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Thyroid hormones promote transcriptional activation of the nuclear gene coding for mitochondrial β-F_1-ATPase in rat liver

José M. Izquierdo and José M. Cuezva

Departamento de Biología Molecular, Centro de Biología Molecular (UAM-CSIC), Universidad Autónoma de Madrid, 28049 Madrid, Spain

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Thyroid hormones acutely regulate gene expression of the β-catalytic subunit of the mitochondrial F_1-ATPase complex in the liver of hypothyroid rat neonates at either a transcriptional and/or post-transcriptional level [(1990) J. Biol. Chem. 265, 9090–9097]. Administration at birth of various thyroid hormone doses to hypothyroid newborn rats promote a rapid (1 h) increase in liver steady-state amounts of both β-F_1-ATPase protein and mRNA. Induction of the β-F_1-ATPase mRNA is coincident with an elevation in gene transcription detected using nascent RNA chains synthesized by isolated nuclei. These results suggest that thyroid hormones induction of postnatal mitochondrial differentiation in the liver of hypothyroid rat neonates is mostly triggered by transcriptional regulation of β-F_1-ATPase gene.

β-F_1-ATPase transcription; Thyroid hormone; Mitochondrial differentiation; Newborn; Liver

1. INTRODUCTION

The rapid switch in metabolic pathways relevant for energy provision in the liver, from the predominant fetal anaerobic glycolysis [1,2] to the efficient neonatal oxidative phosphorylation [3,4], affords an excellent experimental system for the study of mammalian mitochondrial biogenesis [4,5]. Mitochondrial biogenesis in rat liver has a postnatal onset and results from the operation of two processes, proliferation and differentiation of the organelle [4,6], both resulting from a coordinated expression of the two genetic systems in which mitochondrial proteins are encoded [7,8]. Mitochondrial proliferation in rat liver is a continuous process that spans the whole developmental period [6], while differentiation of the organelle, i.e., the acquisition of the ultrastructural, molecular and functional characteristics that define mitochondrial function, precisely takes place during the first hour following birth [3–5]. It has been recently documented that regulation of the expression of nuclear-encoded proteins needed for mitochondrial differentiation is exerted at the translational level [9]. Furthermore, short- and long-term effects of thyroid hormones are known to affect mitochondrial functions, number and molecular structure (for references see [5]). Modulation of gene expression through the control of DNA transcription is the first stage at the molecular level for induction of a specific gene by thyroid hormones [10]. However, additional levels of regulation of gene expression have been reported to be modulated by thyroid hormones [11–15]. Regulation of mitochondrial biogenesis by thyroid hormones [16] has been shown to promote a coordinated up-regulation of all mitochondrial mRNAs [17] and a differential response in gene activation for those nuclear genes coding for mitochondrial proteins [18]. Recently, we have explored the role of thyroid hormones in rat liver postnatal mitochondrial differentiation [5]. We used, as a marker protein of mitochondrial biogenesis during this stage of development, the nuclear encoded β-catalytic subunit of the mitochondrial F_1-ATPase complex [5]. The results obtained indicate that thyroid hormones regulate the basal expression of β-F_1-ATPase gene at either a transcriptional and/or post-transcriptional level [5]. We report herein, using in vitro run-on assays of isolated liver nuclei from hypothyroid rat neonates treated at birth with various thyroid hormones doses, that the hormonal treatment affects transcriptional rates of the β-F_1-ATPase gene, suggesting that nuclear transcription is the major site of thyroid hormone action on F_1-ATPase gene expression for postnatal mitochondrial differentiation.

Correspondence address J.M. Cuezva, Centro de Biología Molecular, Universidad Autónoma de Madrid, 28049 Madrid, Spain. Fax: (34) (1) 397 4799.

Abbreviations. T4, 3,5,3′,5′-tetraiodo-L-thyronine; T3, 3,5,3′-triiodo-L-thyronine; TRE, thyroid hormone-responsive elements; MMI, 1-methylimidazole-2-thiol; SSC, standard saline citrate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride.

