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Hypothyroidism Leads to a Decreased Expression of Mitochondrial F_0F_1-ATP Synthase in Rat Liver

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In liver mitochondria isolated from hypothyroid rats, the rate of ATP synthesis is lower than in mitochondria from normal rats. Oligomycin-sensitive ATP hydrolase activity and passive proton permeability were significantly lower in submitochondrial particles from hypothyroid rats compared to those isolated from normal rats. In mitochondria from hypothyroid rats, the changes in catalytic activities of F_0F_1-ATP synthase are accompanied by a decrease in the amount of immunodetected β-F_1, $F_0$1-PVP, and OSCP subunits of the complex. Northern blot hybridization shows a decrease in the relative cytosolic content of mRNA for β-F_1 subunit in liver of hypothyroid rats. Administration of 3,5,3′-triodo-L-thyronine to the hypothyroid rats tends to remedy the functional and structural defects of F_0F_1-ATP synthase observed in the hypothyroid rats. The results obtained indicate that hypothyroidism leads to a decreased expression of F_0F_1-ATP synthase complex in liver mitochondria and this contributes to the decrease of the efficiency of oxidative phosphorylation.

KEY WORDS: Hypothyroidism; oxidative phophorylation; mitochondria; F_0F_1-ATP synthase.

INTRODUCTION

Thyroid hormones influence oxidative phosphorylation in various tissues: liver, heart, kidney, and brain (Soboll, 1993). Oxidative phosphorylation can be treated as two groups of reactions: those that generate protonmotive force (respiratory chain, dehydrogenases, and mitochondrial metabolic carriers) and those that consume protonmotive force (adenine nucleotide and phosphate carriers, ATP synthase, and proton leak) (Hafner *et al.*, 1990). Kinetic studies have shown that, in mitochondria isolated from liver of hypothyroid rats, only the kinetics of the protonmotive consumers is altered while that of protonmotive generators is unchanged (Hafner *et al.*, 1990). A decrease in the transport of adenine nucleotides (Chen and Hoch, 1977) and of phosphate (Paradies *et al.*, 1991) across the inner mitochondrial membrane of livers of hypothyroid rats has been observed. This decrease has been, at least in part, attributed to changes in the lipid composition of mitochondrial membrane (Paradies *et al.*, 1991) and, in particular, to a decrease in cardiolipin and to an increase in the cholesterol content. Although it has been reported that mitochondrial ATP synthase is one of the sites of thyroid hormone action (Hafner *et al.*, 1990) and that thyroid hormones promote transcriptional activation of the nuclear gene coding for mitochondrial β-F_1-ATP synthase (Izquierdo and Cuezva, 1993), no detailed study on the structure

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and function of mitochondrial F_0F_1-ATP synthase in hypothyroid rats has been reported.[5]

In this paper it is shown that hypothyroidism, induced by 6-*n*-propyl-2-thiouracil (PTU) administration (Blake and Henning, 1985), reduces the amount and the activity of the ATP synthase and the transcript level for β-F_1 subunit, a major component of the catalytic F_1 subunit. *In vivo* administration of 3,5,3′-triiodo-L-tyronine (T_3) to hypothyroid rats tends to restore the normal levels and activities of the complex.

MATERIALS AND METHODS

Materials

6-*n*-Propyl-2-thiouracil and 3-5-3′-triiodo-L-thyronine were purchased from Sigma Chemical Co. Pyruvate kinase, hexokinase, lactate dehydrogenase, and p1,p5-di[adenosine-5′] pentaphosphate (Ap5A) were obtained from Boehringer. SDS, goat anti-rabbit IgG labeled with horseradish peroxidase, horseradish peroxidase color development reagent, molecular mass standards, and agarose were from Bio-Rad; nitrocellulose membranes (0.45 μm pore size) were from Schleicher and Schüll; [α-^{32}P]ATP (specific activity 3000 Ci/mmol) was from Amersham. The rat-cDNA probe for β-F_1 subunit was a generous gift from Prof. P. L. Pedersen (The Johns Hopkins University, Baltimore, Maryland). All other chemicals were of high purity grade.

