ATP synthase subunit c expression: physiological regulation of the *P1* and *P2* genes

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Pre-translational regulation of subunit c has been suggested to control the biosynthesis of mitochondrial ATP synthase (ATPase) in brown adipose tissue (BAT). Subunit c is encoded by the genes *P1* and *P2*, which encode identical mature proteins. We have determined here the levels of *P1* and *P2* mRNAs in different tissues, in response to cold acclimation in rats, during ontogenic development of BAT in hamsters, and following thyroid hormone treatment in rat BAT and liver. Quantitative ribonuclease protection analysis showed that both the *P1* and *P2* mRNAs were present in all rat tissues measured. Their total amount in each tissue corresponded well with the ATPase content of that tissue. While the *P1*/*P2* mRNA ratio is high in ATPase-rich tissues, the *P2* mRNA dominates in tissues with less ATPase. Cold acclimation affects *P1* but not *P2* gene expression in rat BAT. A rapid and transient increase in *P1* mRNA is followed by sustained depression, which is accompanied by a decrease in ATPase content. Similarly, ontogenic suppression of ATPase content in hamster BAT was accompanied by suppression of the *P1* mRNA levels, while *P2* expression was virtually unchanged. Furthermore, when hypothyroid rats were treated with thyroid hormone, the steady-state level of *P1* but not of *P2* mRNA was significantly increased in liver. BAT was unaffected. We conclude that the *P1* and *P2* genes for subunit c are differentially regulated *in vivo*. While the *P2* gene is expressed constitutively, the *P1* gene responds to different physiological stimuli as a means of modulating the relative content of ATP synthase.

INTRODUCTION

Mitochondrial H^+-transporting ATP synthase, a key enzyme of ATP production in mammals [1], consists of two major components, F_1 and F_o, linked together by a slender stalk [2]. The F_o part translocates the protons and transduces the energy of the proton electrochemical gradient, probably via the stalk to the catalytic F_1 part. The membrane-embedded F_o part assembles multiple copies (9–12) of the c-subunit, the mitochondrially encoded subunits a and A6L and the b-subunit, which has a large part protruding to the stalk [3,4]. Additional subunits, e, f and g, isolated from bovine mitochondrial ATP synthase (ATPase) preparations have putative membrane spanning regions and might thus also be a part of F_o [2].

The highly conserved subunit c is an essential constituent of the proton channel [5] and is encoded by two different nuclear transcripts in rodents, cows and humans, designated *P1* and *P2* [6–8]. The two mRNAs code for identical mature proteins, albeit with large differences in the cleaved mitochondrial import sequences and in the 5′ and 3′ non-translated regions. The biological reason for this diversity remains obscure, but presents an intriguing question. Expression of one or other of the *P1* and *P2* genes was shown to be tissue-specific in bovine tissues, since the *P1*/*P2* ratio was approx. 1:1 in heart, kidney and muscle but approx. 1:3 in liver, intestine and lung [6].

Pre-translational regulation of subunit c has been suggested to control biosynthesis of ATPase in the mammalian thermogenic organ brown adipose tissue (BAT) [9], which utilizes a high oxidative capacity to produce heat instead of ATP. The basis for this reaction is a H^+-short-circuiting of the proton-motive force across the inner mitochondrial membrane, via a tissue-specific proton channel, the uncoupling protein (for reviews see [10,11]). In most species, BAT mitochondria contain only very low amounts of ATPase, and the ATPase/respiratory chain stoichiometry is some ten-fold lower than in other tissues [12–15]. The reduction of ATP synthase content is paralleled by a corresponding, low, subunit c expression, while the mRNA levels for most other ATP synthase subunits display an apparent overexpression, indicating that subunit c expression has a key role in regulation of ATPase biosynthesis in BAT [9].

In this paper we have used the ribonuclease protection assay (RPA) to quantify the expression of the two subunit c genes in different tissues and relate it to ATPase content. Furthermore, we have investigated the effect on gene expression of three potential physiological modulators of ATPase biosynthesis: (1) the effect of cold acclimation on BAT, which is known to induce mitochondriogenesis [16,17], but not ATPase activity in BAT [18], (2) the ontogenic development of BAT in the hamster, which is known to be accompanied by a reduced biosynthesis of subunit c and ATPase [9], and (3) the effect of thyroid hormones on rat liver, which have been shown to activate the synthesis of several nuclear-encoded mitochondrial proteins, including subunit c [19].

