Thyroid hormone increases transcription of GA-binding protein/nuclear respiratory factor-2 α-subunit in rat liver

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Abstract Thyroid hormone (TH) regulates mitochondrial respiratory rate by activating coordinated transcription in the nucleus and mitochondria. Whereas TH activates transcription of mitochondrial genes directly, the activation of nuclear-encoded mitochondrial genes is probably executed by indirect unknown mechanisms. Nuclear respiratory factors (NRF)-1 and GA-binding protein (BP)/NRF-2 may function as transacting genes, but regulation of these genes by TH is not demonstrated. We show that TH administration to hypothyroid rats promptly increases GABP/NRF-2 α-subunit mRNA levels in the liver, without significant changes in β, γ subunits. In run-on and time-course experiments, the transcription rate and protein levels increased three-fold in response to TH, indicating GABP/NRF-2 transcriptional regulation. The results also support the notion that ATP synthase β-subunit is regulated by TH through the indirect activation of GABP/NRF-2. **© 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Mitochondria; Thyroid hormone; Nuclear respiratory factor; GA-binding protein/nuclear respiratory factor-2; Transcriptional regulation; ATP synthase β-subunit

1. Introduction

GA-binding protein/nuclear respiratory factor-2 (GABP/NRF-2) is an ubiquitously expressed nuclear transcription factor that has been involved in a broad range of cellular processes, such as the activation of some nuclear-encoded mitochondrial genes [1–5], the induction of interleukin-2 during T-cell activation [6] or, more recently, the regulation of synapse-specific gene expression at the neuromuscular junction [7]. The biogenesis of mitochondria requires the expression of genes both from the nuclear and mitochondrial genomes, the mitochondrial function depending on the precise cross-regulation of the two genomes expression [8]. However, the mechanisms that control this coordinated expression are not well known. In this respect, it is interesting that the thyroid hormone (TH), which is long known to modulate the mitochondrial respiratory rate in response to the varying energy demands [9], stimulates transcription both in the nucleus and mitochondria. The direct regulation of mitochondrial RNA synthesis by TH has been demonstrated recently in vivo [10]. Nevertheless, the overall mechanisms that allow TH-transcriptional activation of the nuclear-encoded respiratory subunits, although known of certain genes for a long time, are still far from being completely understood. It is generally accepted that the low sequence conservation of the TRES present in these genes promoters [11] makes most likely that TH exerts control by indirect mechanisms, regulating the expression of common transcription factors required for the expression of these genes. NRF-1 and GABP/NRF-2 have been implicated in the expression of many respiratory subunits along with key components of the mitochondrial transcription, replication and haem biosynthetic machinery [12]. A transacting gene (for example NRF-1 or GABP/NRF-2) might be activated by TH and in turn transactivate the OXPHOS promoters. However, the transcriptional regulation of NRF-1 and GABP/NRF-2 by TH has not been demonstrated to date. In this paper, we have examined this issue in the liver of hypothyroid and triiodothyronine (T_3)-treated hypothyroid rats and found that T_3 specifically activates the expression of the DNA-binding α-subunit of the heteromeric GABP/NRF-2 nuclear transcription factor.

2. Materials and methods

2.1. Animals treatment and tissue sampling

Adult male Wistar rats (about 200 g) grown in our animal facilities were used. Hypothyroidism was induced by surgical thyroidectomy and the supply of 0.04% 2-mercapto-1-methylimidazole (an inhibitor of TH synthesis) in the drinking water for over 1 month prior starting the experiment. T_3 (20 μg/100 g body weight) was administered daily by intraperitoneal injections for 3 days. In the time-course experiments, animals were killed after a single injection at the times indicated. The animals were killed by decapitation and their livers quickly dissected and frozen in liquid nitrogen.

2.2. RNA extraction and Northern analysis

Total RNA was obtained by guanidine and caesium chloride purification [13]. For Northern blots, 20 μg of total RNA were separated on a 1% (w/v) agarose/formaldehyde gel and transferred onto nylon membranes with 20×SSC (1×SCC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Probes for GABP/NRF-2 α, β, γ-subunits, mitochondrial transcription factor A (Tfam) and ATP synthase β-subunit have been described previously [14]. Human S26 ribosomal protein probe [15] was kindly provided by Dr T. Tanaka and was used to normalise the amount of RNA loaded per lane. Probes were labelled with ^{32}P using a random priming system. Hybridisation and washing were carried out as described by Church and Gilbert [16].