Animals

Male Wistär rats (200–250 g) were housed at a temperature of 22°C with food and water *ad libitum*. Hypothyroidism was induced by administration of 0.1% w/v PTU in drinking water for 21 days. The animals were killed by decapitation, the trunk blood was collected, and the liver was dissected, cleaned, and weighed (wet weight). Where indicated PTU-treated rats were injected intraperitoneally for 3 days with a dose of T_3 of 30 μg/100 g body weight. Animals were killed 24 h after the final administration. Control PTU-treated animals received only the solvent for the same period. No change both in catalytic activity and content of immunodetected subunits of ATP synthase complex was observed in mitochondria of rats treated with solvent compared to mitochondria of hypothyroid rats.

Rat liver mitochondria were prepared according to Bustamante *et al.* (1977). Inside-out submitochondrial particles (ESMP) were prepared by exposure of mitochondria to ultrasonic energy in a Branson Sonifier (Model W 185, output 70 W) for 60 s at pH 8.5 in the presence of EDTA (Lee and Ernster, 1968).

Determination of Thyroid Hormones

Blood collected from animals was quickly mixed with equal volume of ice-cold 0.9% NaCl containing 0.24 mg EDTA·100 ml^{-1}. Plasma was separated by centrifugation in the cold, and the samples stored at −70°C until assayed. Plasma thyroxine (T_4) and triiodothyronine (T_3) were determined using commercial T_4 and T_3 LIA kits (Diagnostic Products BYK-GULDEN, Italy). After incubation, the tubes were thoroughly decanted and the luminescence was determined. Standard curves were constructed by plotting the amount of total luminescence against the hormone concentration.

Electrophoresis and Immunoblotting Procedures

SDS-PAGE of mitochondria (50 μg protein) was performed on slab gels with a linear gradient of polyacrylamide (15–20%) (Guerrieri *et al.*, 1989a). SDS gels were subjected to immunoblot using polyclonal antibodies against bovine F_1 (Guerrieri *et al.*, 1989a), which cross-react with the β subunit of liver complex Guerrieri *et al.*, 1989b), bovine $F_0$1-PVP (Guerrieri *et al.*, 1989c), and bovine OSCP (which was generously provided by Dr. F. Zanotti, University of Bari). Nitrocellulose sheets were scanned at 590 nm with a CAMAG TLC Scanner. The quantity of antigen detected was evaluated from the computed peak area and expressed in arbitrary units.

[5] Abbreviations: F_1, catalytic sector of mitochondrial F_0F_1-ATP synthase; β-F_1, subunit of the catalytic sector of F_0F_1-ATP synthase; F_0, membrane sector of F_0F_1-ATP synthase; $F_0$1-PVP and OSCP, subunits of the membrane sector of F_0F_1-ATP synthase; A_p5A, p1, p5-Di[adenosin-5′-] pentaphosphate; R.C.I., respiratory control index; T_3, 3,5,3′-triiodo-L-thyronine; T_4, 3,5,3′,5′-tetraiodo-L-thyronine (thyroxine); PTU, 6-*n*-propyl-2-thiouracil; ESMP, submitochondrial particles prepared in the presence of EDTA; PAGE, polyacrylamide gel electrophoresis; PK, pyruvate kinase; LDH, lactate dehydrogenase. Enzymes: ATP synthase (E.C.3.6.1.34).

Northern Blot Hybridization

Total liver RNA was extracted from about 250 mg of liver tissue (Chirgwin *et al.*, 1979). The A260/A280 ratios were in the range of 1.8–2.0. For Northern blot hybridization, 20 μg of RNA were loaded on a 1.1% formaldheyde agarose gel (Sambrook *et al.*, 1989). The gel was first photographed under UV light and then blotted onto a nylon filter (Hybond-Amersham). The membrane was incubated with a cDNA labeled by random priming (Sambrook *et al.*, 1989) with [α-^{32}P]ATP. Hybridization and washing conditions were as described in (Cantatore *et al.*, 1987). After autoradiography the relative amounts of β-F_1 mRNA was determined by densitometry, comparing the intensity of the hybrid band with that of 28S rRNA present in the same gel and used as internal standard.

