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Differential 3,5,3′-Triiodothyronine-Mediated Regulation of Uncoupling Protein 3 Transcription: Role of Fatty Acids

Pieter de Lange, Anna Feola, Maurizio Ragni, Rosalba Senese, Maria Moreno, Assunta Lombardi, Elena Silvestri, Ramon Amat, Francesc Villarroya, Fernando Goglia, and Antonia Lanni

Dipartimento di Scienze della Vita (P.d.L., A.F., M.R., R.S., A.La.), Seconda Università degli Studi di Napoli, 81100 Caserta, Italy; Dipartimento di Scienze Biologiche ed Ambientali (M.M., E.S., F.G.), Università degli Studi del Sannio, 82100 Benevento, Italy; Dipartimento delle Scienze Biologiche (A.Lo.), Sezione Fisiologia ed Igiene, Università degli Studi di Napoli "Federico II," 80134 Napoli, Italy; and Departament de Bioquímica i Biologia Molecular (R.A., F.V.). Universitat de Barcelona, and CIBER Fisiopatologia de la Obesidad y Nutrición, Instituto de Salud Carlos III, 08028 Barcelona, Spain

T_3 regulates energy metabolism by stimulating metabolic rate and decreasing metabolic efficiency. The discovery of mitochondrial uncoupling protein 3 (UCP3), its homology to UCP1, and regulation by T_3 rendered it a possible molecular determinant of the action of T_3 on energy metabolism, but data are controversial. This controversy may in part be attributable to discrepancies observed between the regulation by T_3 of UCP3 expression in rats, humans, and mice. To clarify this issue, we studied 1) the induction kinetics of the UCP3 gene by T_3 in rat skeletal muscle, 2) the influence of fatty acids, and 3) the structure and regulation of the various UCP3 promoters by T_3. Within 8 h of single-dose T_3 administration, hypothyroid rats showed a rise in serum fatty acid levels concomitant with a rapid increase in UCP3 expression in gastrocnemius muscle, followed by inductions of peroxisome proliferator activated receptor δ (PPARδ) (within 24 h) and PPAR target gene expression (after 24 h). This T_3-induced early UCP3 expression depended on fatty acid-PPAR signaling because depleting serum fatty acid levels abolished its expression, restorable by administration of the PPARδ agonist L165,041 (4-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy]phenoxy]acetic acid). In transfected rat L6 myoblasts, only the rat UCP3 promoter positively responded to T_3 and L165,041 together in the presence of MyoD, thyroid hormone receptor β1 (TRβ1), PPARδ, or PPARδ plus the TR dimerization partner retinoid X receptor α. All promoters share a response element common to TR and PPAR (TRE 1), but the observed species differences may be attributable to different localizations of the MyoD response element, which in the rat maps to exon 1. (*Endocrinology* 148: 4064–4072, 2007)

IN ADULT MAMMALS, T_3 regulates energy metabolism (1, 2) by stimulating metabolic rate and decreasing metabolic efficiency. T_3 is known to control the rate of transcription of those target genes whose promoters contain thyroid hormone response elements (TREs) by binding to different thyroid hormone receptor (TR) isoforms (TRα and TRβ) (3). However, the molecular mechanisms by which T_3 regulates energy expenditure are only starting to be elucidated, and, over the past 10 yr, opinions have varied as to the nature of the genes involved. Mitochondria, by virtue of their biochemical functions, are key cellular sites for the metabolic effects of thyroid hormones (1). Skeletal muscle constitutes the bulk of the total metabolically active body mass, by virtue of its significant mitochondrial capacity, and it thus represents an important target for the action of T_3 (1). The discovery of mitochondrial uncoupling proteins (UCPs) 2 and 3, which are similar to the UCP1 in brown adipose tissue (BAT), suggested that these proteins might be potential targets for thyroid hormone and indeed may serve as mediators of the effects of thyroid hormone on energy expenditure (4). In contrast to UCP2, which is ubiquitously expressed (5), UCP3 is expressed to some extent in BAT but to a greater extent in muscle (more in skeletal muscle than in heart) (6, 7). The induction of UCP3 expression has been shown to be a clear target for T_3 in skeletal muscle of humans (8, 9) and rats (10–12), although the situation is controversial in mice. Transgenic mice harboring the human UCP3 transgene show no significant T_3-induced stimulation of the transcription of the endogenous UCP3 gene in their muscle, whereas transcription of the human transgene is clearly stimulated (13). In addition, T_3 is able to induce UCP3 protein accumulation and UCP3-mediated uncoupling in skeletal muscle mitochondria in rats (12, 14), but, once again, the situation is different in mice. In a recent report (15), the hypothesis was tested that UCP3 mRNA levels might show a positive correlation with resting metabolic rate (RMR) and proton leak in mice in various thyroid states, and it was concluded that T_3 does not influence the intrinsic mitochondrial properties and that variations in UCP3 mRNA levels may only partly explain the variations in RMR. Another study in mice (16) seems not to support UCP3 serving as one of the determinants of the induction of RMR by T_3 as it does in the rat (12).

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Abbreviations: BAT, Brown adipose tissue; BW, body weight; CPT1b, carnitine palmitoyl transferase 1b; dNTP, deoxynucleotide triphosphate; FFA, free fatty acid; I, iopanoic acid; Luc, luciferase; MTE I, mitochondrial thioesterase I; NA, nicotinic acid; P, 6-*n*-propyl-2-thiouracil; PPAR, peroxisome proliferator activated receptor; RMR, resting metabolic rate; RPS12, ribosomal protein S12; TR, thyroid hormone receptor; TRE, thyroid hormone response element; UCP, uncoupling protein.

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Indeed, during repeated treatment with high doses of T_3, UCP3 knockout mice show a nonsignificantly lower stimulation of RMR compared with their wild-type controls (16).

These discrepancies indicate, as also suggested by the previously cited authors (15), the existence of species differences in the action of T_3 on UCP3 expression, and this has caused considerable uncertainty about the role performed by UCP3 as a thermogenic protein mediating the action of T_3 in skeletal muscle (4).