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (B & K Universal AB) were kept at 28 °C on a 12 h dark/12 h light cycle with food and water *ad libitum*. At the beginning of each experiment, 1-month-old animals (approx. 150 g) were maintained for at least 4 days at 28 °C. Where indicated in the text, animals were moved to a cold room at 4 °C under otherwise identical conditions.

Hypothyroidism was induced by administration of 0.05% methimazole (Sigma) in the drinking water, as described in [20]. 3,5,3′-Tri-iodothyronine (T_3) (Sigma) was dissolved in slightly alkaline saline and pH was adjusted with 0.01 M HCl to 8. T_3 was injected subcutaneously at a dose of 100 μg/kg body weight.

Abbreviations used: BAT, brown adipose tissue; cox, cytochrome *c* oxidase; RPA, RNase protection assay; T_3, 3,5,3′-tri-iodothyronine.

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Mitochondria and protein analysis

Animals were killed by CO_2 and tissues were dissected into a medium consisting of 0.25 M sucrose, 10 mM Tris/HCl (pH 7.2), 1 mM EDTA, 2 mM PMSF and 2 μg/ml of each of aprotinin, leupeptin, antipain and pepstatin A. Homogenates (5 %) were prepared with ten strokes in a glass–teflon homogenizer and mitochondria were isolated according to standard procedures [21]. Protein concentration was determined by the Bradford method [22].

SDS/PAGE was performed on 10 % (w/v) polyacrylamide slab gels according to [23], and proteins were transferred by semi-dry electroblotting onto Hybond® C-extra membranes (Amersham). For immunodetection, rabbit antisera to bovine heart F_1-ATPase (1/10000) and ovine c-subunit (1/1000) were used as described previously [9]. Immune complexes were visualized by a chemiluminescence method using a peroxidase-conjugated Fab fragment of goat-anti rabbit IgG (Bio-Rad) and an ECL kit (Amersham). Exposures made on KODAK X-OMAT RP films were quantified on a Molecular Dynamics Computing Densitometer.

Blue native electrophoresis was performed according to [24], on a 6–15 % (w/v) gradient gel (1 mm). Aliquots (25 μg) of lauryl maltoside-solubilized protein were analysed.

RNA analysis

Total RNA was isolated using Ultraspec® (Biotecx lab.) from 50–100 mg (w/w) of each tissue. For Northern blot analysis, 20 μg of total RNA was separated on a 1.2 % (w/v) formaldehyde agarose gel and blotted onto Hybond®-N membrane. The prehybridization and hybridization were performed as described [9]. The membranes were washed 3 × 20 min in 0.1 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate) and 0.2 % SDS at 55 °C and exposed to a Molecular Dynamics PhosphorImager.

RPA analysis of β-actin, *P*1 and *P*2 mRNAs was performed essentially according to [25]. Briefly, 20 μg of total RNA was dried down in a SpeedVac and redissolved in 20 μl of hybridization buffer [40 mM Pipes/400 mM NaCl/1 mM EDTA in 80 % (v/v) formamide] containing 10^5 c.p.m. of each riboprobe (> 10-fold molar excess of the specific mRNA), denatured for 5 min at 85 °C and incubated for 16 h at 42 °C. The RNA was then digested for 60 min at 37 °C in 200 μl of RNase digestion buffer (10 mM Tris/Cl, 300 mM NaCl and 5 mM EDTA) with 10 μg/ml RNase A and 200 units/ml RNase T_1 (Ambion). The reaction was stopped with 220 μl of 4 M guanidinium isothiocyanate/0.1 M mercaptoethanol/0.5 % sodium *N*-lauroylsarcosine/25 mM sodium citrate (pH 7.0). The RNA was then precipitated with 450 μl of propan-2-ol together with 25 μg of *Escherichia coli* tRNA (Sigma) and pelleted for 20 min at 15000 ***g***. The RNA pellet was dried and resuspended in 12 μl of gel loading buffer (80 % formamide, 0.1 % (w/v) Bromophenol Blue, 0.1 % (v/v) xylene cyanol and 1 mM EDTA, pH 8.0), incubated for 5 min at 85 °C and a 5 μl aliquot was separated on 6 % denaturing PAGE. The gel was dried and exposed to a Molecular Dynamics PhosphorImager and specific mRNA signals were quantified on MD ImageQuant Software v. 3.3.