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Abbreviations: GABP, GA-binding protein; TH, thyroid hormone; T_3, triiodothyronine; NRF, nuclear respiratory factor; Tfam, mitochondrial transcription factor A

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2.3. Run-on analysis

To measure the transcriptional activity of the NRF-2 α-subunit gene, we isolated nuclei from the livers of hypothyroid or T_3-treated hypothyroid rats. Briefly, nuclei from 1 g of liver were isolated through a 2 M-sucrose cushion as described [4]. Nuclei storage, in vitro run-on transcription assays, and hybridisation conditions were carried out as described [17]. The following undigested plasmids were denatured and immobilised on nylon filters: rat GABP/NRF-2 α-subunit and ATP synthase β-subunit, and human β-actin. Rat ATP synthase β-subunit and human β-actin cDNAs were kindly provided by Dr J.M. Cuezva [4].

2.4. Protein extraction and Western analysis

Total liver extracts were obtained either by homogenising in medium 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM HCl–Tris, pH 7.5 [18], plus protease inhibitors (phenylmethylsulfonyl fluoride, E-64, pepstatin A) or in boiling medium 65 mM Tris–HCl, pH 6.8, 10% glycerol, 0.14 M mercaptoethanol, 2% SDS, followed by centrifuging at $17\,400\times g$ for 20 min [19]. Proteins (50 μg) were separated in 10% SDS–polyacrylamide gels and transferred to PVDF membranes. The Western blots were probed with GABP/NRF-2 α-subunit antibody at 1:2000 dilution; the antibody was produced for us by Isogen Bioscience BV (The Netherlands) against peptide IS-DETSEQVTRWAA. Protein disulfide-isomerase antibody (kind gift of Dr J.G. Castaño) [20] was used to control load; the expression of this protein is not affected by the different thyroid status of the animal. As secondary antibody, an anti-rabbit peroxidase conjugate (Bio-Rad) at 1:3300 dilution was employed with both primary antibodies. The immunoreactive proteins were visualised by chemiluminescence (ECL detection system; Amersham)

3. Results

3.1. T_3 increases expression of GABP/NRF-2 α-subunit mRNA in hypothyroid liver

The expression of NRF-1 and GABP/NRF-2 genes was studied in parallel with those of Tfam, the mitochondrial transcription factor, and ATP synthase β-subunit, a nuclear-encoded mitochondrial gene known to respond to THs. Whereas ATP synthase β-subunit, Tfam and NRF-1 are encoded each by one gene, GABP/NRF-2 is encoded by three genes giving α, β, and γ-subunits, in the NRF nomenclature [21] or otherwise α, β1, and β2-subunits, in the GABP one [22]. NRF-2 is the human homologue of the mouse GABP [21]. Only the α-subunit, that belongs to the ETS family of regulatory proteins [23], has DNA-binding activity. Although the α-subunit binds DNA weakly, the heterodimerisation with the β-subunit provides the active form of GABP/NRF-2, an α2β2 heterotetramer, with the ability to bind avidly to tandem GGAA (ets) sites [21,22]. The analysis of the mRNA levels in the liver of control, hypothyroid and T_3-treated hypothyroid rats (Fig. 1) indicated that T_3 increased significantly ($P < 0.001$) the mRNA levels of GABP/NRF-2 α-subunit without changing those of β or γ-subunits. The presence of two transcripts in the GABP/NRF-2 α-subunit mRNA was described previously [19,22]. Smaller increases ($P < 0.05$) were observed in the NRF-1 or Tfam levels upon T_3 treatment. In accordance with previous reports [24,25], T_3 increased significantly ($P < 0.001$) the level of ATP synthase β-subunit mRNA. In our experiments, a three-fold increase over control livers was observed for both GABP/NRF-2 α-subunit and ATP synthase β-subunit. Therefore, T_3 increased specifically the expression of GABP/NRF-2 α-subunit.