Sequence comparisons between human and rodent UCP3 promoters has indicated a rapid phylogenetic evolution, suggesting functional and regulatory diversification (17). Human UCP3 contains two tissue-specific transcription start sites for skeletal muscle and BAT, respectively, whereas rat and mouse transcripts initiate at the same site for BAT and for muscle tissue (17). Mechanistically, transcription of the human UCP3 gene in skeletal muscle is dependent on the presence of the transcription factor MyoD, which has been shown to bind to a multiple E-box present in the proximal promoter region (18). The human UCP3 gene is regulated by both fatty acids and retinoic acid, through a response element in the UCP3 promoter described previously (18, 19), and T_3 directly stimulates human UCP3 expression through the same element, termed TRE 1 (13). This element is a nonperfect direct repeat with one nucleotide spacing (DR+1) of the sequence AGGTTTCAGGTCA. Although the proximal promoter-exon 1 structures of rodent and human UCP3 diverge, the TRE1 element and its surrounding sequences, as well as the MyoD E-box, are 100% conserved in the mouse UCP3 promoter (13). However, unlike in humans, T_3 only weakly affects mouse UCP3 expression (13, 15). Thus, it seems increasingly evident that T_3 regulates the UCP3 gene in a species-dependent manner and that simply collating all available data without taking into account the animal models from which they were derived may hinder our understanding of the actual role of UCP3. To our knowledge, no published data are available concerning the mechanism by which T_3 regulates the rat UCP3 gene. Because the rat UCP3 is a clear target for T_3, studying the regulation of the rat UCP3 gene may help to clarify this issue. UCP3, together with several genes involved in lipid metabolism, such as carnitine palmitoyl transferase 1b (CPT1b) and mitochondrial thioesterase I (MTE I), is a target of transcriptional regulation by fatty acids through their binding to peroxisome proliferator activated receptors (PPARs) (20–22). Because T_3 induces lipolysis (2), we measured the stimulation of the UCP3 gene by T_3 and verified the dependence of this action of T_3 on the presence of fatty acids both *in vivo* (in rat skeletal muscle) and *ex vivo* (in transfected rat L6 myoblasts). Next, because of the relatively high expression of PPARδ in skeletal muscle [PPARδ is expressed in skeletal muscle at 10- and 50-fold higher levels than PPARα and PPARγ, respectively, with a relative high expression in oxidative muscle fibers (see Ref. 20)], we used the same *in vivo* and *ex vivo* models to investigate whether PPARδ influences T_3-induced UCP3 expression through its activation by the specific ligand L165,041, known to induce UCP3 expression (23). Finally, in transfected L6 cells, we analyzed the responses of the human, rat, and mouse UCP3 promoters to T_3 via TRβ, retinoic acid receptor α (RXRα), and PPARδ, and we also compared the structure of the proximal promoter among the different species.

Materials and Methods

Experimental animals

All animal experimentation was conducted in accord with accepted standards of humane animal care.

Materials

T_3, 6-*n*-propyl-2-thiouracil (P), iopanoic acid (I), and the PPARδ agonist L165,041 (4-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)propoxy-]phenoxy]acetic acid) were purchased from Sigma (St. Louis, MO). Nicotinic acid (NA) was from Fluka Biochimica (Buchs, Switzerland). The pSG5-hPPARδ construct (24) was kindly provided by Dr. Bart Staels (Department of Atherosclerosis at the Institut Pasteur de Lille, Institut National de la Santé et de la Recherche Médicale, Lille, France). The r-AOX-602-Luc construct was kindly provided by Dr. Ronald Evans (The Salk Institute, La Jolla, CA).

Animals

Male Wistar rats (220–250 g) were kept, one per cage, in a temperature-controlled room at 28 C under a 12-h light, 12-h dark cycle. A commercial mash and water were available *ad libitum*. To determine the dose of the PPARδ agonist L165,041 that effectively induced UCP3 transcription, 500 μg/100 g or 1 mg/100 g body weight (BW) were administered intraperitoneally to euthyroid control rats. Hypothyroidism was induced by simultaneous injection of P and I, as described previously (12) (P+I rats). For time-course experiments, hypothyroid rats were injected with 25 μg T_3/100 g BW. This dose was used because it produces a clear-cut effect on RMR and indeed restores it to the level observed in euthyroid controls (25). This dose given acutely is not a large dose; in fact, it has been established that 200 μg/100 g BW, given iv, is the acute dose needed to obtain at least 95% nuclear receptor saturation for 24 h (26). Animals were killed at 6, 8, 12, 24, and 48 h after T_3 injection. In a subgroup of rats (derived from the 8 h group and the P+I controls), the antilipolytic agent NA was administered at 10 mg/100 g BW intraperitoneally every 2 h for 8 h before the animals were killed, in the presence or absence of L165,041, which was administered at 500 μg/100 g BW. At the end of the treatments, rats were anesthetized using an ip injection of chloral hydrate (40 mg/100 g BW) and were then killed by decapitation. Gastrocnemius and soleus muscles, as well as hearts, were excised, immediately frozen in liquid nitrogen, and stored at −80 C for later processing.

RNA isolation

Total RNA was isolated using the TRIZOL standard protocol (Invitrogen, Milan, Italy). Tissue/TRIZOL mixtures were homogenized using a polytron, keeping the viscosity of the solution to a minimum to ensure effective inactivation of endogenous ribonuclease activity.

RT-PCR assays

One microgram of total RNA was reverse transcribed using 100 pmol random hexamers (Invitrogen), 2.0 U Superscript reverse transcriptase, 0.5 U ribonuclease inhibitor, and 1 mM deoxynucleotide triphosphates (dNTPs) in reverse-transcriptase buffer (all from HT Biotechnology, Cambridge, UK). The total volume was adjusted to 20 μl with distilled H_2O, and the reaction was performed for 1 h at 40 C. One quarter of the RT reaction mixture was used directly for the PCR reaction in a total volume of 25 μl, containing 0.25 U SuperTaq polymerase, 0.25 mM dNTPs, SuperTaq PCR buffer (all from HT Biotechnology), 5% (vol/vol) dimethylsulfoxide (Sigma), and 0.38 pmol of the relevant oligonucleotide primers (Sigma Genosys, Cambridge, UK). Gene expression signals were normalized with respect to the signal for the nonregulated 40S ribosomal protein S12 (RPS12), because this gene did not vary its expression in the tested conditions, in contrast to the usually applied β-actin gene. The primers used had the following sequences: RPS12 sense, 5′-GCTGCTGGAGGTGTAATGGA-3′; RPS12 antisense, 5′-CTA-