2. MATERIALS AND METHODS

2.1 *Animals and treatments*

Timed-pregnant albino Wistar rats weighing 200 g were housed under controlled conditions [3]. Maternal and fetal hypothyroidism

were induced by administration of 0.02% MMI in the drinking water of pregnant rats from day 14 of gestation [5]. Term hypothyroid fetuses were delivered and maintained at 37°C without feeding as described [5]. At the time of delivery, hypothyroid newborns were injected intraperitoneally with 50 μl of a 0.9% NaCl solution containing 0 + 0 (0 × dose); 4.5 + 16.6 (1 × dose) or 45 + 166 (10 × dose) μg/100 g body weight of T3 + T4, respectively.

2.2. *Nuclear transcription assays*

Nuclei from 1 g of one hour-old hypothyroid and (T3 + T4)-treated hypothyroid neonatal rat livers were prepared by centrifugation through a 2 M sucrose cushion as described in [19] and modified in [20]. Washed isolated nuclei were resuspended in 25% glycerol, 50 mM Tris-HCl; pH 8, 5 mM magnesium acetate, 0.1 mM PMSF, 0.1 mM EDTA and 5 mM DTT. Aliquots of diluted nuclei were (i) stained with 4% Giemsa solution, visualized and counted under light microscopy and (ii) used for the determination of $A_{260\,nm}$. A linear correlation ($m = 0.33$, $b = 0.004$, $r = 0.976$, $n = 19$, $P < 0.001$) between the number of nuclei ($0–9 \times 10^6$) and $A_{260\,nm}$ (0–3 O.D.) was obtained (not shown). Nuclei (20×10^6) were used in 'run-on' assays immediately after isolation. The transcription reaction was carried out in the presence of 200 μCi of [^{32}P]UTP (3,000 Ci/mmol), RNase inhibitor (Boehringer Mannheim, 40 U/μl) and 0.5 mM each of ATP, CTP and GTP as described in [21] The reaction was incubated at 25°C for 60 min and terminated by addition of 10 μl ribonuclease-free DNase I (Boehringer Mannheim, 23 U/μl) and 10 μl of 20 mM $CaCl_2$. Total nuclear RNA was isolated [22]. For specific detection of radioactive nascent RNA transcripts, 10×10^6 cpm of the isolated RNA was hybridized for 60 min at 60°C and 72 h at 42°C with various plasmid DNAs immobilized on nylon filters (see Fig. 1). Undigested plasmid DNAs (10 μg) were denatured with 3 M NaOH at 65°C for 60 min and neutralized with 2 M NH_4Ac, pH 7. Denatured DNAs were applied to nylon membranes using a slot blot apparatus (Schleider and Schuell, Keene, NH, USA). After hybridization, membranes were washed according to the following protocol (i) 1 × SSC containing 0.1% SDS at 65°C for 15 min (2 times) and (ii) 0.1 × SSC containing 0.1% SDS at 65°C for 10 min (1 time). Membranes were exposed to X-ray films and analyzed by densitometric scanning

2.3. *Other methods*

Preparation of liver homogenates, 'Western blot' analysis of mitochondrial β subunit of the F_1-ATPase complex, RNA isolation and nucleic acid hybridization of β-F_1-ATPase mRNA have been previously reported in detail [5,9].