3.2. Time-course of GABP/NRF-2 α-subunit mRNA induction by T_3

In order to study the kinetics of GABP/NRF-2 α-subunit mRNA activation, hypothyroid rats were treated with a single T_3 dose. Livers from individual rats were dissected at various times after T_3 injection. Total RNA was isolated and analysed by Northern blot. Fig. 2 shows that T_3 increased the accumulation of GABP/NRF-2 α-subunit transcripts shortly after the hormone administration. The autoradiographic signals were corrected for load control with a probe from the ribosomal protein S26, a gene expressed at a constant level in mammalian tissues in different physiological contexts [15]. The quantitative analysis showed that GABP/NRF-2 α-subunit induction took place in two peaks, the first detected 30 min after T_3 injection with a two-fold increase in the mRNA level, and a second one after 16 h with a three-fold increase. The expression kinetics of Tfam and ATP synthase β-subunit mRNAs were also analysed in the same samples. The Tfam mRNA levels showed a negligible response to T_3, thus confirming the results obtained in the steady state (Fig. 1). In contrast, ATP synthase β-subunit mRNA increased steadily, reaching a maximum at 16 h. The data therefore indicated that the GABP/NRF-2 α-subunit induction preceded that of ATP synthase β-subunit, thus suggesting that the control of this gene by T_3 was mediated by GABP/NRF-2. This is in agreement with the previous demonstration that the ATP synthase β-subunit promoter contains response elements for GABP/NRF-2 [26].

3.3. T_3 activates GABP/NRF-2 α-subunit transcription

The rapid accumulation of GABP/NRF-2 α-subunit transcripts following T_3 administration suggested that the regulation was due to the transcriptional activation of the gene. To determine whether T_3 modulates GABP/NRF-2 α-subunit transcription rate, we performed run-on assays with isolated nuclei from the livers of hypothyroid or hypothyroid T_3-treated rats (Fig. 3). The transcription rate of the GABP/NRF-2 α-subunit gene was compared with that obtained for β-actin, a gene whose expression is not affected by T_3, and for ATP synthase β-subunit, that is transcriptionally activated by TH in liver [27]. The relative rate of GABP/NRF-2 α-subunit gene transcription increased 3.4-fold after 30 min of T_3 injection and 2.2-fold, 19 h later. Interestingly, the transcription rate of the ATP synthase β-subunit gene was increased 2.4-fold only after 19 h of injection, with no change observed at the 30 min time. These data provide clear evidence for a direct effect of T_3 on the transcription rate of the GABP/NRF-2 α-subunit gene in vivo, and support the notion that the transcriptional regulation of the ATP synthase β-subunit gene by T_3 is mediated by GABP/NRF-2 activity.

3.4. GABP/NRF-2 α-subunit protein level increases in response to T_3

The time-course expression of GABP/NRF-2 α-subunit protein was also analysed by Western blotting in the livers of hypothyroid rats after T_3 injection. The immunoreactive signals were corrected for load control by the signal given at each time by protein disulfide-isomerase antibody, which recognises a protein whose expression in the liver is not affected by the thyroid status of the rat. As shown in Fig. 4, the quantitative analysis indicated that the level of GABP/NRF-2 α-subunit protein increased steadily from the administration time up to 60 min, when nearly a three-fold increase was observed. Afterwards, it returned to the control level after about 24 h of hormone administration. The kinetics of protein accumulation (Fig. 4) differed somewhat from that of mRNA