Assays

Respiratory rate was measured by a Clark oxygen electrode. 750 μg mitochondrial proteins were suspended in 1.5 ml of a solution containing sucrose 200 mM, $MgCl_2$ 3 mM, EDTA 1 mM, K-Pi 10 mM (pH 7.4), glucose 20 mM, bovine serum albumin 0.25%,5 units of hexokinase, Ap5A 300 μM, and rotenone 2 $\mu g \cdot ml^{-1}$. After 2 min of equilibration, K-succinate (20 mM, pH 7.4) was added and the respiratory rate in state 4 was measured. After 4 min Mg-ADP 300 μM was added and the respiratory rate in state 3 was followed for 4 min. The ATP-stimulated respiratory rate was measured in the presence of the regenerative system for ADP (glucose *plus* hexokinase) and was linear until oxygen is exhausted (not shown). After 4 min of respiration in state 3, either 4 μM FCCP was added to measure the respiratory rate in the uncoupled state or 500 μl of the suspension was taken, treated with 500 μl of 28% $HClO_4$, and the rate of synthesis of ATP determined spectrophotometrically as described in Papa *et al.* (1969).

To measure the oligomycin-sensitive proton permeability of the inner mitochondrial membrane, ESMP (3 mg protein/ml) were incubated in a mixture containing 250 mM sucrose, 0.2 mg/ml purified catalase, valinomycin (1 μg/mg protein), and 20 mM succinate (final volume 1.5 ml; pH 7.5). Incubation was carried out in a glass vessel, under a constant stream of N_2, thermostated at 25° by a glass jacket connected to a water bath, until anaerobiosis was reached. Respiration-driven proton translocation was activated by repetitive pulses of 3% H_2O_2 (5 μl/ml). The pH of the suspension was monitored potentiometrically with a Beckman combination electrode (No. 39030 Beckman Instrument) connected to a Keitley Electrometer and from this to a strip chart recorder. The overall response time of the pH recording system was about 400 ms at 25°C (Papa *et al.*, 1979). The ATP hydrolase activity of ESMP was determined in the presence of added PK, phosphoenolpyruvate, and LDH by following NADH oxidation at 340 nm (Guerrieri *et al.*, 1989a).

RESULTS

In hypothyroid rats the serum levels of T_3 and T_4, as well as the body weight and the liver weight, were significantly reduced as compared to the normal animals ($p < 0.01$), which confirms the hypothyroid state (Table I) (see also Verhoeven *et al.*, 1985; Horrum *et al.*, 1990). After treatment of hypothyroid rats with T_3 for 3 days, a significant increase in the liver weight ($p < 0.02$) was observed.

Consistent with data already reported (Verhoeven *et al.*, 1985; Horrum *et al.*, 1990; Paradies *et al.*, 1991), the respiratory rates, both in state-3 and state-4, using succinate as substrate, were significantly lower in liver mitochondria isolated from hypothyroid rats compared to liver mitochondria isolated from normal rats. However the decline of respiratory rate in state-3 was greater (79% inhibition) than that of respiratory rate in state-4 (44% inhibition), resulting in a decrease of R.C.I. ($p < 0.001$) (Table II) (cf. Hoch, 1968; Chen and Hoch, 1977). A significant decline of the uncoupler-stimulated respiratory rate was also observed in liver

Table I. Body Weight, Liver Weight, and Serum Levels of T_3 and T_4 in Normal, Hypothyroid, and T_3-Treated Hypothyroid Rats[a]

	Normal rats	Hypothyroid rats	T_3-treated hypothyroid rats
Body weight (g)	235 ± 8.2	200 ± 6.1[b]	215 ± 7.3
Liver weight (g)	11 ± 0.6	7.3 ± 0.7[b]	9.1 ± 0.1[c]
T_3 (ng/dl)	185 ± 26	87 ± 8[b]	>800
T_4 (μg/dl)	4.1 ± 0.5	1.87 ± 0.22[b]	7.86 ± 0.5

[a] For treatment of rats with PTU or with T_3 (to PTU-treated rats) and determination of serum levels of T_3 and T_4, see Materials and Methods. Data represent the mean ± S.E.M. of 12 differnt rats.
[b] $p < 0.01$ vs. normal rats.
[c] $p < 0.02$ vs. hypothyroid rats.