CAACGCAACTGCAACCA-3′; CPT1b sense, 5′-CTCAGCCTCTACGGCAAATC-3′; CPT1b antisense, 5′-CTTCTTGATCAGGCCTTTGC-3′; PPARδ sense, 5′-AACATCCCCAACTTCAGCAG-3′; PPARδ antisense, 5′-GGAAGAGGTACTGGCTGTCG-3′; UCP3 sense, 5′-ATGGATGCCTACAGAACCAT-3′; UCP3 antisense, 5′-CTGGGCCACCATCCTCAGCA-3′; MyoD sense, 5′-CTGCTCTGATGGCATGATGG-3′; MyoD antisense, 5′-GGACACTGAGGGGTGGAGTC-3′; MTE I sense, 5′-CCTCGTCTTTCGCTGTCCTG-3′; MTE I antisense, 5′-GTGTCCGTCCAGCACCTCCA-3′; TRβ1 sense, 5′-GTTCAAGAGGAGCCACACTG-3′; and TRβ1 antisense, 5′-CAGGCTTCGGACATTCCTAC-3′. For all genes tested, parallel amplifications (20, 25, and 30 cycles) of the same cDNA were used to determine the optimum number of cycles. After 30 cycles, a readily detectable signal within the linear range was observed. For the actual analysis, samples were heated for 5 min at 94 C and then 30 cycles were performed, each consisting of 1 min at 94 C, 1.5 min at 61 C, and 1.5 min at 72 C. This was followed by a final 10-min extension at 72 C. The quantities of the PCR products were determined in separate preparations from three rats. Separation of the PCR reaction products was performed on a 2% agarose gel containing ethidium bromide, and the products were readily visualized. Reverse-image signals of the RT-PCR bands were quantified by means of a Bio-Rad (Hercules, CA) Molecular Imager FX using the supplied software. Primary, reverse-image RT-PCR data are shown in the figures, with quantities being displayed for each gel. The accuracy of the RT-PCR method has been confirmed by confronting the obtained data with those of Northern analysis of UCP3 expression using the same treatments (12).

Measurement of circulating free fatty acid (FFA) levels

Serum fatty acid levels were measured using a Wako NEFA C kit (Wako Chemicals, Neuss, Germany).

Construction of the rat UCP3-promoter transfection plasmid

A fragment from −2134 to +43 of the rat UCP3 gene was amplified by PCR from 200 ng of genomic DNA using the following oligonucleotides: sense, 5′-CCCCTCGAGCCAGGTCATGGACAGTTG-3′; and antisense, 5′-CCCCTCGAGCATTCACTGTTGTCTCTG-3′. The PCR amplification protocol was as described above, with the following amendments: the final dNTP concentration was 0.3 mM, the final $MgCl_2$ concentration was 1.75 mM, the cycle number was 40, the annealing temperature was 50 C, and the extension period was 2 min at a temperature of 68 C. The obtained fragment was digested with *Xho*I and cloned into pGL3 basic (Promega, Milan, Italy), which contains the cDNA for firefly (*Photinus pyralis*) luciferase (Luc) as a reporter gene. The integrity of the fragment was verified by direct DNA sequencing performed using a commercially available sequencing kit (USB Sequenase PCR product sequencing kit; GE Healthcare, Little Chalfont, UK) using an antisense oligo complementary to the Luc gene ranging from nucleotide positions 120 to 139 of the pGL3 plasmid (Promega) of the sequence 5′-CCAGCGGATAGAATGGCGCC-3′.

Cell culture and transient transfection assays

Rat myoblastic L6 cells were obtained from the Cell Bank (Interlab Cell Line Collection) of the National Institute for Cancer Research (Genoa, Italy), cultured in DMEM containing 10% fetal bovine serum (Hyclone, Logan, UT). Transfection experiments were performed using L6 cells seeded at 50% confluence in DMEM containing 10% dextran/charcoal-treated fetal bovine serum (Hyclone) (using Lipofectamine 2000 in accordance with the instructions of the manufacturer; Invitrogen). For L6 transfection, each point was assayed in triplicate in a 12-well plate. Cells were transfected with 750 ng/well −2134/+43rUCP3-Luc, −1946/+60mUCP3-Luc (14), or −1588/+47hUCP3-Luc reporter vectors (17), 150 ng/well of the mammalian expression vectors pCMV-MyoD, pRSV-hTRβ1, pSG5 hPPARδ (23), and PRSV-hRXR, together with 1.5 ng/well phRL-TK-Luc (Promega), with an expression vector for the sea pansy (*Renilla reniformis*) Luc being used as an internal transfection control. Cells were incubated for 48 h after transfection and treated with T_3 and/or the PPARδ agonist L165,041 for 24 h before harvest. T_3 was added in the concentration range of 0–100 nM, as indicated in the figures, or at a fixed concentration of 100 nM in the presence or absence of 50 μM L165,041. Luminescence was measured in a Turner Biosystems Luminometer (model TD20/20) using the Dual Luciferase Reporter assay system kit (Promega).

Statistical analysis

Results are expressed as means ± SEM. The statistical significance of differences between groups was determined using a one-way ANOVA followed by a Student-Newman-Keuls test.

Results

UCP3 expression in gastrocnemius muscle is rapidly up-regulated by a single administration of T_3 to hypothyroid rats, followed by induction of PPARδ and PPAR-responsive genes

Within 6 h after administration of a single dose of T_3 (25 μg/100 g BW) to hypothyroid (P+I) rats, UCP3 mRNA levels were increased around 3.5-fold (Fig. 1). At the 0-h time point, PPARδ mRNA was already at a clearly detectable level. At 12 h, the PPARδ mRNA level was doubled, and it reached a maximal increase of around 6-fold at 24 h. The mRNA levels for the PPAR target genes MTE I and CPT1b were significantly increased only at the 48-h time point after T_3 administration. At first glance, these data favor direct regulation of UCP3 by T_3, followed by up-regulation of genes involved in fatty acid oxidation through PPAR signaling, an effect at-