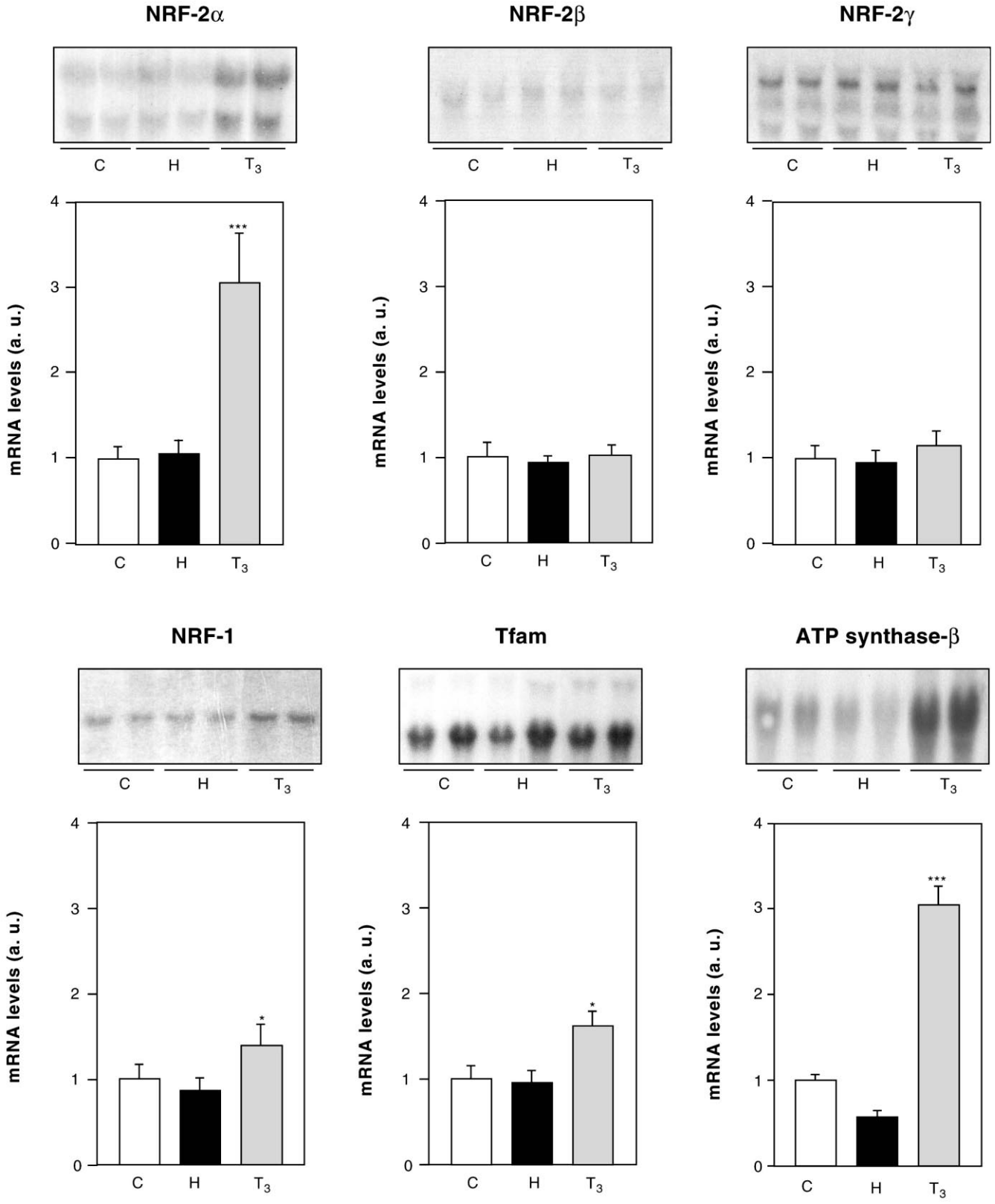

Fig. 1. T_3-treatment increases GABP/NRF-2 α-subunit mRNA levels in hypothyroid liver. Total RNA was extracted from the liver of untreated control (C), hypothyroid (H) and hypothyroid rats previously injected with a dose of T_3 for 3 days (T_3), which leads to a severe hyperthyroid state. Northern analysis was performed as described in Section 2. Representative autoradiograms after hybridisation with probes for the different GABP/NRF-2 α, β and γ-subunits are shown. The same Northerns were hybridised with probes for Tfam, NRF-1 and ATP synthase β-subunit. The autoradiographic signals from four different animals per experimental group and two different experiments were corrected for loading with the β-actin probe and expressed as fold-stimulation (means ± S.D.) over control levels. The significant increases are marked, $*P < 0.05$; $***P < 0.001$.