Table II. Respiratory Activities and ATP Synthesis of Hepatic Mitochondria from Normal, Hypothyroid, and T_3-Treated Hypothyroid Rats[a]

	Respiratory rate in state 4	Respiratory rate in state 3	R.C.I.	FCCP-stimulated respiratory rate	Rate of ATP synthesis	~P/O
Normal rats	25 ± 2	213 ± 17	8.5 ± 0.3	253 ± 30	278 ± 18	1.3 ± 0.1
Hypothyroid rats	14 ± 1[b]	44 ± 7[b]	3.1 ± 0.4[b]	102 ± 14[b]	71 ± 8[b]	1.6 ± 0.1
T_3-treated hypothyroid rats	19 ± 1	170 ± 8	8.9 ± 0.4	192 ± 14	263 ± 13	1.5 ± 0.04

[a] The succinate-dependent oxygen consumption was measured polarographically as described under Materials and Methods. Respiratory activities are expressed as ng-at. O · min^{-1} · mg protein.$^{-1}$ Rate of ATP synthesis is expressed as nmol ATP formed min^{-1} · mg $protein^{-1}$. Data represent the mean ± SEM of 12 different experiments.
[b] $p < 0.001$ vs normal rats.

mitochondria from hypothyroid rats (Table II). Treatment of hypothyroid rats with T_3 tended to reverse all the impairments observed in mitochondria isolated from hypothyroid rats (Table II).

A decrease in the respiratory rates in state 4 and in the uncoupled state could suggest alteration of respiratory complex functions in liver mitochondria from hypothyroid rats. Although the data of this paper cannot exclude such possibility, it should be considered that no significant changes in hemes *a*, *b*, and c_1 were observed in hypothyroid rats (Table III, see also Chen and Hoch, 1977) and that the increased cholesterol level in mitochondria from hypothyroid rats (Paradies *et al.*, 1991) could alter the membrane permeability to the uncoupler. When hypothyroid rats were injected for 3 days with 30 μg/100 g of body weight no significant increase of the heme's content was observed (Table III). This is in agreement with the observation by Mutvei and Nelson (1989) who showed no significant changes of cytochrome amounts and of activities of respiratory chain complexes following injection to hypothyroid rats of low doses of T_3 for short periods of time.

Table III. Cytochrome Contents in Liver Mitochondria from Normal, Hypothyroid, and T_3-Treated Hypothyroid Rats[a]

	Heme a	Heme *b*	Heme c_1
Normal rats	0.48 ± 0.03	0.24 ± 0.02	0.09 ± 0.01
Hypothyroid rats	0.42 ± 0.03	0.18 ± 0.02	0.09 ± 0.01
T_3-treated hypothyroid rats	0.42 ± 0.05	0.21 ± 0.04	0.11 ± 0.01

[a] For treatment of rats with PTU or with T_3, see Materials and Methods. Heme content was determined by spectral differential analysis of dithionite reduced vs. oxidized liver mitochondria as reported by D. J. Williams (1964). Data represent the mean ± SEM of eight different preparations of mitochondria and are expressed as μmol·mg $protein^{-1}$.

The significant decrease of R.C.I. in mitochondria from hypothyroid rats (Table II) suggests changes in the mitochondrial oxidative phosphorylation.

Direct measurements of the rate of ATP synthesis in succinate-supplemented mitochondria showed a decline (74%) in liver of hypothyroid rats Vs. liver of normal rats (Table II). No decline in the ~P/O ratio is observed (Table II), indicating that the mitochondria from liver of hypothyroid rats are well coupled. In liver mitochondria from T_3-treated hypothyroid rats the rate of ATP synthesis tended to be restored (Table II).

In order to see whether hypothyroidism affects directly the activities of the F_0F_1-ATP synthase, inside-out submitochondrial particles (ESMP), which expose directly the catalytic sites to the suspension medium, from liver mitochondria of normal and hypothyroid rats were prepared. Figure 1 shows a decrease in V_{max} of oligomycin-sensitive ATP hydrolase activity, with no change in K_m for ATP, in ESMP prepared from livers of hypothyroid rats. In T_3-treated hypothyroid rats the ATP hydrolase activity of ESMP tended to recover (Fig. 1).

In ESMP, anaerobic release of transmembrane proton gradient, set up by respiration, with succinate as substrate, is 70–80% inhibited by the F_0 inhibitor oligomycin (Fig. 2, see also Pansini *et al.*, 1978). In ESMP from liver of hypothyroid rats this process was 60% depressed and almost restored in ESMP from T_3-treated hypothyroid rats (Fig. 2). The residual oligomycin-insensitive passive proton permeability was about the same in the three kinds of ESMP and amounted to about 25% of the control value (Fig. 2).