2.4. *DNA probes*

Both a 1,577 and a 1,218 bp cDNA probe for rat liver [23] and *D melanogaster* β-F_1-ATPase, generously provided by Dr. P.L. Pedersen (The John's Hopkins University, Baltimore) and Dr R. Garesse (Universidad Autonoma de Madrid, Spain), were used. The full-length human β-actin cDNA clone pHF5 [24] was kindly provided by Dr. J. Ortín. Vector DNA (pUC18) [25] was included as a negative control for non-specific hybridization

3. RESULTS AND DISCUSSION

We have recently reported that administration of thyroid hormones to hypothyroid neonates promote a rapid increase and similar response in magnitude in liver (i) mitochondrial ATPase activity, (ii) amount of β-F_1-ATPase protein, (iii) steady-state β-F_1-ATPase mRNA content and (iv) in vivo relative rates of β-F_1-ATPase synthesis [5]. These provided evidence for a rapid thyroid hormone-mediated regulation of the nuclear coded β-subunit of the mitochondrial β-F_1-ATPase complex at either a transcriptional and/or post-tran-

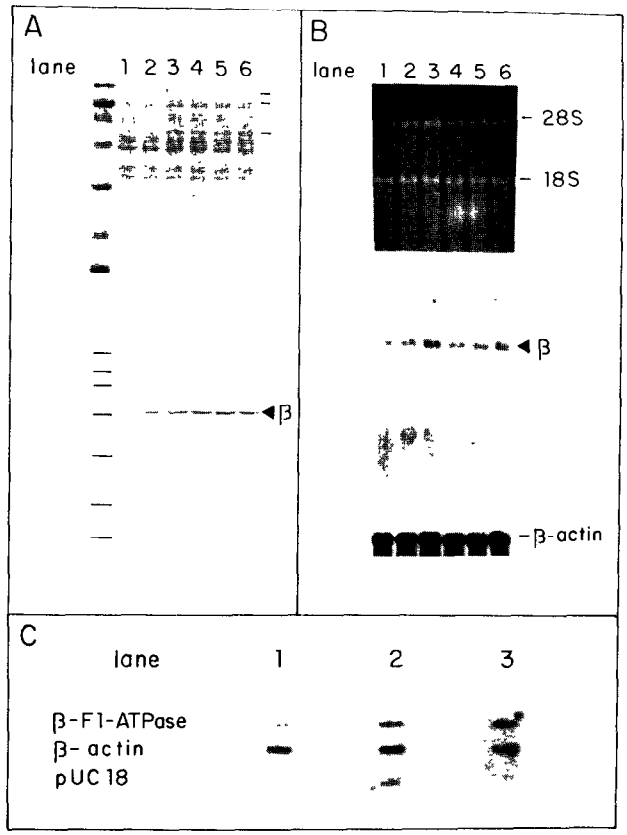

Fig. 1. Effect of thyroid hormones on the induction of β-F_1-ATPase protein, mRNA content and on the transcriptional rate of β-F_1-ATPase gene in the liver of hypothyroid rat neonates. At the time of delivery, hypothyroid rat neonates were intraperitoneally injected with various T3 + T4 doses (0 × dose, 0 + 0, 1 × dose: 4.5 + 16.6 and 10 × dose: 45 + 166 μg/100 g body weight of T3 + T4, respectively). 1 h after birth, newborns were decapitated and liver homogenates, total liver RNA and liver nuclei isolated as described in section 2 The rates of gene transcription were determined in nuclear run-on assays by the level of incorporation of [α-^{32}P]UTP into nascent RNA transcripts after hybridization of 10×10^6 cpm of isolated RNA to 10 μg of immobilized rat liver β-F_1-ATPase and human β-actin cDNAs. Vector DNA (pUC18) was included as a negative control for non-specific hybridization. (A) Upper panel, Coomassie blue stained gel of 50 μg of liver proteins fractionated on 12% SDS-PAGE. For each dose of T3 + T4 administered, two different liver preparations are shown. Lanes 1,2 for 0 × dose; lanes 3,4 for 1 × dose and lanes 5,6 for 10 × dose. Molecular weight markers are shown in the left. Dashes on right side of the gel denote liver proteins that increase as a result of thyroid hormone treatment to the hypothyroid neonates. Lower panel, 50 μg of fractionated liver proteins were transferred to PVDF membranes and probed with affinity purified anti-β-F_1-ATPase antibodies. Samples are as indicated above. Migration of the β subunit of the F_1-ATPase complex is indicated by a closed arrowhead. (B) Autoradiogram (middle panel) of β-F_1-ATPase end-labeled oligonucleotide to 30 μg of total liver RNA from one-hour-old hypothyroid neonates treated at birth with various T3 + T4 doses. For each dose of T3 + T4 administered, two different RNA preparations are shown. Lanes 1,2 for 0 × dose, lanes 3,4 for 1 × dose and lanes 5,6 for 10 × dose. Above the autoradiogram a picture of a parallel ethidium bromide-stained gel is shown. 18 S and 28 S indicate the migration position of the corresponding ribosomal RNA bands. Lower panel, autoradiogram showing liver RNA hybridization signals to labeled human β-actin cDNA Sample in lanes as indicated above. (C) Autoradiograms of in vitro synthesized β-F_1-ATPase and β-actin mRNAs in isolated liver nuclei of hypothyroid rat neonates treated at birth with various thyroid hormone doses (lane 1, 0 × dose; lane 2, 1 × dose and lane 3, 10 × dose)

