposed for autoradiography at −70ºC with a DuPont screen intensifier. Quantification of the amount of protein was carried out in the autoradiograms, as described above for the DNA and RNA measurements.

Statistical analysis

Differences between means were evaluated by ANOVA and unpaired Student's *t* test using the Stat View 5.0 software.

Results

The thyroid status affects COX activity/cell

Cytochrome c oxidase (COX) and citrate synthase (CS) activities as well as total protein, mtDNA and nDNA content were determined in liver and heart samples from control, hypothyroid and hypothyroid animals injected with thyroid hormones, as described in "Materials and methods".

In the liver, COX-specific activity or COX/CS activity ratio did not show significant changes with any of the treatments, pointing to a parallel change in mitochondrial and total cell protein content. However, COX activity per cell (normalized by the nDNA content) was reduced to around 50% in the hypothyroid situation when compared to the euthyroid situation. The treatment of hypothyroid animals with hormones partially recovered this activity (72% compared to controls) (Table 1). In the heart, COX-specific activity or normalized by CS showed a significant increase with thyroid hormone treatment (Table 1). When the nDNA content in the heart homogenate was used to normalize, a decrease of around 30% was found in the case of the hypothyroid situation which was reduced to less than 10% in the case of the treated animals (Table 1). In summary, COX

activity per cell, as an index of respiratory capacity, is affected in both tissues by the thyroid hormone status.

Differential effects of the thyroid status on heart and liver mtDNA and mtRNA levels

The relative level of mtDNA per cell was estimated in each sample from the hybridization signal ratio between the mitochondrial and the nuclear probes. As shown in Table 1, the hypothyroid hearts presented a significant reduction in their mtDNA content per cell (mtDNA/nDNA) to around 65% when compared with controls, which was partially restored (to 72%) in the treated animals. On the other hand, none of the treatments significantly modified the mtDNA levels in the liver (Table 1).

Total RNA from control and hypothyroid samples was run on denaturing gels and blotted to nylon membranes for hybridization with specific probes for 12S and 16S rRNAs and for Cyt b and COI mRNAs (Fig. 1a). In both tissues, the steady-state levels of these mt-RNAs, were significantly decreased in the hypothyroid situation when compared to the controls. In the heart, the average level of both the mRNAs and rRNAs, normalized by the mtDNA content, was reduced to around 50% (Fig. 1b). When considering the transcript levels on a "per cell" basis, an even larger reduction, to about 35% of the control levels was found in hypothyroid heart. Since this decrease was similar in both kinds of transcripts, the mRNA/rRNA ratio in heart hypothyroid mitochondria was not affected (Fig. 1c). On the other hand, in hypothyroid liver, an even bigger drop in the level of mRNAs per mitochondria (and per cell) was found (to around 20%) but not for the rRNAs, which were reduced only to around 35% of the control levels (Fig. 1b). Thus, contrarily to heart organelles, in liver mitochondria the mRNA/rRNA ratio was significantly diminished in the hypothyroid status (Fig. 1c).

Effect of the thyroid status on heart *in organello* transcription capacity and RNA stability

Using isolation and incubation conditions previously optimized for heart mitochondria (Asín-Cayuela et al. 2001; Fernández-Vizarra et al. 2006) we have analyzed the transcription capacity of isolated organelles obtained from the three groups of animals, under the same conditions. The results were normalized, for comparative purposes, by the mtDNA content of each mitochondrial preparation.

Hypothyroid organelles showed a marked reduction in total RNA accumulation capacity as compared to the controls (47% of control values) and there was a significant recovery, up to 90% of control values in the organelles from hypothyroid hormone-treated animals (Fig. 2a, b). The decrease in the total RNA synthesis in the hypothyroid mitochondria was not the same for the different RNA types. When the relative labeling proportion was compared, taking three mRNAs (Cyt b and ND4 + COI) and one rRNA (16S) as representatives, a higher reduction for the rRNAs than for the mRNAs became evident (Fig. 2a, c). Thus, if the labeling ratio mRNA/rRNA was set to 1 for the control organelles, the hypothyroid mitochondria showed an increase in the mRNA/rRNA average ratio to 1.7, while the mitochondria from thyroid hormone-treated animals showed an mRNA/rRNA relative synthesis rate of 1.3 (Fig. 2c).