Immunoblot analysis showed that the content of the β-F_1 subunit, $F_0$1-PVP, and OSCP decreased in

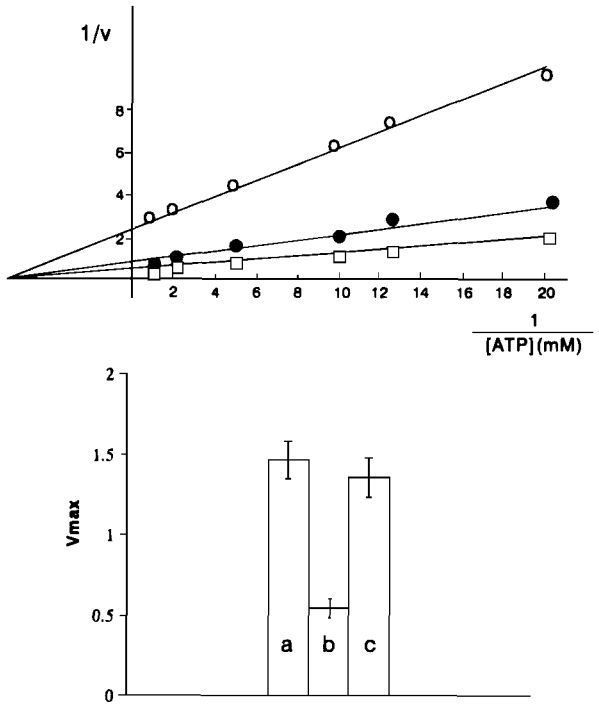

Fig. 1. Effect of hypothyroidism on the kinetics of ATP hydrolase activity in ESMP. Upper panel: Lineweaver–Burk plot of a typical experiment, symbols: □, control ESMP; ○, ESMP from hypothyroid rats, and ●, ESMP from T_3-treated hypothyroid rats. $1/v$ represents the reciprocal values of the rates of ATP hydrolysis expressed in μmol ATP hydrolyzed·min^{-1}·mg particle $protein^{-1}$. Lower panel: ATP hydrolase V_{max} values in ESMP from normal (a), hypothyroid (b), and T_3-treated hypothyroid rats (c). The values reported in the figure are the means ± S.E.M. of experiments using livers of eight different rats.

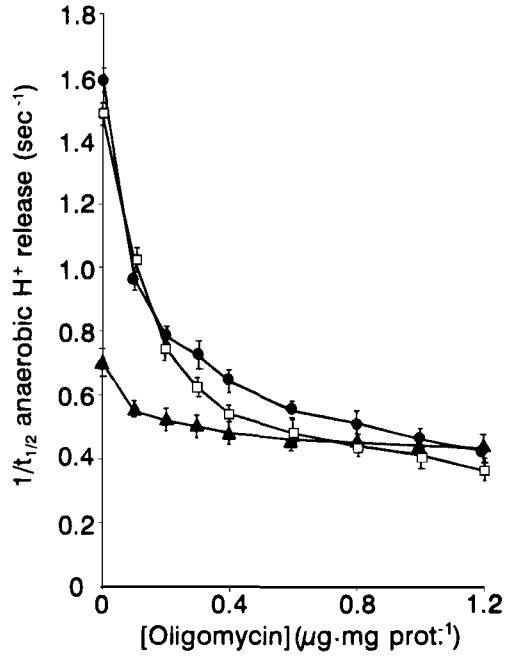

Fig. 2. Oligomycin-sensitive proton permeability in ESMP from normal (●), hypothyroid (▲), and T_3-treated hypothyroid (□) rats. For ESMP preparation and determination of oligomycin-sensitive proton permeability, see Materials and Methods. The values reported in the figure are the means ± S.E.M. of experiments using livers of eight different rats.

liver mitochondria of hypothyroid rats (Fig. 3). In liver mitochondria from T_3-treated hypothyroid rats the content of immunodetected ATP synthase subunits was almost completely recovered (Fig. 3).

Many nuclear-encoded components of oxidative phosphorylation complexes show multiple isoforms encoded by several different genes (Villena *et al.*, 1994), but the β-F_1 subunit is encoded by a single copy gene which is ubiquitously expressed in mammalian cell (Neckelmann *et al.*, 1989). Therefore, in order to see whether the changes in the β-F_1 content of mitochondria were associated with changes in the transcription of the gene, we analyzed the level of β-F_1 mRNA in liver of normal, hypothyroid and T_3-treated hypothyroid rats.