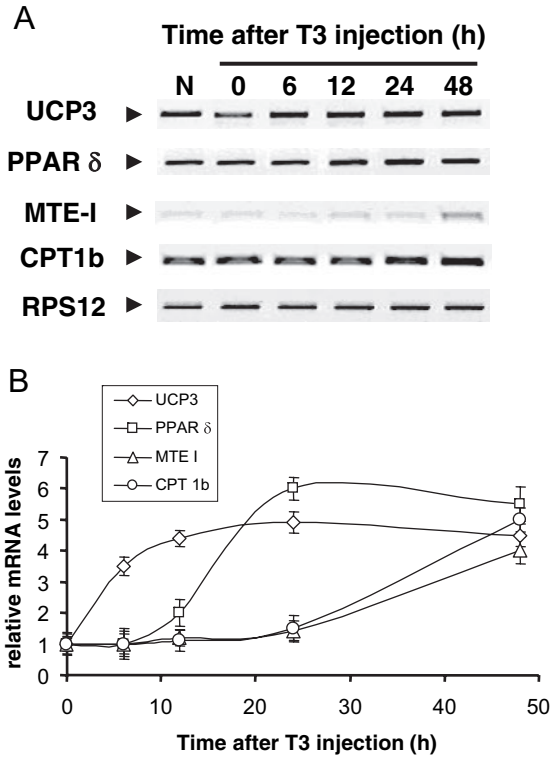

FIG. 1. *In vivo* time course of changes in expression of UCP3 and PPARδ and of genes involved in mitochondrial fatty acid oxidation in the gastrocnemius muscles of hypothyroid (P+I) rats. Animals were killed at different time points after receiving a single dose of T_3 (25 μg/100 g BW). A, Representative RT-PCR-based measurements of UCP3, PPARδ, CPT1b, and MTE I mRNAs at the indicated time points (0–48 h) after injection of T_3. The RPS12 mRNA level was measured as the internal standard. For RT-PCR analysis, each lane contains PCR product derived from cDNA, for which 250 ng total RNA was used. B, Graphic representation of the data shown in A. Means ± SEM are of three independent treatments. N, Euthyroid rats used as controls.

tributable to the lipolytic activity of T_3 that is secondary to its direct transcriptional effect through its binding to TRs.

The PPARδ agonist L165,041 up-regulates UCP3 expression in skeletal muscle with similar kinetics to T_3

A single administration of 500 μg/100 g or 1 mg/100 g BW of the PPARδ agonist L165,041 increased UCP3 expression in gastrocnemius muscle within 8 h, by 2.5- and 3-fold, respectively, whereas PPARδ expression was not increased (Fig. 2A). These data show that activation of the residing PPARδ can induce UCP3 expression, without the need for a preceding increase in the PPARδ mRNA level. In hypothyroid gastrocnemius and soleus muscles, a single administration of T_3 up-regulated UCP3 expression 3-fold, whereas PPARδ expression was affected only slightly, if at all (Fig. 2, B and C). After P+I treatment, PPARδ expression in gastrocnemius muscle was not different from that in the euthyroid controls (Fig. 2B), although it was increased 1.8-fold in soleus muscle (Fig. 2C). The TRβ1 responses to P+I and T_3 treatment were similar to those shown by UCP3 in both gastrocnemius and soleus muscle, with that of TRβ1 being more pronounced (Fig. 2, B and C).