Since the differences observed in RNA accumulation can be affected by changes in RNA stability, in addition to changes in synthesis rate, we estimated the half-lives of the transcripts synthesized *in organello* by performing pulse–chase experiments using control and hypothyroid heart

Table 1 Effect of the thyroid hormone status on cytochrome c oxidase (COX) and citrate synthase (CS) activities and on mtDNA levels per cell (mtDNA/nDNA)

	COX-specific activity (UI/g protein)	COX/CS ratio	COX/nDNA (% control)	mtDNA/nDNA (% control)
(a) Heart				
Control ($n = 5$)	748 ± 116	0.262 ± 0.041	100 ± 30	100 ± 9
Hypothyroid ($n = 5$)	748 ± 177	0.362 ± 0.097	72 ± 15	65 ± 4***
Treated ($n = 3$)	1273 ± 32**	0.544 ± 0.027***	92 ± 13	72 ± 7**
(b) Liver				
Control ($n = 5$)	172 ± 57	1.248 ± 0.343	100 ± 22	100 ± 32
Hypothyroid ($n = 5$)	141 ± 59	1.458 ± 0.414	52 ± 9 **	96 ± 19
Treated ($n = 3$)	150 ± 25	1.574 ± 0.326	72 ± 25	80 ± 15

Results (mean ± SD) for the COX-specific activity and the ratio between COX/CS-specific activities, as well as for the COX activity normalized per cell (per amount of nuclear DNA, expressed as the percentage of the mean control value ± SD), obtained in heart (a) and liver (b) total homogenates from animals in the indicated thyroid state. mtDNA/nDNA ratio for the heart and liver samples, expressed as the percentage of the mean control value ± SD

Asterisks represent the level of statistical significance of the differences with respect to the control samples according to Student's *t* test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$