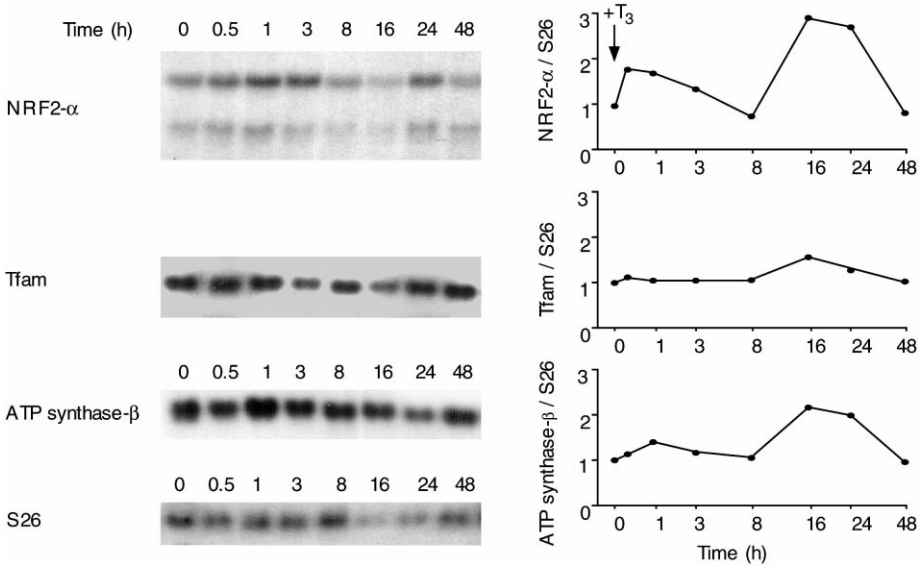

Fig. 2. Time-course of mRNAs induction in the liver after T_3-treatment of hypothyroid rats. Hypothyroid rats were injected with a single dose of T_3 and killed at the indicated times. RNA was extracted and analysed by Northern blot by hybridising with GABP/NRF-2 α-subunit, Tfam, ATP synthase β-subunit probes and the ribosomal protein S26 probe. On the right, the quantitative analysis is shown after correction for loading with the S26 signal and normalisation for the signal at time 0. The data are from one out of two independent experiments carried out with similar results.

(Fig. 2), probably indicating that post-transcriptional mechanisms are also involved in the maintenance of the protein level, as suggested previously [19]. Therefore, GABP/NRF-2 α-subunit protein concentration also increased in response to T_3.

4. Discussion

We have investigated the expression of NRF-1 and GABP/NRF-2 in the liver of control, hypothyroid and T_3-treated hypothyroid rats. Two other genes were also studied in parallel, the mitochondrial transcription factor Tfam, which has promoter recognition sites for both NRF-1 and GABP/NRF-2, and ATP synthase β-subunit, whose transcription is regulated by TH and has sites for GABP/NRF-2. We found

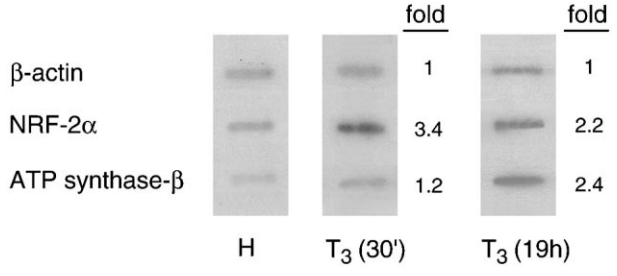

Fig. 3. T_3-stimulation of GABP/NRF-2 α-subunit and ATP synthase β-subunit genes transcription rates in isolated nuclei of hypothyroid rats. Nuclei from livers of hypothyroid (H), or hypothyroid rats killed after 30 min (T_3, 30′) or 19 h (T_3, 19 h) of a single injection of T_3 were used to generate ^{32}P-labelled run-on transcription probes, as described in Section 2. The labelled RNA probes were hybridised to nylon membranes containing 10 μg of plasmid DNA for β-actin, GABP/NRF-2 α-subunit, and ATP synthase β-subunit. The signals obtained are shown together with the fold stimulation of the transcription rates calculated from the densitometric analysis. The data are from one out of two independent experiments carried out with similar results

that the mRNA steady-state level of GABP/NRF-2 α-subunit increased three-fold in the liver of T_3-treated hypothyroid rats after hormone injection (Fig. 1). In contrast, the transcripts of β and γ-subunits were not affected. NRF-1 and Tfam showed little accumulation of their mRNAs. In accordance with previous results [24,25], the ATP synthase β-subunit mRNA was found increased in the liver of T_3-treated hypothyroid rats. The time-course experiments (Fig. 2) indicated that the accumulation of GABP/NRF-2 α-subunit transcripts in response to T_3 was very rapid, and the run-on experiments (Fig. 3) indicated that this accumulation was due to increased transcription rates induced by the hormone. The time-course experiments (Fig. 4) also showed a rise in the protein level of GABP/NRF-2 α-subunit 1 h after T_3 injection, that returned to control levels after 24 h. Thus, our results indicate that TH specifically increases GABP/NRF-2 α-subunit expression and this is probably enough to activate GABP/NRF-2 (see below). We found previously in rat tissues [14] that the mRNA steady-state level of α-subunit is much lower than those of the β and γ-subunits, what may make α-subunit limiting for complex assembly and a subject of regulation. The results presented here also indicate that of the two nuclear respiratory factors, TH specifically activates GABP/NRF-2 when compared with NRF-1. Whether TH activates via direct or indirect mechanisms remains to be established. The Tfam data were quite unexpected in light of the presence of GABP/NRF-2 recognition sites (in addition to NRF-1) in its promoter. In adaptive thermogenesis, the induction of PGC-1, a transcriptional co-activator of nuclear receptors, leads to mitochondrial proliferation, with induction of NRF-1 and GABP/NRF-2, which in turn activate Tfam promoter activity [28]. These authors observed that Tfam activation is abolished by mutation of NRF-1-binding site but only slightly affected by that of GABP/NRF-2. Their results and ours may suggest that, in the absence of a certain level of NRF-1, GABP/NRF-2 plays only a marginal role in Tfam in vivo expression.