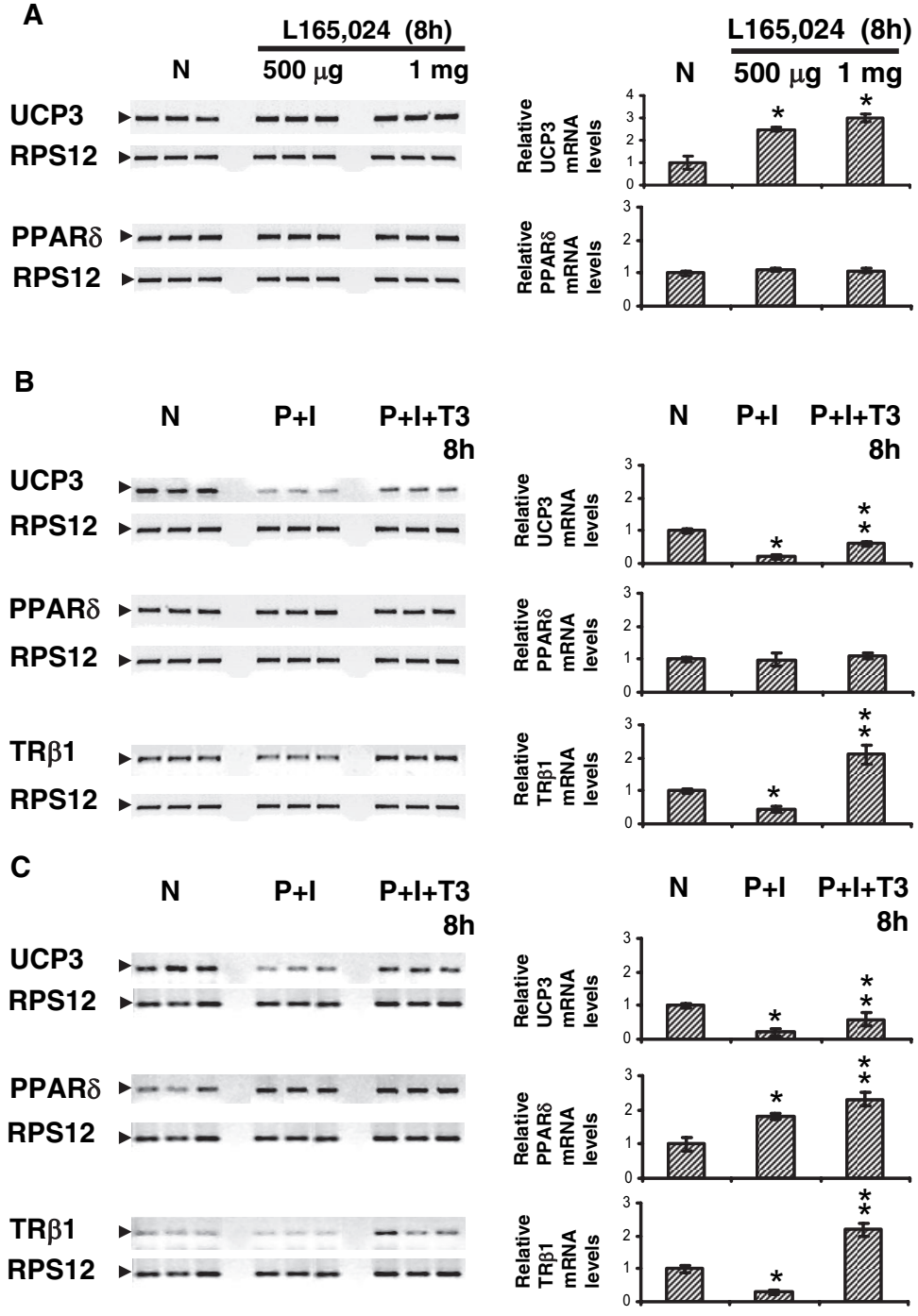

FIG. 2. Short-term effect on UCP3 expression in skeletal muscle induced by either the PPARδ agonist L165,041 or T_3. A, RT-PCR-based measurements of gastrocnemius muscle UCP3 and PPARδ at 8 h after administration of the PPARδ agonist L165,041 at the indicated doses (expressed per 100 g BW) to euthyroid rats. B, RT-PCR-based measurements of gastrocnemius muscle UCP3, PPARδ, and TRβ1 mRNAs at 8 h after administration of T_3 to hypothyroid (P+I) rats [with euthyroid (N) rats as controls]. C, As in B but for soleus muscle. For RT-PCR analysis, each lane contains PCR product derived from cDNA, for which 250 ng total RNA was used. The RPS12 mRNA level was measured as the internal standard. Quantified data are the means ± SEM from three independent treatments. *, $P < 0.05$, significant differences *vs.* untreated euthyroid (N) controls. **, $P < 0.05$, significant differences *vs.* N and hypothyroid (P+I) controls.

Rat UCP3 expression depends on the presence of fatty acids, whereas in their absence T_3-induced UCP3 expression is rescued by L165,041-activated PPARδ

Although the data described above showed that administration of the PPARδ agonist L165,041 regulated UCP3 with rapid kinetics through the residing PPARδ, they suggested that UCP3 expression was directly regulated by T_3 (through TRs), preceding PPAR signaling. To test whether UCP3 can indeed be regulated by T_3 without the need for fatty acids, a subgroup of hypothyroid rats was treated with T_3 alone or cotreated with T_3 plus NA at 8 h before the animals were killed, and the effects on the expressions of UCP3 and TRβ1 were measured in soleus muscle. Surprisingly, NA abolished the T_3-mediated induction of UCP3 mRNA level, whereas TRβ1 expression remained inducible by T_3 in the absence of fatty acids, showing that TRβ1 is directly regulated by T_3 independently of fatty acids (Fig. 3A). Simultaneous administration of L165,041 (500 μg/100 g BW) and T_3 to P+I+NA-treated rats restored the increase in the UCP3 mRNA level to the equivalent of that seen in the P+I+T_3 controls (Fig. 3A). In contrast, in the absence of T_3, L165,041 did not increase the UCP3 mRNA level in P+I+NA-treated rats. These results show that T_3-mediated induction of UCP3, unlike its induction of TRβ1, depends on fatty acids acting through PPAR signaling and that PPARδ activation rescues T_3-mediated UCP3 expression in rat skeletal muscle, in the absence of fatty acids. PPARδ and MyoD mRNA levels remained unaltered by all treatments (results not shown). The short-term lipolytic effect of T_3 and the efficiency of the NA treatment was verified by measurement of serum fatty acid levels (Fig. 3B). The resulting data showed that, within 8 h, T_3 increased serum fatty acid levels around 4-fold, whereas NA treatment resulted in serum fatty acid levels that, regardless of the other treatment(s), were not significantly different from those of the P+I controls.

The human UCP3 promoter, unlike the rat and mouse UCP3 promoters, is strongly activated by T_3 in L6 cells cotransfected with MyoD and TRβ1

Rat L6 myoblasts, cultured overnight in 12-well plates in culture medium supplemented with dextran/charcoal-treated serum, were cotransfected with Luc reporter vectors driven by UCP3 promoters of human, mouse, or rat origin (termed −1588/+47hUCP3-Luc, 1946/+60mUCP3-Luc, and −2134/+43rUCP3-Luc, respectively), together with pCMV-MyoD and pRSV-hTRβ1 and the control plasmid phRL-TK-Luc. This was followed by incubation in the same medium with increasing concentrations of T_3 (from 0 to 100 nM) (Fig. 4). Each of the three promoters revealed a dose-dependent response to T_3, reflected by increased Luc activity, up to around 5.5-fold for the human promoter, but to around only 1.7-fold for the two rodent promoters. Thus, human UCP3 promoter activity was markedly more induced than that of either of the rodent promoters. It should be noted that transfection of rat L6 myoblasts with the rat UCP3 promoter-Luc reporter vector resulted in substantially higher basal expression levels than to those seen when reporter vectors from the two other species were used, the basal expression of the −2134/+43rUCP3-Luc reporter vector being significantly ($P < 0.05$) higher than those of the 1946/+60mUCP3-Luc or −1588/+47hUCP3-Luc reporter vectors. Another Luc reporter vector, containing a 602 bp sequence of the rat acyl coenzyme A oxidase promoter (r602-AOX-Luc), also showed a significantly higher basal expression ($P < 0.05$) (data not