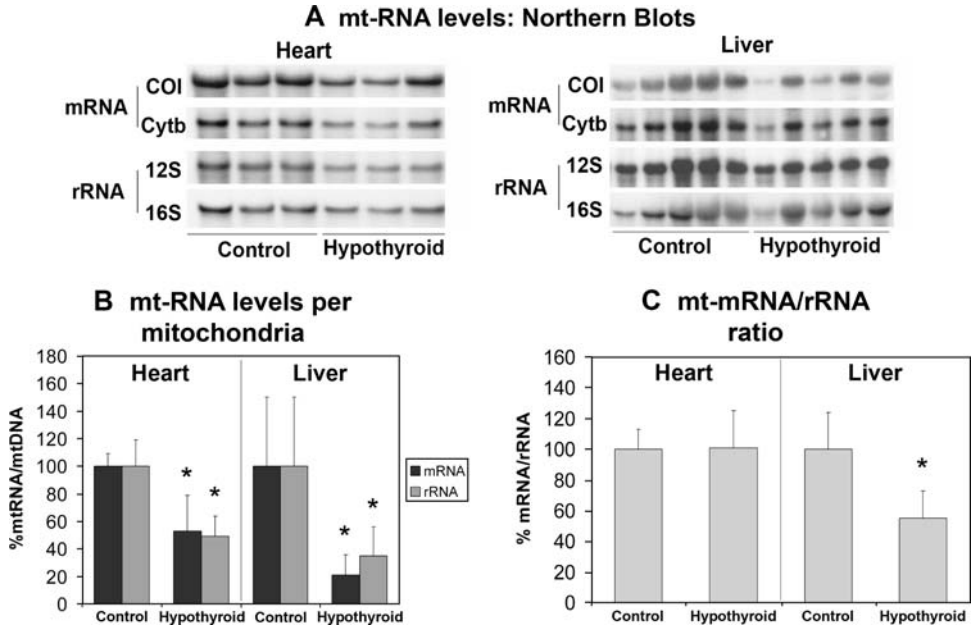

Fig. 1 Effect of the thyroid status on mtRNA steady-state levels. Quantification of mitochondrial RNAs in control and hypothyroid samples by Northern blot hybridization, **a** hybridization of total heart and liver RNA from control and hypothyroid animals, using specific probes for two mt-mRNAs (COI and Cyt b) and for the two mt-rRNAs (12S and 16S). **b** Levels of mitochondrial mRNA and rRNA: the plotted values represent the mean rRNA (12S and 16S) and mRNA (COI and Cytb) signals, in control and hypothyroid hearts and livers, normalized per mitochondria (per amount of mtDNA), and **c** steady-state mRNA/rRNA ratio expressed as percentage of the mean control value ± SD in control (liver $n = 6$; heart $n = 3$) and hypothyroid (liver $n = 6$; heart $n = 3$) samples. *Asterisks* represent the level of statistical significance of the differences with respect to the control samples according to Student's t test (as in Table 1)

mitochondria. After the initial pulse, the labeled RNAs were extracted at different times of chase and analyzed by electrophoresis and autoradiography (Fig. 2d). The labeling of the different RNA species decreased linearly with the chase time allowing to calculate their half-lives (Table 2). Thus, the overall half-life of the transcripts was 186 min for the control and it increased to 290 min for the hypothyroid mitochondria (ratio hypothyroid/control 1.56). However, this increase in stability was not uniform between the different RNAs, being much more marked for the rRNAs (1.7-fold for 16S rRNA) than for the mRNAs (1.1-fold for COI and Cyt b) and hence in the direction of compensating the changes observed in transcription rate (Fig. 2d; Table 2).

In conclusion, in mitochondria isolated from hypothyroid heart there is a general decrease in transcriptional rate, more pronounced for the rRNAs, combined with a stabilization of the *in organello* synthesized transcripts more marked for the rRNAs that may explain the maintenance of the mRNA/rRNA ratio found in the in vivo steady state.

In vitro addition of TH to isolated heart mitochondria has no effect on transcription

We have previously described the existence of a direct TH action on liver mitochondrial transcription, more precisely on the relative mRNA/rRNA transcriptional rate (Enriquez et al. 1999a). Here, we analyzed the possibility of a similar effect by adding different concentrations of T_3 to the incubation buffer of isolated control and hypothyroid heart mitochondria. Contrarily to what was found in liver, the direct addition of hormone (even at concentrations up to 500 pg/ml) had no effect on heart mitochondria, neither on the total transcription capacity nor on the relative mRNA/rRNA transcriptional rate (Fig. 2e).

Effect of the thyroid status on the *in organello* mitochondrial translation capacity

As described earlier for the transcription capacity, isolated organelles can be used to measure the protein synthesis activity. The optimization of the *in organello* incubation conditions (see "Materials and methods" for details), allowed us to obtain a pattern of mitochondrial-labeled peptides very reproducible and very similar to that obtained in vivo (Fig. 3a). Mitochondria from the hypothyroid heart showed only a 30% reduction in their total translation capacity (normalized by mtDNA content) as compared to control organelles, while for liver hypothyroid mitochondria this reduction was around 50% (Fig. 3a, b).