Cloning and labelling of *P1* and *P2* cDNAs

The riboprobes for RPA of *P1* and *P2* were prepared as follows: subtype-specific rat *P1* and *P2* cDNAs were amplified using the PCR from total rat liver cDNA using primers corresponding to positions 51–68 and 238–255 of the *P*1 mRNA and positions 107–124 and 218–235 of the *P2* mRNA, according to the sequences in [8]. A 30 bp sequence corresponding to the T3 RNA polymerase promoter was attached at the 5′-ends of the reverse primers, as described in [26]. The resulting 235 and 159 bp fragments were subcloned into pBluescript SK^+ (Stratagene) with T-overhangs at the *Eco*RV site, created with *Taq* polymerase as described in [27]. The inserts were subsequently cleaved out with *Xba*I and *Sal*I and subcloned into M13mp18 and sequenced with the dideoxy chain termination method [28] to confirm identity with the predicted sequence. Template DNA for *in vitro* transcription was PCR amplified, with relevant primers, from the single-stranded DNA used in sequencing, and used for riboprobe synthesis with T3 RNA polymerase (Stratagene) using [α-^{32}P]CTP (Amersham) with a specific activity of 800 mCi/mmol. The synthesized riboprobes were subsequently gel purified as described in [27]. pTRI-β-Actin-Mouse (Ambion), labelled as above, was routinely used in RPAs as an internal standard.

cDNAs for rat β-F1 and cytochrome *c* oxidase (cox)I and human coxIV [19] were labelled and used for Northern blot analysis as described previously [15].

Statistical analysis

Statistical analysis was performed with Student's unpaired *t*-test, with statistical significance set at $P > 0.05$.

RESULTS

Correlation between *P1* and *P2* mRNA levels and ATPase content in different tissues

Tissue-specific expression of the *P1* and *P2* genes has been observed previously by Northern and dot-blot analyses [6]. These results indicated that *P2* mRNA was present in all tissues, whereas *P1* mRNA was preferentially expressed in heart, brain and kidney and little expressed in other tissues investigated. To quantify the distribution and absolute ratio of *P1* and *P2* mRNA in different tissues in rat, we performed RPAs with uniformly labelled riboprobes for *P1* and *P2* mRNA (see the Experimental section), which allowed quantitative analysis of the levels of the different mRNA subtypes.

As is evident from Figure 1, substantial amounts of both the *P1* and *P2* mRNAs are present in all rat tissues. The level of *P1* mRNA varies greatly between tissues, being highest in heart, intermediate in liver and kidney and low in lung and BAT (Figure 1, upper band). *P1* mRNA could also be detected, at a low level, in other tissues with a low content of ATPase, such as

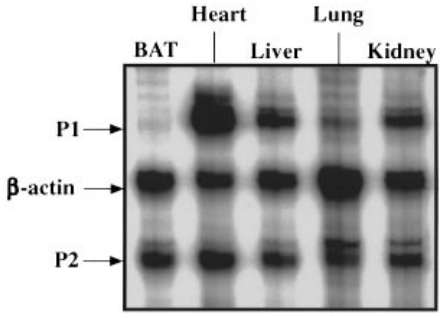

Figure 1 Expression of ATPase subunit c in different tissues of rat

Total RNA (20 μg) from interscapular BAT, heart, liver, lung and kidney was assayed for *P1* and *P2* mRNAs of ATPase subunit c and β-actin by RPA using uniformly labelled specific antisense riboprobes. The positions of the RNase-protected *P1* (199 bp), β-actin (140 bp) and *P2* (123 bp) products are indicated.

Table 1 Quantification of the relative content of *P1* and *P2* mRNA levels and c- and β-subunit content in different tissues of rat

The levels of ATPase subunit c mRNAs (*P1* and *P2*) were determined by phosphor imaging of RPAs with serial dilutions of total RNA (5–20 μg), and normalized to the corresponding β-actin mRNA level. The relative concentration of the sum of *P1* + *P2* transcripts in the different tissues was related to the level found in heart, which was set to 100. The content of ATPase was densitometrically determined in aliquots of homogenates (5–60 μg) as the amount of $\alpha+\beta$ F_1 and c-subunit per μg of protein by Western blotting and related to the level found in heart, which again was set to 100.

	mRNA levels				Protein content	
Tissue	*P1*	*P2*	*P1*/*P2*	*P1* + *P2*	c-Subunit	β-Subunit
BAT	0.75	14	0.05	15	8	9
Lung	1.3	4.3	0.30	5.6	2	5
Liver	14	14	1.00	28	33	37
Kidney	17	17	1.00	34	47	40
Heart	69	31	2.23	100	100	100