Figure 4A shows a representative Northern-blot hybridization between RNA, extracted from the liver of control, hypothyroid, and T_3-treated hypothyroid rats, and a radioactive β-F_1 probe (Houstek *et al.* 1991). The hybridization signal was normalized relatively to the corresponding 28S rRNA signal on the same ethidium bromide-stained gel (Fig. 4A). Statistical analysis of results obtained from eight independent experiments show a significant reduction (about 75%) of the level of β-F_1 mRNA in hypothyroid with respect to normal rats (Fig. 4B). In T_3-treated hypothyroid rats the level of β-F_1 mRNA tended toward the normal value (Fig. 4B).

DISCUSSION

The data reported in this paper show that in liver mitochondria from hypothyroid rats the rate of ATP synthesis is lower than that of liver mitochondria from normal rats. No change in the ~P/O ratio is observed (see also Hafner and Brand, 1988; Paradies *et al.*, 1991), indicating that the liver mitochondria from hypothyroid rats are well coupled.

It has been reported that thyroid hormones influence the oxidative phosphorylation process acting on the phosphorylation machinery rather than on the respiratory chain (Hafner *et al.*, 1990).

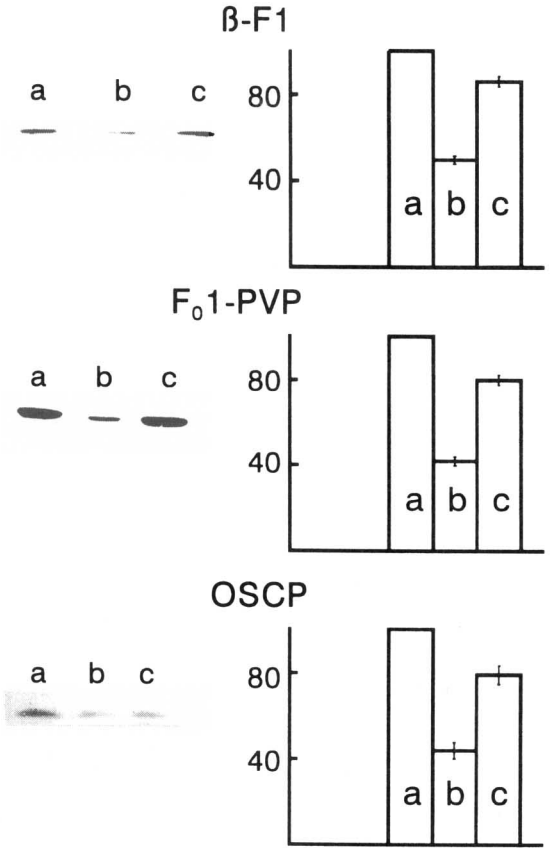

Fig. 3. Immunoblot analysis of content of β-F_1, $F_0$1-PVP, and OSCP subunits of ATP synthase complex in liver mitochondria isolated from normal (a), hypothyroid (b), and T_3-treated hypothyroid rats (c). Columns represent the semiquantitative analysis by densitometry. Data represent the means ± S.E.M. of experiments using eight different mitochondrial preparations.

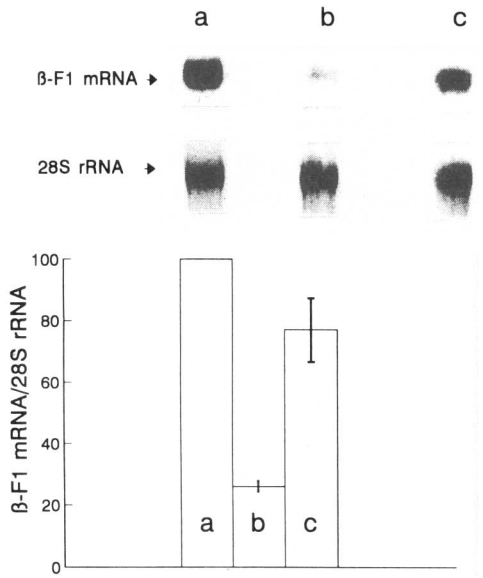

Fig. 4. Northern-blot analysis of βF_1-mRNA levels in liver from normal, hypothyroid, and T_3-treated-hypothyroid rats. Upper panel shows a representative blot-hybridization between 20 μg of RNA, extracted fron the the liver of normal (a), hypothyroid (b), and T_3-treated hypothyroid rats (c), and a radioactive β-F_1 probe. The 28S rRNA signal on the same ethidium bromide-stained gels is also shown. Lower panel shows the semiquantitative analysis by densitometry. Data represent the means ± S.E.M. of results using eight different mitochondrial preparations.