Since we had determined the amount of two mRNAs (COI and Cyt b) in the steady state, we could estimate their relative translation efficiency, as the ratio between mRNA

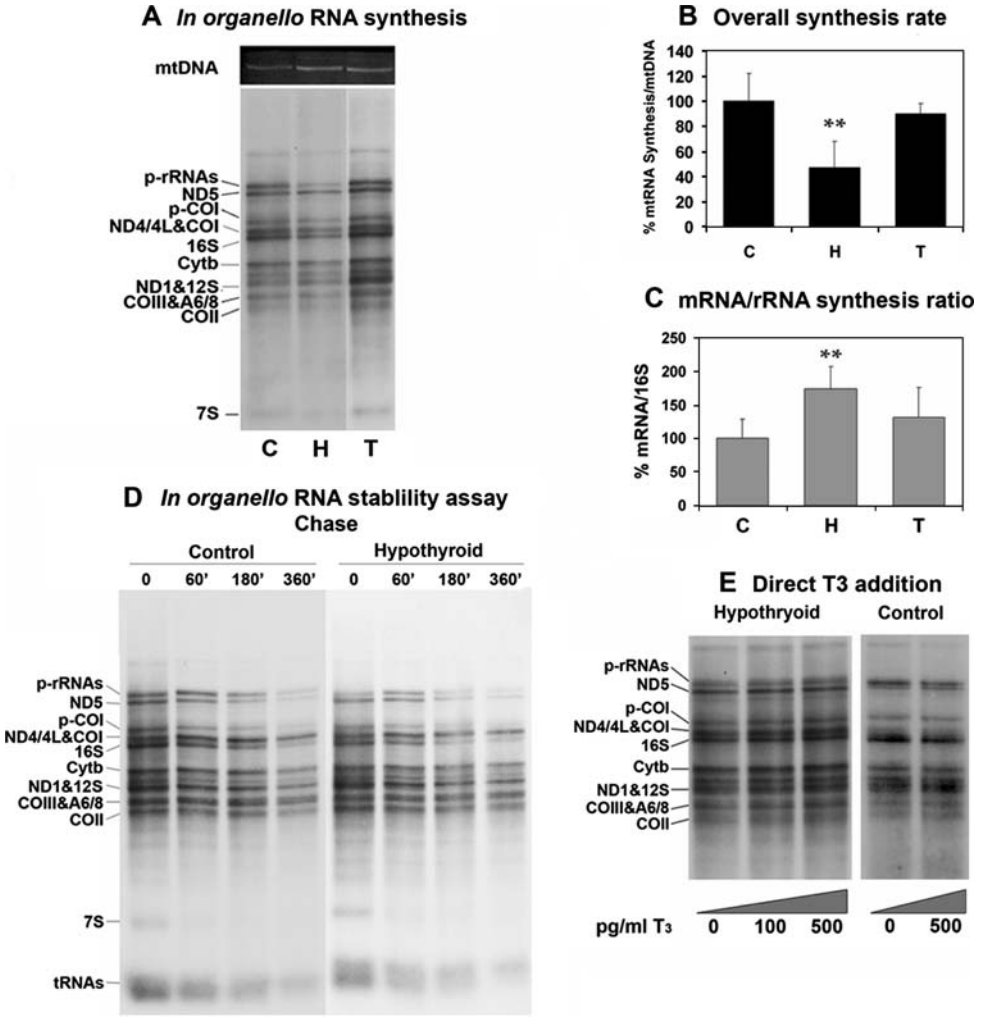

Fig. 2 *In organello* RNA synthesis and stability using isolated heart mitochondria obtained from animals in different thyroid status. **a** Example of the electrophoretic pattern obtained with the heart *in organello* transcription assays. **b** Mt-RNA overall synthesis rate in isolated heart mitochondria, expressed as the percentage of the mean control value ± SD, and normalized by mtDNA. **c** control ($n = 7$); *H* hypothyroid ($n = 7$); *T* hypothyroid treated ($n = 3$). **c** mt-mRNA/rRNA synthesis ratio expressed as the percentage of the mean control value ± SD. *C* control ($n = 7$); *H* hypothyroid ($n = 7$); *T* hypothyroid treated ($n = 5$). *Asterisks* represent the level of statistical significance of the differences with respect to the control samples according to Student's *t* test. **d** Stability of the mitochondrial transcripts in mitochondria from hearts of control and hypothyroid animals. Electrophoretic pattern of a pulse–chase *in organello* transcription assay using isolated heart mitochondria. The chase times are read in the figure: 0 (pulse), 60, 180 and 360 min. **e** Effect of in vitro T_3 addition on isolated heart mitochondria transcription activity. Autoradiogram of a representative *in organello* transcription assay using mitochondria isolated from hypothyroid and from control heart in the presence of the indicated T_3 concentrations. ND1, ND5, and ND4/4L, are the mRNAs for subunits 1, 5 and 4 plus 4L of the NADH Dehydrogenase; *Cytb* Cytochrome b; *p-COI* COI, COII and CO III are the precursor (p-) and mature mRNAs for subunits I, II and III of Cytochrome c oxidase subunits; A6/8, is the mRNA encoding ATP synthase F0 subunit 6 and 8, p-rRNAs is a precursor of the mature rRNAs; 7S is a noncoding transcript from mtDNA L-strand. Other symbols are as in Fig. 1