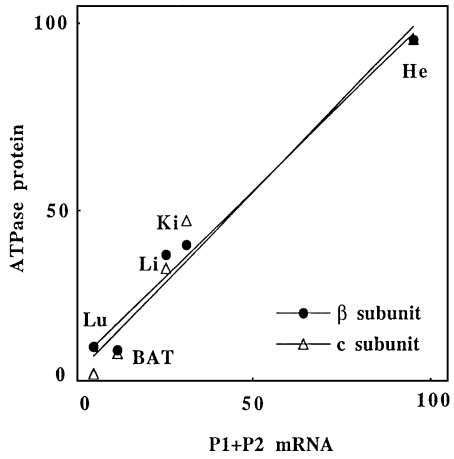

Figure 2 Correlation between the *P1* + *P2* mRNA levels and content of ATPase

The levels of ATPase subunit c mRNAs (*P1* and *P2*) and the content of ATPase β- and c-subunits was determined in lung (Lu), BAT (BAT), liver (Li), kidney (Ki) and heart (He) as described in Table 1.

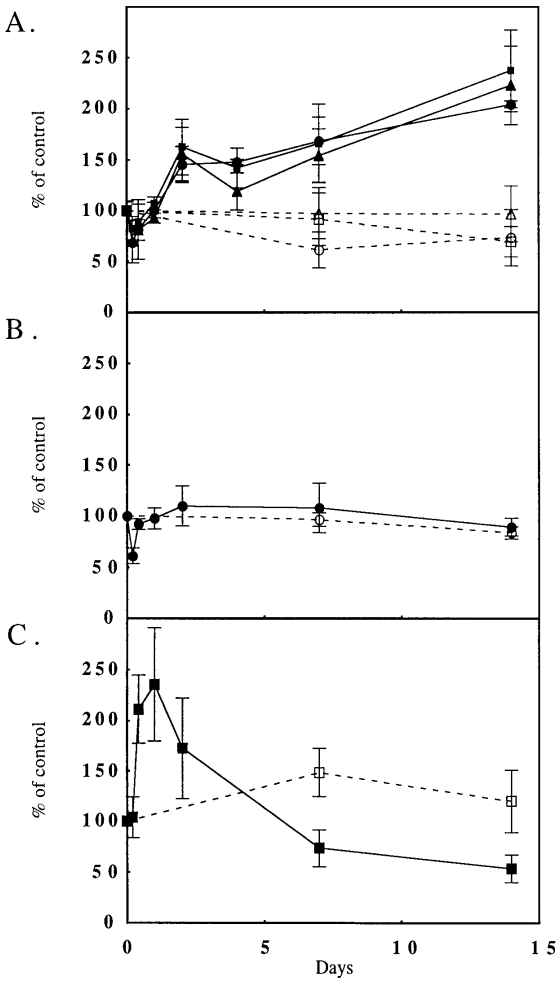

Figure 3 Effect of cold exposure on the expression of subunits of ATPase and cytochrome *c* oxidase in BAT

(**A**) Quantification of Northern blot analyses of total RNA from BAT of rats exposed to 4 °C (solid symbols) or 28 °C (open symbols), probed with ^{32}P-labelled cDNAs for (▲) ATPase subunit β, (■) coxIV and (●) coxI. Determination of (**B**) *P2* and (**C**) *P1* mRNA levels with RPA of 20 μg of total RNA from BAT of rats exposed to 4 °C (solid symbols) or 28 °C (open symbols). Results are means ± S.E.M. of four rats and are expressed as percentages of control.

white adipose tissue and testis (not shown). The *P2* mRNA, however, was expressed to a more similar extent in all tissues tested, in agreement with [6]. Accordingly, the specific suppression of c-subunit expression in BAT is only conferred by selective depression of *P1* mRNA levels. In our previous report, we underestimated the contribution of the *P2* transcript in BAT because the probe preferentially recognized the *P*1 mRNA [9]. In order to quantify the specific contribution of the *P1* and *P2* mRNAs in different tissues, serial dilutions of total RNA were assayed for linearity response and normalized to β-actin mRNA. The differences in specific activity of the *P1* and *P2* protected hybrids in the assay, due to their different lengths, were also compensated for. As shown in Table 1, the *P*1/*P*2 mRNA ratio varies by almost 50-fold in these tissues. Furthermore, it is evident that the total expression of subunit c genes, i.e. the sum of *P1* + *P2* mRNA, best reflects the tissue content of ATPase. Equally good correlation between the sum of the c-subunit mRNA levels and protein (Figure 2) was obtained when using either anti-F_1 or anti-c-subunit antibodies for quantification of ATPase in homogenates of different tissues ($r = 0.988$ and $r = 0.981$ respectively). These data thus further support a pivotal role for the expression of the subunit c genes in the biosynthesis of the complex.