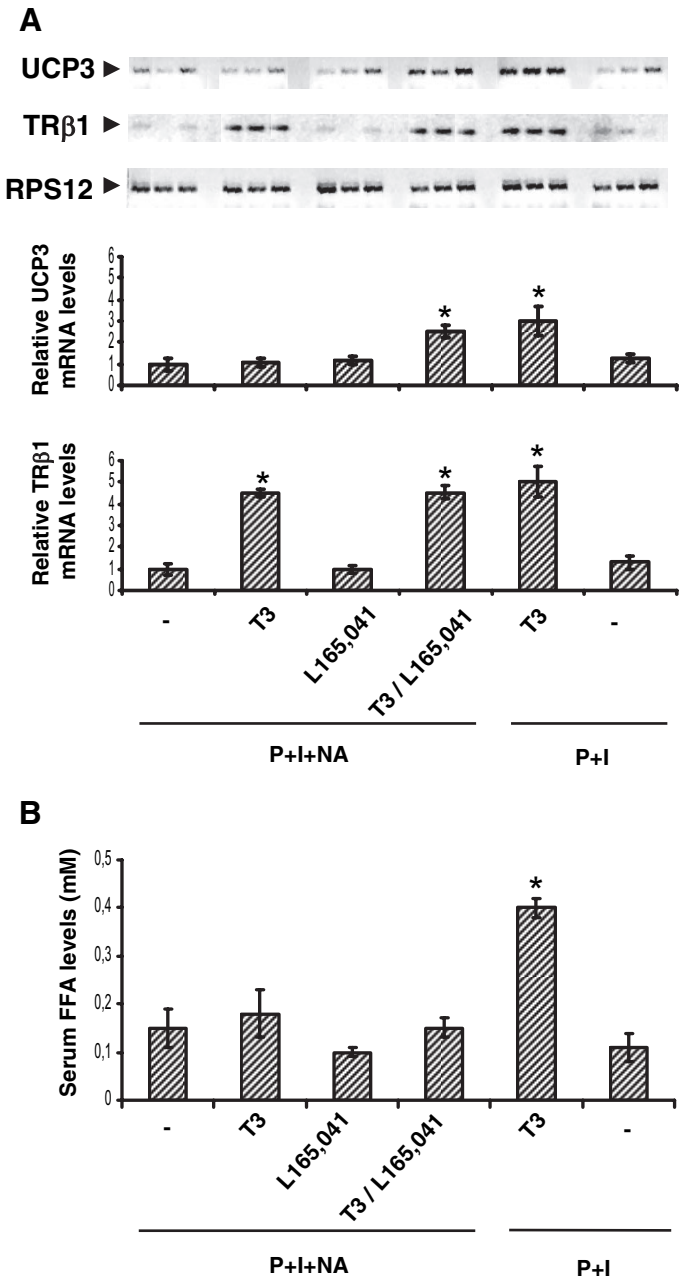

FIG. 3. *In vivo* effects of fatty acids, through PPARδ, on the T_3-induced transcriptional control of UCP3 mRNA in skeletal muscle. Soleus muscle UCP3 and TRβ mRNA levels (A) and serum fatty acid levels (B) were measured in hypothyroid (P+I) rats at 8 h after treatment with T_3 (25 μg/100 g BW), L165,041 (500 μg/100 g BW), or both in the presence or absence of NA (10 mg/100 g BW). For RT-PCR analysis, each lane contains PCR product derived from cDNA, for which 250 ng total RNA was used. The RPS12 mRNA level was measured as the internal standard. Quantified data are the means ± SEM from three independent treatments. *, $P < 0.05$, significant differences *vs.* hypothyroid (P+I) controls within each NA-treated or untreated group.

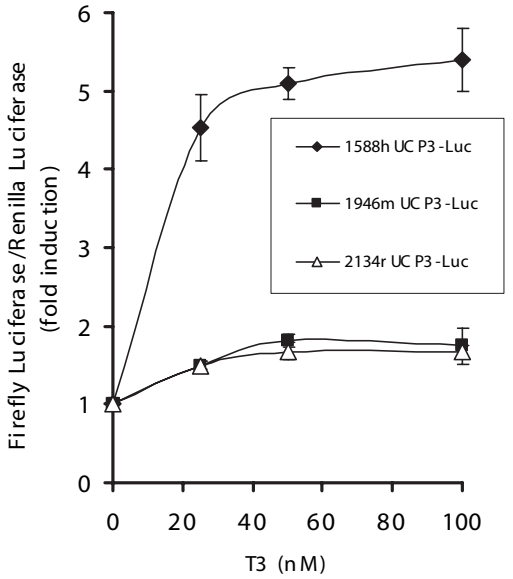

FIG. 4. *Ex vivo* effects of T_3 on the UCP3 promoter regions from rat, mouse, and human. Rat L6 myoblasts were cotransfected with −2134/+43rUCP3-Luc, −1946/+60mUCP3-Luc, or −1588/+47hUCP3-Luc, together with 0.15 μg MyoD and TRβ expression vectors plus 1.5 ng of the phRL-TK-Luc control vector. Cells were then cultured in DMEM containing dextran/charcoal-treated serum for 24 h and subsequently treated with T_3 at the indicated concentrations in the same medium for 24 h. The results are expressed as the fold induction of Luc activity achieved by the addition of T_3 and are the means ± SEM from at least three independent experiments performed in triplicate.

shown). This may relate to the cellular context, because L6 cells originate from the rat.

Differential response of the rat UCP3 promoter with respect to that from mouse and human to cotransfection of PPARδ and RXRα

L6 cells were cultured as described above and then cotransfected with the above-described plasmids, in combination with either pSG5-hPPARδ alone or pSG5-hPPARδ plus PRSV-hRXR. Cotransfection of the PPARδ expression plasmid diminished only slightly the response to T_3 shown by the rat UCP3 promoter, whereas it completely blunted that of the mouse UCP3 promoter and strongly suppressed that of the human UCP3 promoter. The mouse and human UCP3 promoters each failed to respond to L165,041 (Fig. 5). However, compared with controls lacking cotransfected PPARδ, cotransfection of PPARδ and addition of L165,041 caused a weak, but significant, increase in the mouse UCP3 promoter (data not shown). The only activating effect of L165,041 administration was on the rat UCP3 promoter (by 1.3-fold), but this agonist did not enhance the effect of T_3 in this condition (T_3-induced Luc activity being enhanced by around 1.5-fold in both the presence and absence of L165,041). During cotransfection with both the PPARδ and RXRα expression plasmids, 1) the mouse UCP3 promoter failed to increase in response to any of the treatments, whereas 2) the activating effect of T_3 on the human UCP3 promoter (which was still present when PPARδ alone was cotransfected) was now completely blunted. In contrast, rat UCP3 promoter activity was increased 1.4-fold by T_3 and 1.5-fold by L165,041 but 1.8-fold by T_3 plus L165,041 (significantly more than with T_3 treatment alone). Thus, only the rat UCP3 promoter was activated by T_3-bound TRβ1 as well as by L165,041-bound PPARδ, and it showed a slight additive increase in activity when both TRβ and PPARδ were activated by their respective ligands. It should be noted that cotransfection of RXRα without ligand significantly ($P < 0.05$) decreased basal UCP3 promoter activity from all species, which we interpret as being a nonspecific squelching effect, probably caused by competition for common transcription factors between the different expression vectors.

The MyoD response element of the rat UCP3 gene is localized in exon 1

Sequence analysis of the rat UCP3 proximal promoter-exon fusion revealed a difference from the mouse and human promoters. The MyoD response element was completely located in exon 1 of the rat UCP3 gene (nucleotide positions +17/+37 with respect to the transcription start site), whereas in the human promoter this same element is located at nucleotide positions −29/−9 and in the mouse promoter at nucleotide positions −12/+9 (both with respect to the transcription start site). This implies that the rat promoter is structurally different from mouse and human promoters. Figure 6 shows a schematic overview of the UCP3 promoters of the various species and their proposed differential regulation by T_3.

Discussion

In this study, we determined the prerequisites for T_3-induced expression of the UCP3 gene in rat skeletal muscle *in vivo*, as well as the putative mechanisms underlying T_3-mediated transcriptional regulation of the rat UCP3 promoter (*vs.* the mouse and human UCP3 promoters) in rat L6 cells.