The phosphorylation machinery is the result of integrated functions of F_0F_1-ATP synthase complex and of the adenine nucleotide and phosphate carriers. The present study was addressed to investigate whether, in addition to the altered function of the carriers involved in the phosphorylation machinery (Chen and Hoch, 1977; Paradies *et al*, 1991), the F_0F_1-ATP synthase is affected by hypothyroidism. For this purpose we analyzed the oligomycin-sensitive ATP hydrolase activity and the oligomycin-sensitive passive proton permeability in inside-out submitochondrial particles in which these activities of F_0F_1-ATP synthase complex are not dependent from the activities of other component of the phosphorylation machinery. In submitochondrial particles isolated from hypothyroid rats, the oligomycin-sensitive ATPase activity and passive proton permeability decrease.

These observations suggest that hypothyroidism affects directly the mitochondrial F_0F_1-ATP synthase. In addition, in agreement with the observation that the effect of hypothyroidism on oxidative phosphorylation is not due to an increase of proton leak (see also Harper *et al.*, 1993), our experiments show that the oligomycin-insensitive passive proton permeability in ESMP is not affected by thyroid hormones (see Fig. 2).

These experiments, for the first time, clearly indicate that hypothyroidism directly affects the functions of F_0F_1-ATP synthase. This is associated with a decrease in the immunodetected amount of the β-F_1, $F_0$1-PVP, and OSCP subunits of the complex. This suggests a lower content of the whole enzyme in mitochondria from hypothyroid rats, which is recovered after T_3 treatment of the hypothyroid rats.

It has been reported (Nelson, 1987; Nelson *et al.*, 1995) that thyroid hormones regulate the *in vivo* expression of a selected set of rat nuclear genes encoding inner mitochondrial membrane proteins. Luciakova and Nelson (1992) reported that T_3 treatment of hypothyroid rats increased the levels of $mRNA_s$ for cytochrome c_1, adenine nucleotide translocase, and subunit

c of the F_0F_1-ATP synthase. The same group (Luciakova *et al.*, 1992; and Nelson et al., 1995) suggested that the mRNA for the β-F_1 subunit of F_0F_1-ATP synthase does not respond to the thyroid hormone although they reported a twofold increase in β-F_1 mRNA levels after T_3 administration to hypothyroid adult rats. The data here reported (Fig. 4) show that in the liver of hypothyroid rats there is a 75% reduced content of the level of the nuclear encoded β-F_1 transcripts. T_3 treatment of the hypothyroid rats increased about three times the β-F_1 mRNA level (Fig. 4). Thus thyroid hormones appear to regulate gene expression of the nuclear encoded β-F_1 (see also Izquierdo *et al.*, 1995).

The decrease of intramitochondrial content of β-F_1 subunit (50%), observed in hypothyroid rats, is lower than the decrease of the level of β-F_1 mRNA (75%). These data agree with previous studies on mitochondrial biogenesis during postnatal differentiation of normal and hypothyroid rats (Luis *et al.*, 1993; Izquierdo *et al.*, 1995) and on liver regeneration (Guerrieri *et al.*, 1995), where it was observed that the changes in the level of β-F_1 mRNA do not correlate with its translational efficiency. The higher reduction of the β-F_1 mRNA content with respect to that of the protein may be explained by a compensatory mechanism operating either at the translation or at the post-translation level (i.e., control of the protolysis of the protein). The effect of thyroid hormones on the expression of nuclear genes may be due to a unique control mechanism or, more probably, a multiple regulatory circuit could exist. Although it has been shown that, in some nuclear genes, thyroid hormones affect gene expression by directing the binding of nuclear proteins to sequences located in the promoter of the gene (Chung *et al.*, 1992), it is still unknown whether the β-F_1 ATPase gene contains functional thyroid hormone-responsive elements. Recently Almeida *et al.* (1995), studying the effect of hypothyroidism on postnatal changes in rat liver mitochondria, suggested that thyroid hormones regulated the synthesis of some nuclear encoded protein involved in the correct assembly of the F_0F_1-ATP synthase complex.

In conclusion our data show that hypothyroidism leads to a decreased expression of the subunits(s) of the F_0F_1-ATP synthase which results in a low level of the enzyme in the mitochondria, contributing to the alteration of the oxidative phosphorylation machinery.

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