Steady-state levels of *P1*, but not *P2*, transcripts in BAT are influenced by ambient temperature and ontogenic development

Mitochondrial content increases in BAT after cold acclimation when the thermogenic, but not the ATP-producing, capacity increases [16–18]. In order to investigate how changes in ambient temperature influence the expression of nuclear genes for ATP synthase in BAT, we analysed the steady-state levels of the *P1* and *P2* mRNAs, as well as the mRNA for the β-subunit of F_1-ATPase, during cold acclimation of rat. For comparison, we followed the mRNA levels of cox subunits of nuclear (coxIV) and mitochondrial (coxI) origin as markers for mitochondrial biogenesis.

Table 2 Effect of ambient temperature on *P1* and *P2* mRNA levels in BAT

P1 and *P2* mRNAs were measured in 20 μg of total RNA extracted from rat interscapular BAT. (WA) Rats acclimated to 28 °C for 1 month, (CA) rats acclimated to 4 °C for 1 month and (C-W) rats first adapted to 4 °C for 1 month and then re-acclimated to 28 °C for 1 week. Results are means $\pm$ S.E.M. of quantifications of four experiments. Data expressed as percentages of *P1* and *P2* mRNA levels in warm-acclimated animals: *difference statistically significant compared with (C-W).

Rats	*P1* mRNA	*P2* mRNA
WA	100 (4)	100 (4)
CA	31 $\pm$ 6 (4)*	91 $\pm$ 4 (4)
C-W	106 $\pm$ 32 (4)	129 $\pm$ 13 (4)

As shown by Northern analysis in Figure 3(A), the levels of coxI, coxIV and ATPase-β mRNAs increase during the first 2 weeks of cold acclimation, which is in accord with similar investigations on the effect of cold exposure on mitochondrial content in BAT [16,29–31].

There is evidence suggesting that some of the genes encoding proteins involved in mitochondrial function have common DNA elements that bind transcriptional activators that subsequently may co-ordinate mitochondrial biogenesis [32–34]. It is plausible that these mechanisms might be involved in the co-ordinated increase in the mRNAs of coxI, coxIV and ATPase-β as a response to cold acclimation. However, post-transcriptional responses may also be involved in modulating the steady-state levels of these transcripts [35–37].

Expression of the subunit c mRNAs was then analysed with RPA after different times of cold exposure. The *P2* mRNA level remained unaltered during the course of cold acclimation, the only exception being a small transient drop during the first 6 h (Figure 3B). Similar transient drops in mRNA levels during the first day of cold acclimation were also observed for the ATPase-β and coxIV mRNA analysed in Figure 3(A). This effect may be explained by an increase in the concentration of mRNA in relation to total RNA and is probably not related to a specific transcriptional response. On the other hand, *P1* mRNA levels responded dynamically as an effect of cold acclimation. The *P1* mRNA levels transiently increased up to 2.5-fold within the first 24 h at 4 °C and declined afterwards. Subsequently, during 2 weeks of cold exposure, the *P1* expression became steadily suppressed until a level 40 % of that of control animals (Figure 3C) was reached. This effect of cold exposure on *P1* expression was specific to BAT and could not be demonstrated in liver or heart (results not shown).

Comparative analysis of *P1* and *P2* mRNA levels of animals acclimated to cold and warm for prolonged periods (1 month at 4 °C and 28 °C respectively) showed that the level of *P1* mRNA was 3-fold lower in the cold-acclimated than that in warm-acclimated controls (Table 2). When cold-acclimated animals were returned to 28 °C, *P1* expression was fully restored within 1 week. Again, no significant difference in expression could be observed for the *P2* transcript. The β-subunit mRNA levels, however, remained slightly higher in cold-acclimated animals than in thermoneutral controls (results not shown). The expression of the *P1* gene thus seems to be under both positive and negative control, in response to changes in ambient temperature.