In vivo, induction of UCP3 expression by T_3 in hypothyroid gastrocnemius muscle clearly preceded the inductions of the PPARδ, CPT1b, and MTE I genes (Fig. 1) [both CPT1b and MTE I genes are known to be PPAR regulated (see Refs. 21, 22)]. The presence of TREs in the promoter of the UCP3 gene may explain the difference in kinetics observed between the up-regulation of UCP3 on the one hand and CPT1b and MTE I (not containing TREs but containing PPAR response element) on the other. The T_3-induced regulation of the latter two genes is thus very likely mediated by PPARs, which are activated by the increased FFA levels elicited by T_3 (Fig. 3B). This would seem to suggest that UCP3 can be directly regulated by T_3 without the need for fatty acid-mediated PPAR signaling. However, 1) the PPARδ agonist L165,041 up-regulated UCP3 expression within 8 h after its administration to euthyroid rats (Fig. 2A), and 2) in the hypothyroid state, PPARδ mRNA was already present at high levels (*i.e.* before its up-regulation by T_3) in both gastrocnemius and soleus muscle (Fig. 2C) (in soleus muscle, PPARδ mRNA levels were even higher in P+I rats than in the euthyroid controls). This suggests that, in the hypothyroid state, the residing PPARδ would be sufficient for the induction of short-term T_3/FFA-mediated transcriptional effects and thus may play a functional role in the short-term effects of T_3. Indeed, additional analysis revealed that the rat UCP3 gene was clearly dependent on fatty acids for its short-term up-regulation by T_3 in

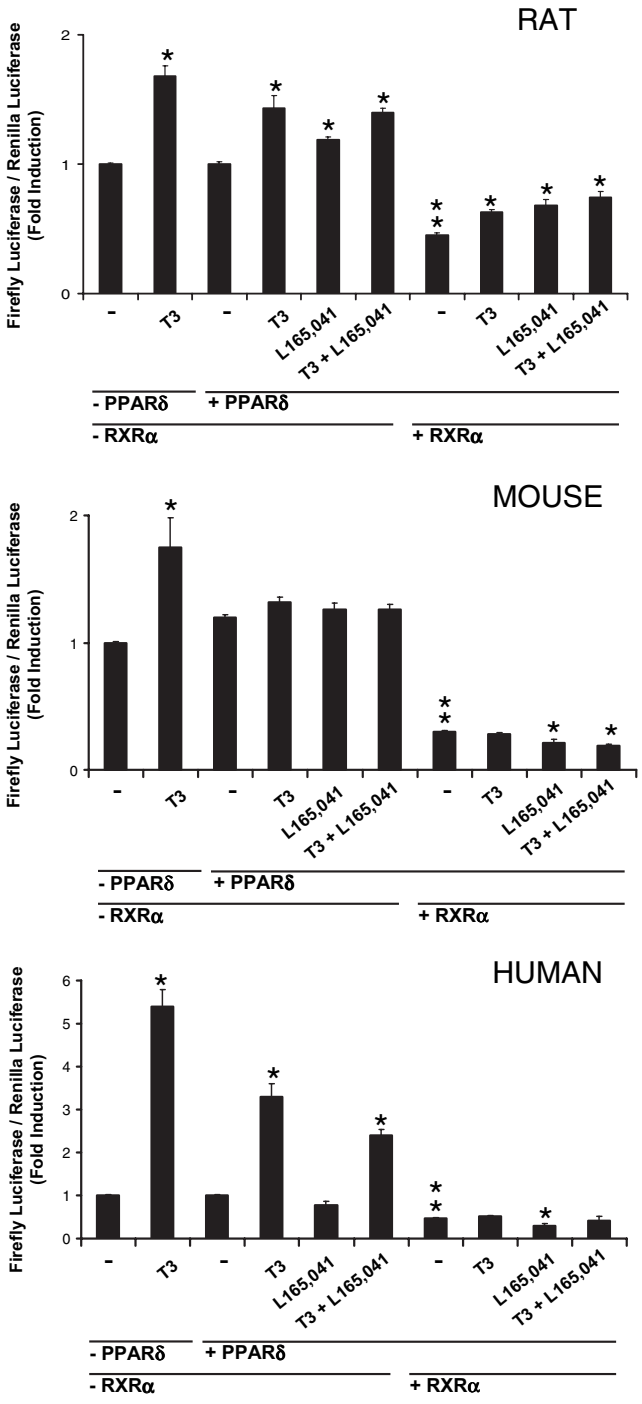

FIG. 5. *Ex vivo* effects of T_3, L165,041, or both on the UCP3 promoter regions from rat, mouse, and human. Rat L6 myoblasts were cotransfected with −2134/+43rUCP3-Luc, −1946/+60mUCP3-Luc, or −1588/+47hUCP3-Luc, together with 0.15 μg MyoD and TRβ expression vectors plus 1.5 ng of the phRL-TK-Luc control vector. When indicated, RXRα and/or PPARδ and expression vectors were cotransfected. Cells were cultured in DMEM containing dextran/charcoal-treated serum for 12 h and subsequently treated with T_3 (100 nM), L165,041 (50 μM), or both in the same medium for 24 h. The results are expressed as the fold induction of Luc activity achieved by the addition of T_3 and are the means ± SEM from at least three independent experiments performed in triplicate. *, $P < 0.05$, significant differences *vs.* corresponding ligand-untreated (−) controls. **, $P < 0.05$, significant differences between controls (−) cotransfected with and without RXRα.