abundance and polypeptide labeling, in control versus hypothyroid organelles. Thus, if the translation efficiency for the average of COI and Cyt b in the control situation was set to 1, in hypothyroid heart mitochondria the value was of 1.35, while in hypothyroid liver it was 2.05 (Fig. 3b). These results suggest an increase in translation efficiency, which, if operating in vivo, would partially compensate the drop in mRNA levels and thus reduce the final effect on OXPHOS capacity caused by the hypothyroid situation.

Discussion

The energetic demands and the metabolic role of the mtETC can vary substantially between different cell types and in different physiological situations requiring an adaptation in mitochondrial biogenesis and particularly in OXPHOS complexes biogenesis and function. OXPHOS complexes biogenesis depends on the expression of more than 100 nuclear genes and of 13 protein products of the mtDNA.

Table 2 Effect of the thyroid hormone status on mitochondrial transcript stability

	Control	Hypothyroid	*H*/*C* ratio
rRNA			
16S	156.3 ± 73.8	263.3 ± 95.6	1.68
mRNAs			
ND4/4L&COI	448.0 ± 176.8	492.7 ± 100.1	1.10
Cytb	306.3 ± 60.1	330.0 ± 71.6	1.08
Average (total RNA)	186.0 ± 46.8	290.3 ± 113.2	1.56

Half-life values in minutes (mean ± SD), determined from three independent *in organello* pulse–chase experiments (see Fig. 3d), expressing the stability for the overall RNA synthesis (average) as well as for several individual transcripts in control and hypothyroid heart mitochondria; *H*/*C* ratio: relative half-life values between the hypothyroid and control samples