An explanation for the effect of prolonged cold exposure on *P1* expression, although this expression accounts for only a part of the total expression of subunit c mRNAs, might be to maximally reduce the already low biosynthesis of ATP synthase in BAT. In this context it should be noted that the total reduction

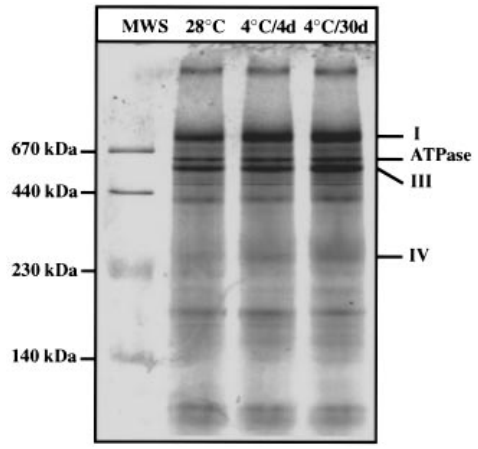

Figure 4 The content of respiratory chain components and ATPase in BAT of cold- and warm-acclimated rats

Lauryl maltoside-solubilized proteins of BAT mitochondria (25 μg protein aliquots) of cold- (4 °C) and warm-acclimated (28 °C) rats were subjected to blue-native PAGE, as described in the Experimental section. The gel was stained with Coomassie Brilliant Blue. The positions of NADH dehydrogenase (I), bc_1 complex (III), cytochrome *c* oxidase (IV), ATPase and of molecular mass standards (MWS) are indicated. The results are representative of two independent analyses.

of subunit c mRNAs in BAT of cold-acclimated rats, as an effect of decreased *P1* expression (Table 2), is only around 20 % (not shown), as BAT predominantly expresses the *P2* mRNA. Nevertheless, electrophoretic analysis under non-dissociating conditions of the assembled oxidative phosphorylation complexes in BAT mitochondria shows that the content of ATP synthase, relative to other respiratory complexes (cf. complex I and ATPase), decreases in cold-acclimated rats compared with warm-acclimated controls (Figure 4). Densitometric analysis of the staining intensity in two independent experiments revealed that if the ratio between complex I and ATPase in BAT mitochondria of 28 °C control animals was arbitrarily set to 1.00, cold acclimation significantly decreased this ratio to 0.61 $\pm$ 0.02. This could reflect a maximal stimulation of the thermogenic potential (capacity) of BAT, as has been found in BAT of the developing hamster [38], in which the lowest ATPase content and thus the minimal phosphorylating capacity coincides with the highest content of the thermogenic uncoupling protein and vice versa. We have previously shown that the decreased content of ATPase during hamster ontogeny occurs concurrently with a reduced expression of subunit c mRNAs [9]. We have now extended this analysis in order to distinguish between the expression of the *P1* and *P2* genes. As shown in Figure 5, in a Northern blot analysis of hamster BAT total RNA isolated from post-natal days 3 and 30, only the *P1* mRNA level was developmentally suppressed during the time when ATPase biosynthesis is reduced. The *P2* mRNA levels, during the same period of time, were only slightly elevated.

Effect of T_3 on *P1* and *P2* expression

It has been shown that the expression of subunit c mRNAs is markedly induced in liver by T_3 treatment of hypothyroid rats [19]. This study [19], however, was limited to the analysis of the combined expression of both the *P1* and *P2* genes. In order to assess the relative contribution of the two subunit c mRNAs in the inducibility by T_3, we treated hypothyroid rats

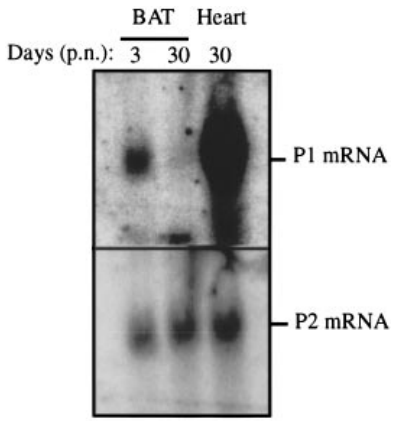

Figure 5 Ontogenic suppression of *P1* mRNA levels during BAT development in hamster

Northern-blot analysis of 10 μg total RNA isolated from post-natal (p.n) day 3 and day 30 of hamster BAT and from heart at day 30, hybridized with subtype-specific *P1* and *P2* riboprobes. The results are representative of two independent analyses.