scriptional level. To further analyze the mechanism by which thyroid hormones regulate β-F_1-ATPase gene expression, we have administered to hypothyroid neonates at the time of birth, various T3 + T4 doses (see section 2) and both the steady-state amounts of β-F_1-ATPase protein and mRNA were determined in their livers after 1 h of receiving the hormonal treatment (Fig. 1). The doses (1 × and 10 ×) of T3 + T4 administered were in the range of other in vivo studies of thyroid hormone effects in hypothyroid rats [11,12,18]. Usually, a pharmacological dosage (50–200 μg of T3/100 g body weight) is injected to promote saturation of nuclear receptors. In our study, both moderate (1 ×) and pharmacological (10 ×) administration of T3 + T4 to hypothyroid neonates significantly increased the amount of the mitochondrial β-subunit of the F_1-ATPase complex (Fig. 1A, lower panel). In addition, the hormonal treatment promoted the appearance and/or increase in the relative amount of various selected liver polypeptides (most notorious are a 135 kDa, a 110 kDa and 56 kDa protein) (Fig. 1A, upper panel). Similarly, both moderate (1 ×) and pharmacological (10 ×) doses of T3 + T4 promoted a rapid increase in the steady-state amounts of β-F_1-ATPase mRNA in the liver of one-hour-old hypothyroid neonates (Fig. 1B, middle panel). These results confirmed and extended our previous findings [5], revealing that administration at birth of thyroid hormones to hypothyroid neonates induced a rapid increase in liver β-F_1-ATPase protein and mRNA content (Table I).

The rapid increase in liver mRNA levels for the β-F_1-ATPase protein that occurs in T3 + T4-treated hypothyroid neonates, could either result from an increase in the rates of transcription for the β-F_1-ATPase gene and/or from an increase in the stability of the cytoplasmic mRNA, since both mechanisms for the expression of liver proteins are known to be controlled by thyroid hormones [11,12,14]. Hence, to answer the question if thyroid hormones affect transcriptional rates of the β-F_1-ATPase gene, we employed the in vitro approach of the nuclear 'run-on' assays using nuclei prepared from one-hour-old hypothyroid neonates treated at birth with various T3 + T4 doses. As shown in Fig. 1C and Table I, relative transcriptional rates of β-F_1-ATPase gene (normalized to the β-actin densitometric reading) were stimulated in a dose-dependent way by administration of thyroid hormones to hypothyroid neonates. Comparison of fold-stimulation (over 0 × dose) in liver steady-state amounts of β-F_1-ATPase protein and mRNA after treatment of the neonates with the two thyroid hormone doses (Table I), revealed a similar fold-stimulation to that of 'in vitro' determined relative transcriptional rates for the 1 × dose and a slightly lower stimulation for the 10 × dose (Table I). This apparent discrepancy between liver steady-state β-F_1-ATPase protein and mRNA levels with the in vitro determined transcriptional rates at high T3 + T4 dosage (10 × dose) (Table I) could reflect an unphysiological imbalance between transcriptional activation of the gene and post-transcriptional events involving the newly synthesized β-F_1-ATPase mRNA precursor. In any case, the findings reported in this study most likely suggest that the increase in liver β-F_1-ATPase mRNA after thyroid hormones administration to hypothyroid neonates [5] is mainly due to the effect of the hormones in promoting transcriptional activation of the gene and thus, that negligible or no effect could be ascribed to a hormonal effect in the stability of liver β-F_1-ATPase mRNA.