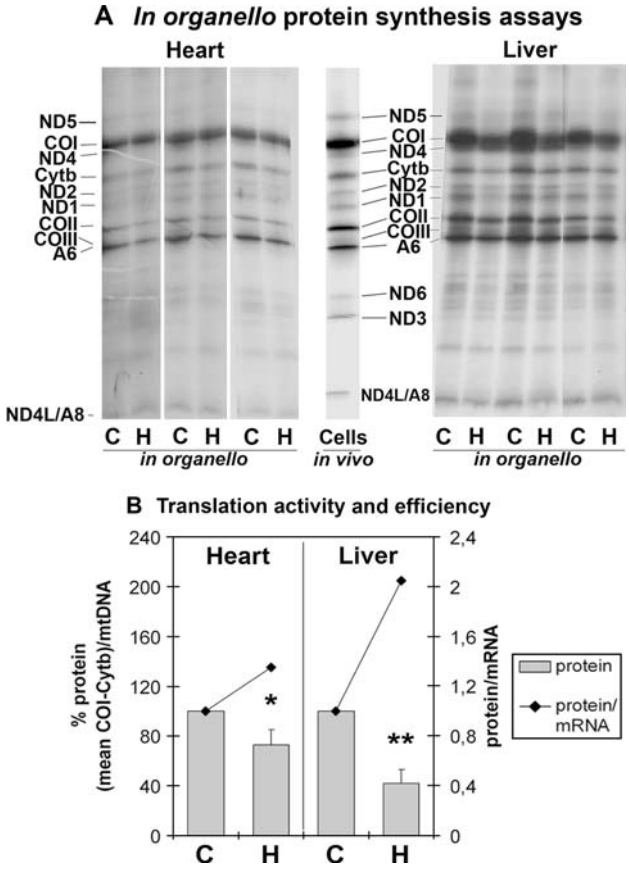

Fig. 3 *In organello* protein synthesis using isolated heart and liver mitochondria from control and hypothyroid animals. **a** Electrophoretic patterns obtained in the *in organello* protein synthesis assays. The result of a mitochondrial protein in vivo labeling using cultured mouse whole cells is also shown for comparison. *C* control; *H* hypothyroid. ND1, ND2, ND3, ND4, ND5, ND6, and ND4L are subunits 1 to 6 and 4L of the NADH dehydrogenase; *Cytb* cytochrome b; *COI*, COII and COIII are cytochrome c oxidase subunits 1 to 3; A6, A8, ATP synthase F0 subunits 6 and 8. **b** Total mitochondrial translation activity (*columns*) and efficiency (*lines*) estimated for the average of two individual proteins (COI and Cyt b) considering their mRNA steady-state concentration in control ($n = 3$) versus hypothyroid ($n = 3$) mitochondria. The results are expressed as the percentage ± SD of controls, normalizing by the amount of mtDNA present in each sample determined by slot–blot and hybridization (not shown). *Asterisks* represent the level of statistical significance of the differences between the hypothyroid and the control samples according to the ANOVA test (*$P < 0.05$)

Overall, the present evidence supports the existence of a positive correlation between the thyroid hormone levels and the respiratory capacity of the cells, although contradictory results can be found in the literature, either for liver (Irrcher et al. 2003; Lanni et al. 1993; Sheehan et al. 2004; Soboll 1993; Wiesner et al. 1992) or for heart (Das and Harris 1991; Goldenthal et al. 2005; Irrcher et al. 2003; Paradies et al. 1993; Sheehan et al. 2004; Stevens et al. 1995; Tanaka et al. 1985; Wiesner et al. 1994). In addition to the methodological differences, one of the problems in the origin of such heterogeneous results is the use of different normalization criteria to express the mitochondrial activities (gram of tissue, total protein, mitochondrial protein, etc.). To minimize these problems, we have chosen to normalize the mitochondrial activities and other parameters by an index of the cell number (nDNA) or by an index of mitochondrial content (mtDNA). This kind of normalization seems to us more adequate when looking at changes in gene expression since it allows the comparison between tissues and within the same tissue in situations in which the protein content and the cell size and structure can vary, such as under different thyroid states (Calamita et al. 2007; Jakovcic et al. 1978; Tata et al. 1963).

There are several reports in the literature describing effects of the thyroid hormones on mtDNA expression (Enriquez et al. 1999a; Goglia et al. 1999; Wiesner et al. 1994; Garstka et al. 1994; Leung and McKee 1990) being transcription the parameter analyzed by most studies. However, few reports correlate the changes in mtDNA expression with enzyme activity and check simultaneously different levels of gene expression to determine their hierarchy and relevance. We have summarized our results for the hypothyroid versus control situation in liver and heart in Fig. 4. Thus, the first level of regulation analyzed, mtDNA dosage, responds to thyroid hormone in the heart but not in the liver. This result contrasts with a previous report where the mtDNA levels in heart were found unaffected in hypothyroidism (Wiesner et al. 1994), although a more indirect normalization method was used and a much larger range of variation (over fourfold) in the mtDNA values was found there. It should be noted that in skeletal muscle, even if it is different from cardiac muscle, mtDNA dosage has been proposed as the main mechanism involved in mitochondrial biogenesis during endurance training (Williams 1986). On the other hand, the absence of responses in liver for this regulation level is in agreement with previous results (Goldenthal et al. 2004; Van Itallie 1990; Wiesner et al. 1992).