with T_3 at receptor-saturating concentrations [39]. As shown in Table 3, T_3 increased both *P1* and *P2* mRNA levels, as well as β-actin, in agreement with the report referred to above [19]. However, when the changes evoked by T_3 are related to the β-actin mRNA levels, the *P1* mRNA level was specifically increased, to an approx. 1.4- and 1.7-fold level of the β-actin mRNA level, after 24 and 48 h respectively, as compared with saline-injected controls. This not only corrects for variations in loading, but also reveals the degree of responsiveness to T_3 treatment in relation to genes unrelated to ATPase biosynthesis. In contrast, the expression of the *P*2 gene relative to the β-actin RNA was not significantly altered by hormone treatment. Surprisingly, hypothyroidism itself did not induce any major changes in the levels of any of the three analysed transcripts, with the exception of a small but significant increase in *P2* mRNA levels (Table 3). However, there are no previous reports indicating that methimazole treatment would either increase or decrease subunit c expression in liver, compared with euthyroid animals.

In BAT, the tissue content of T_3 is mainly regulated by intracellular conversion of T_4 into T_3 and this conversion is exclusively catalysed in rodents by type II thyroxine 5′-deiodinase [40,41]. Furthermore, it has been demonstrated in several rodent species that type II 5′-deiodinase in BAT is highly activated during cold exposure [41,42], which consequently results in a marked increase in nuclear T_3 [43].

Given the above indications that thyroid hormones can influence *P1* gene expression and might therefore play a role in the transient increase in *P1* gene expression in response to cold exposure, we investigated the effect of thyroid hormone status on *P1* and *P2* gene expression in BAT. However, neither hypothyroidism nor hyperthyroid concentrations of T_3 affected the expression of the *P1* and *P2* genes compared with that of β-actin (Table 3). A small increase in all three investigated mRNAs was detected in BAT of T_3-treated animals, presumably reflecting a general increase in transcription of many genes, which occurs also in other tissues [44]. In addition, treatment of euthyroid animals with T_3 did not evoke any significant change in *P1* expression in the manner seen after transient cold exposure (results not shown). It is thus unlikely that the transient cold-inducibility of the *P1* gene is mediated via the known activation of 5′-deiodinase in BAT and subsequent increases in nuclear T_3 levels. In conclusion, although T_3 specifically induces the *P1* gene in liver, a similar T_3-mediated activation is not able to circumvent the specific suppression of the *P*1 gene in BAT, a suppression most likely further maintained during prolonged cold exposure via a different regulatory mechanism.

DISCUSSION

Regulation of the multi-subunit, genetically mosaic ATPase complex consists of several control mechanisms, which include selective transcriptional control of genes for some subunits, coordinate expression of others and different post-transcriptional events that affect mRNA stability, location and efficacy of ribosome binding [19,35,45–47] (for a review, see [49]). Additional mechanisms seem to be involved at the post-translational level to remove unassembled subunits in different physiological and pathological conditions [50,51]. In particular, there are many reports of post-transcriptional regulation of the ATPase β-subunit [15,35,45–48], based on a lack of correlation between β-subunit mRNA levels and contents of enzyme.

We have, however, recently shown that in BAT, the steady-state level of the mRNAs encoding subunit c correlate well with the content of ATPase, in contrast with the mRNAs encoding all other subunits of ATPase investigated, and we therefore suggested that the expression of subunit c appears to regulate the level

Table 3 Effect of T_3 on *P1* and *P2* expression

Hypothyroid rats (see the Experimental section) were injected twice subcutaneously with T_3 (100 μg/ml) or with vehicle at 24 h intervals. Total RNA was isolated 24 and 48 h after the first injection and RPA of *P1*, *P2* and β-actin mRNAs was performed on 20 μg of total RNA from liver and BAT. Data are from 4–8 animals as indicated in parentheses, performed in two individual experiments. Results are means ± S.E.M. and expressed as percentages of mRNA levels in untreated euthyroid animals of similar age; *difference statistically significant compared with hypothyroid animals; †difference statistically significant compared with euthyroid animals.