Table I

Induction by thyroid hormones of β-F_1-ATPase protein, mRNA content and transcriptional rates of β-F_1-ATPase gene

Parameter	T3 + T4 dose		
	0 ×	1 ×	10 ×
β-F_1-ATPase protein	(8) 1.0 ± 0.1	(8) 2.0 ± 0.1***	(8) 2.5 ± 0.1***
β-F_1-ATPase mRNA	(5) 1.0 ± 0.1	(5) 1.8 ± 0.1**	(5) 2.2 ± 0.3*
Rates β-F_1-ATPase transcription	(5) 1.0 ± 0 2	(4) 2.0 ± 0.2*	(2) 3.1 ± 0.1**

For details see legend to Fig. 1. Quantitation (arbitrary units, a.u.) of β-F_1-ATPase protein and mRNA content in the liver of hypothyroid neonates treated at birth with various T3 + T4 doses was carried out by laser densitometric scanning of the immunoreactive band or hybridization signal, respectively. Relative transcriptional rates were expressed as arbitrary units of the values obtained from quantitation by densitometric scanning of the β-F_1-ATPase/β-actin hybridization signals. The three parameters were relatively compared with those obtained in liver or isolated nuclei from non-T3 + T4-treated neonates (0 × dose), arbitrarily fixed to a value of 1. The results shown are means ± S.E.M. The number of samples processed (liver proteins and mRNA levels) or experiments (transcription) are indicated in parentheses. *$P < 0.01$; **$P < 0.0025$ and ***$P < 0.0005$, when compared with 0 × dose, by Student's t-test.

It has been shown that several nuclear-encoded 'housekeeping' genes coding for mitochondrial proteins are regulated by thyroid hormones [5,18,26,27] and, in those cases in which the mechanism has been studied – cytochrome c [27] and β-F_1-ATPase (this study) – this is as a result of transcriptional activation of the gene by thyroid hormones. Transcriptional activation of rat liver β-F_1-ATPase gene by thyroid hormones (Fig. 1 and Table I) could result from (i) a direct effect of the T3-receptor complex with short DNA sequence (cis-acting) elements located adjacent to the promoter or in enhancers of the gene and/or (ii) mediated through hormonal regulation of the activity of certain essential transcriptional factors (*trans*-acting) that could interact with the gene [28]. The existence of three putative TREs (A1, A2 and A3) has been described in the human β-F_1-ATPase gene [5] in a DNA sequence region that has been shown to have certain promoter activity [28,29]. This might suggest that activation of rat liver β-F_1-ATPase gene transcription after thyroid hormone administration to hypothyroid neonates is the result of a

direct interaction of the T3-receptor complex with any of these *cis*-acting elements. However, the possiblity still exists, as reported for the expression of other thyroid hormone regulated genes [30–32], that thyroid hormones could affect the activity of certain trans-acting factor(s) involved in transcriptional activation of nuclear-encoded mitochondrial 'housekeeping' genes. In this regard, it is worth noting that thyroid hormones have been recently shown to affect the binding of tissue-specific and ubiquitous nuclear proteins to certain specific DNA sequence elements located nearby (450 bp) the promoter of the human ATP synthase β subunit gene [33]. In conclusion, our findings indicate that activation of β-F_1-ATPase gene transcription after thyroid hormones administration to hypothyroid neonates is the primary mechanism by which these neonates could rapidly promote differentiation of their liver mitochondria.

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