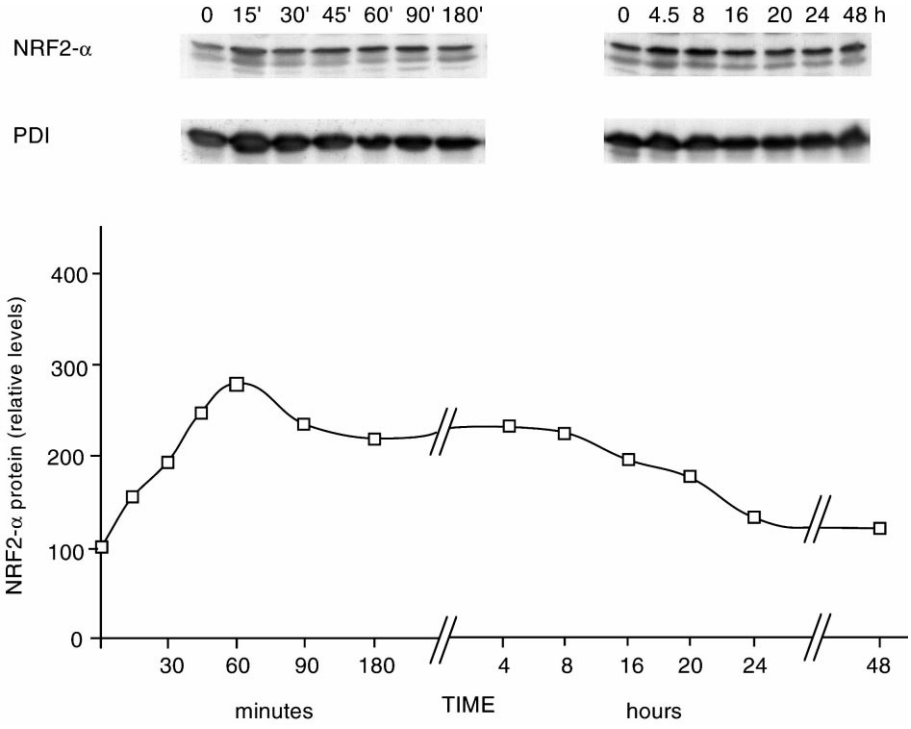

Fig. 4. Time-course of liver GABP/NRF-2 α-subunit protein expression after T_3-treatment of hypothyroid rats. Total liver extracts were obtained from hypothyroid rats (time 0) or hypothyroid rats killed at the indicated times after a single injection of T_3. The Western blots were probed with GABP/NRF-2 α-subunit and PDI antibodies, as indicated in Section 2. The various time signals were corrected for protein loading with the PDI signal and the final quantification normalised for the signal at time 0.

On the contrary, the data regarding ATP synthase β-subunit expression were straightforward. Our results from the time-course and run-on experiments (Figs. 2 and 3) showed for the first time that the induction of GABP/NRF-2 α-subunit preceded the increase in ATP synthase β-subunit expression, thus supporting that the TH-transcriptional activation of ATP synthase β-subunit is indirect, exerted via GABP/NRF-2 activation. In support of these results, previous analysis of TH-binding sites in the human promoter [29] indicated that the putative response elements were non-functional.

In summary, we show that TH in vivo activates expression of GABP/NRF-2, one of the two nuclear respiratory factors suggested to coordinate expression of the nuclear and mitochondrial genomes. The activation is produced via the increase in expression of GABP/NRF-2 α-subunit. We also show that this accumulation precedes activation of ATP synthase β-subunit, a gene known to be regulated by TH and GABP/NRF-2. The results thus support the notion that the TH activation of nuclear-encoded mitochondrial genes is exerted through indirect means.

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