The steady-state concentration of mitochondrial transcripts is reduced in hypothyroidism in both tissues,

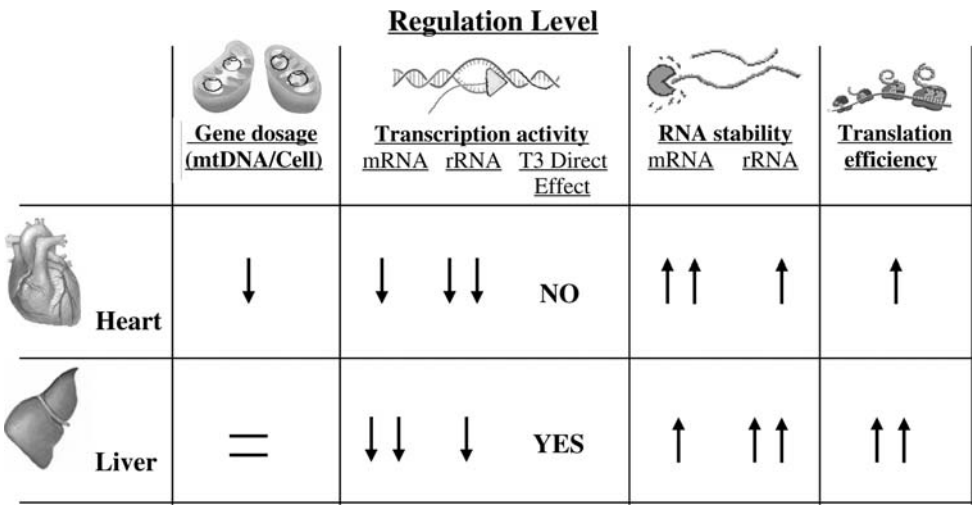

Fig. 4 Differential effects of hypothyroidism on mtDNA expression levels in the heart and liver. Summary of the relative changes in the regulatory levels analyzed for heart and liver mtDNA expression. The direction and scale of the changes induced in the hypothyroid situation, compared to the control, is shown. *Equal symbol* no change, *down arrow* decrease, *up arrow* increase

although more markedly in the liver, which is in agreement with earlier reports (Enriquez et al. 1999a; Gadaleta et al. 1990; Mutvei et al. 1989; Sheehan et al. 2004; Van Itallie 1990; Wiesner et al. 1992, 1994). The reduction in mtRNAs in hypothyroid organelles can be brought about by a general decrease in the transcription activity normalized per mtDNA as suggested by the *in organello* assays. Furthermore, in both organs there was a differential effect on the two types of transcripts but with an opposing sign, being the synthesis of the mRNAs preferentially decreased in liver (Enriquez et al. 1999a) while in the heart the rRNAs were more affected (Fig. 4). In addition to the transcriptional rate, we have observed, also *in organello*, changes in RNA stability in both tissues, namely, a stabilization of all transcripts in the hypothyroid situation compared to controls. However, in hypothyroid heart the rRNAs were preferentially stabilized over the mRNAs, while in liver the opposite was observed (Enriquez et al. 1999a). The RNA stability can be a relevant regulatory step since it affects both the steady-state concentration of transcripts and the mRNA/rRNA ratio, which in turn may condition protein synthesis (Garstka et al. 1994; Polosa and Attardi 1991).