Rats	Tissue	*P1* mRNA	*P2* mRNA	β-Actin mRNA	*P1*/β-Actin	*P2*/β-Actin
Euthyroid	Liver	100 ± 5 (6)	100 ± 7 (6)	100 ± 6 (6)	1.0 ± 0.05	1.0 ± 0.04
Hypothyroid		113 ± 13 (8)	128 ± 14 (8)	110 ± 6 (8)	1.0 ± 0.06	1.2 ± 0.07†
Hypo + T_3 (24 h)		230 ± 36 (6)*	163 ± 26 (6)	167 ± 25 (6)	1.4 ± 0.1*	1.0 ± 0 .1
Hypo + T_3 (48 h)		335 ± 41 (8)*	190 ± 16 (8)*	238 ± 24 (8)*	1.7 ± 0.07*	1.2 ± 0.1
Euthyroid	BAT	100 ± 4 (4)	100 ± 4 (4)	100 ± 15 (4)	1.0 ± 0.1	1.0 ± 0.2
Hypothyroid		127 ± 21 (4)	116 ± 17 (4)	140 ± 32 (4)	0.97 ± 0.2	0.86 ± 0.07
Hypo + T_3 (24 h)		145 ± 13 (4)	120 ± 14 (4)	134 ± 13 (4)	1.1 ± 0. 2	0.92 ± 0.1
Hypo + T_3 (48 h)		146 ± 16 (4)	136 ± 6 (4)	146 ± 18 (4)	1.0 ± 0.1	0.96 ± 0.1

of the entire ATPase complex [9]. Therefore a thorough analysis of the physiological regulation of the expression of the two genes that encode subunit c of mitochondrial ATP synthase is of great interest.

Previously, we used Northern blotting techniques and were unable to distinguish between *P1* and *P2* gene expression, and we also have indications that the probe used did not hybridize equally well with both mRNAs. Therefore in the present investigation we have used the RPA to enable quantitative evaluation of *P*1 and *P*2 gene expression and concentrated on BAT, where there is conspicuous regulation of ATPase biosynthesis. In addition, we show that the correlation between the level of subunit c mRNAs and ATPase can be extended to a number of tissues, indicating that subunit c expression may be a determinative factor for ATPase levels in general.

Our data unequivocally show that *P1* gene expression is highly regulated under two related physiological conditions affecting ATPase biosynthesis in BAT, namely the ontogenic development of BAT and during cold acclimation. In contrast, neither of these conditions significantly affect the *P2* mRNA levels. The mRNA levels of the ATPase β-subunit consistently increase at times when ATPase biosynthesis declines (but when the total content of mitochondrial proteins increases), indicating that the β-subunit gene, but not the subunit c genes, is co-ordinately expressed with the bulk of mitochondrial proteins in BAT. These experiments also suggest that the role of the *P2* mRNA is mainly to serve the cell with a constitutive expression of subunit c and thus to maintain minimum levels of ATP synthase, whereas *P1* gene expression reflects a differential physiological requirement for ATP synthase capacity in a given cell or state of differentiation.

Although the physiological nature of the transient increase in *P1* mRNA levels in BAT of cold-exposed rats (Figure 3C) is enigmatic, it may reflect the changes in the cell population which occur in the interscapular BAT depots during the first days of cold exposure, involving an increased blood flow (with a concomitant increase in the number of leucocytes in the tissue) [52] and a proliferation of preadipocytes and interstitial cells [30]. A transient increase in *P1* expression would thus be expected to occur in the tissue if any of these cells have higher levels of *P1* mRNA than have mature brown adipocytes. Indeed, primary cultures of proliferating preadipocytes from mouse BAT do exhibit 3-fold higher *P1* mRNA levels compared with the tissue (U. Andersson, J. Houštěk and B. Cannon, unpublished work) and also a several-fold increased content of ATP synthase [53].

Thyroid hormones are known to promote mitochondrial biogenesis and exert their action by influencing transcription of a selected set of mitochondrial proteins [54]. It was previously shown that the mRNA of subunit c of the ATPase complex was one of the most responsive to thyroid hormone treatment in rat liver [19]. In accordance with the proposed general role for the *P*1 gene outlined in this study, we have shown that this T_3 sensitivity in rat liver is primarily conferred by affecting *P1* mRNA levels.

In summary, we conclude that the *P1* and *P2* genes for subunit c are differentially regulated *in vivo*. While the *P2* gene is apparently expressed constitutively regardless of physiological challenge, the *P1* gene responds to different physiological stimuli as a means of modulating the relative content of ATP synthase. These, and our previous data [9], thus suggest a pivotal role for subunit c in ATP synthase biosynthesis in BAT and probably also in other tissues.

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