Isolated hypothyroid mitochondria show a reduced translation capacity in both tissues. This is most probably a consequence of the drop in the concentration of transcripts, ribosomal and messenger RNAs (and probably tRNAs as well). However, when we estimated the translation efficiency (ratio between a given mRNA abundance and its polypeptide labeling), a significant increase was found in the hypothyroid situation, especially in the liver (around twofold). This increase in efficiency would act to partially compensate the drop in RNA levels, and could be attributed in liver to a higher relative availability of ribosomes for each messenger to be translated. However, in heart we have also observed an increase in translation efficiency, although more moderate (1.5-fold), and since in this organ the mRNA/rRNA ratio remains unchanged, a different mechanism must be invoked. Again, this step of translation efficiency represents a relevant regulatory point not very often considered in mtDNA gene expression studies, and shows quantitative differences among the two tissues (Figs. 3b, 4). All these changes in gene expression are associated with a parallel modification of the complex IV activity, as an index of the function of the mtETC. Thus, hypothyroidism induced a drop in COX activity and the treatment of the hypothyroid animals with thyroid hormones partially recovered the COX activity per cell in both tissues. However, while in heart this was paralleled by an increase in the mtDNA levels, as well as in the transcription capacity, in liver only the transcription was recovered in parallel (Enriquez et al. 1999a) and there was no change in mtDNA levels.

Finally, an important difference in the response of both tissues to TH is the lack of a direct effect of T_3 on isolated heart mitochondrial transcription. Part of the effect of the hormone on transcription in liver was exerted directly on the mitochondria (Enriquez et al. 1999a), probably through receptors located in the organelle (Wrutniak et al. 1995; Casas et al. 1999) and mediating the selection of the two H-strand promoters (Enriquez et al. 1999a). This difference between tissues could be explained by a different distribution of thyroid receptor isoforms (Brent 1994; White and Dauncey 1999) and would reflect the diverse metabolic roles of the mtETC in each cell type (Goffart and Wiesner 2003; Moyes and Hood 2003). In this respect, it has been reported that the cardiac tissue is less responsive than liver to thyroid hormones in the expression of mtDNA-encoded COX subunits (Irrcher et al. 2003; Sheehan et al. 2004). This fact could be related to the presence in heart mitochondria of versions of the TRα2 receptor, the dominant negative isoform lacking a functional ligand-binding domain (Morrish et al. 2006).

Although the *in organello* results cannot be taken as a quantitatively exact indication of the in vivo situation, we and others have previously demonstrated that isolated mitochondria from several sources can maintain their biogenetic activities, showing differences according to the organ and physiological situation of origin, and that are able to respond to changes in the incubation conditions (Leung and Mckee 1990; Enriquez et al. 1996; Enriquez et al. 1999a; Asín-Cayuela et al. 2001; Fernández-Vizarra et al. 2002).

In summary, our results show that mtDNA expression is a tightly regulated process where several levels of control can take place simultaneously for the fine-tuning of energy metabolism, illustrating the complexity of the response to a metabolic signal and the need to analyze those different levels of control to better understand the final effect in cell function. Furthermore, we find that the steps and mechanisms to control mtDNA expression and their relevance clearly differ between liver and heart (Fig. 4). Briefly, in the thyroid hormone control of mtDNA expression, gene dosage and transcription are the more relevant points in heart while transcription rate (partly controlled by a direct effect on the organelles) and translation efficiency have more weight in liver. All these differences contribute and are part of the mechanisms that establish the physiological diversity of the OXPHOS system in the various cell types (Benard et al. 2006).

Concerning the mechanisms that could mediate the observed changes, it is probable that several regulatory circuits and feed-back mechanisms controlling gene expression, some of them tissue-specific, are involved. Still, regulatory steps downstream from gene expression like allosteric or covalent modification of enzyme activities can also contribute to the final OXPHOS output (Franco et al. 2006; Harper and Brand 1993). More studies are needed to identify the factors and signaling pathways that trigger and coordinate those effects.

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