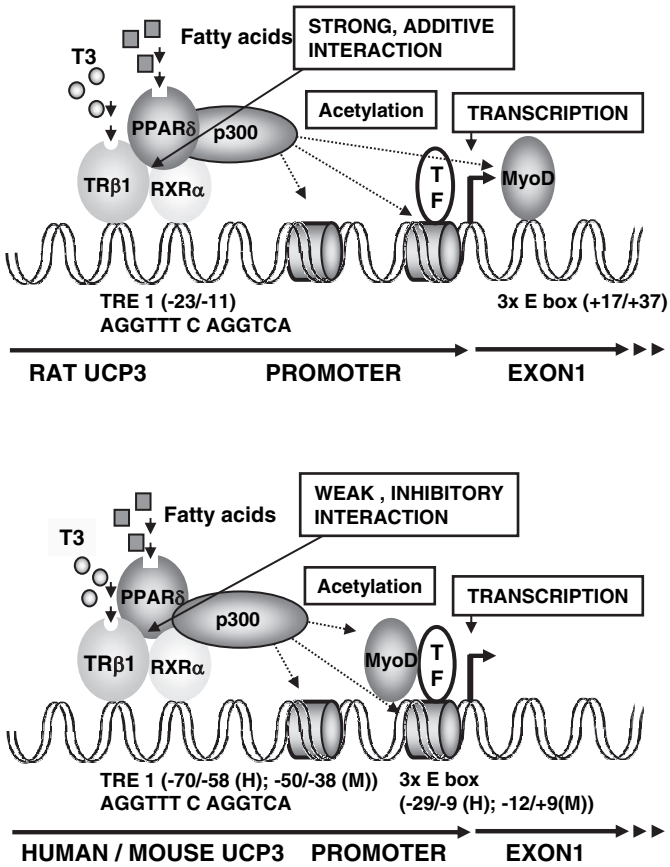

FIG. 6. Schematic representation of the possible mechanism for the regulation of the rat UCP3 gene by MyoD, TRβ, PPARδ, and RXRα with that for the mouse and human UCP3 genes shown for comparison. Based on the present data plus data from previous work (19). Note the difference in the localization of the MyoD response element (3x E box) between the rat UCP3 gene (exon 1) and those of mouse and human (proximal promoter).

skeletal muscle, because NA treatment completely abolished the induction of its expression (Fig. 3A). This was underlined by the finding that T_3 treatment increased serum fatty acid levels within 8 h, well before its up-regulation of PPARδ expression (Fig. 3B). It has been shown recently that NA inhibits white adipose tissue lipolysis through binding to receptors termed PUMA-G and HM74 (27). Consequentially, short-term treatment with this compound decreases triglyceride content in liver, heart muscle, and oxidative-fibered skeletal muscle without any change in cholesterol content (28). We used the oxidative-fibered soleus muscle to examine the effects of NA on short-term T_3-induced UCP3 transcription, because NA treatment was ineffective at inhibiting short-term UCP3 transcription in mixed-fibered gastrocnemius muscle (data not shown). This is probably attributable to the considerable intramuscular fatty acid reserves in the latter muscle.

PPARs are necessary for the T_3/TR-mediated induction of rat UCP3 expression, and TRs are necessary for the FFA/PPAR-mediated induction of UCP3 expression. Evidence for this comes from our observation that the PPARδ agonist L165,041 alone did not induce UCP3 expression in skeletal muscles from P+I rats (Fig. 3A), which had low TRβ1 mRNA

levels (Fig. 2, B and C), whereas administration of the same dose of L165,041 caused a 2.5-fold increase in UCP3 mRNA levels in euthyroid rats, in which TRβ1 levels are higher (Fig. 2A). In addition, in L6 cells cotransfected with TRβ, MyoD, and PPARδ (in the presence or absence of RXRα), L165,041 increased rat UCP3 promoter activity (Fig. 5). Although PPARδ is the predominant isoform in skeletal muscle, we cannot exclude the possibility that PPARα can mediate the described effects as well. Indeed, it has been shown that PPARα can bind to the TRE1 element of the human UCP3 promoter (19). However, on the basis of the results obtained in the hypothyroid state (in which a selective downregulation of TR β1, with respect to TRα, abolished the PPARδ-induced stimulation of UCP3 transcription), the effects described *in vivo* very likely involve TRβ1. Support for this assumption comes from transient transfection experiments showing that a stimulatory interaction between TRβ1 and PPARα on a direct repeat with two nucleotide spacing (DR+2) TRE stimulated gene expression, whereas in contrast, TRα-PPARα interactions negatively influenced gene expression (29). In addition, (repressing) interactions between TRα and PPARα on natural TREs have been demonstrated recently *in vivo* (30). In these studies, however, PPARδ was not taken into consideration.

Unlike UCP3, TRβ1 appears to be directly regulated by T_3 in soleus muscle, with or without suppression of fatty acids (compare the P+I/T_3 with the P+I+NA/T_3 levels of TRβ1 mRNA in Fig. 3A), whereas TRα1 expression remained unaltered (results not shown). However, it is conceivable that TRα1 functions as a T_3-dependent inducer of TRβ1 in skeletal muscle; indeed, it has been demonstrated that, in the mouse heart, up-regulation of TRβ1 by T_3 is mediated through ligand-bound TRα (31).

In L6 cells transiently transfected with MyoD and TRβ1 plus one of the UCP3 promoter constructs, 1) the human UCP3 promoter responded much more strongly to T_3 treatment than either the mouse or rat UCP3 promoter, whereas 2) the rat UCP3 promoter was unique in responding positively to T_3 and/or PPARδ agonist treatment when PPARδ was cotransfected and even more so when PPARδ and RXRα were cotransfected (Fig. 5). Thus, the data from the transfection studies are in line with the observed strong, direct effect of T_3 on the human UCP3 transgene promoter as opposed to the weak response of the endogenous UCP3 promoter obtained in skeletal muscle of transgenic mice (13). In addition, they underline the strong response to T_3 treatment shown by skeletal muscle in hypothyroid rats *in vivo* in the current and a previous study (12), a response that we show here to depend on the presence of FFA acting through PPAR signaling.

Phylogenetic analysis has revealed that mouse and rat sequences are nearly the same distance from a putative common ancestor sequence (with a nucleotide substitution rate of $<$10% per site), whereas the human UCP3 promoter-exon 1 region is highly divergent (with a nucleotide substitution rate of $>$30% per site) (17). The additional comparison performed in this study between the mouse and the rat proximal UCP3 promoters, however, has revealed an important species difference: namely, a differential localization of the MyoD response element in the different species (it is located in exon 1 of the rat UCP3 gene, whereas it surrounds the transcription start site in the mouse UCP3 gene and is positioned within the promoter of the human UCP3 gene). This may imply a different folding of the rat promoter around the basic transcription factor-RNA polymerase II complex than that present in the human and mouse UCP3 genes, and this may explain the different response to T_3 and fatty acids seen for the rat UCP3 gene than for the mouse and human UCP3 genes. Because TRβ can form trimers when bound to natural response elements comprising reiterated half-sites (32), it is conceivable that PPARδ and TRβ1, in the presence of RXRα, interact in an additive manner with the natural TRE1 element of the rat UCP3 promoter. The more upstream position of the MyoD response element in the mouse and human UCP3 promoters may not allow this interaction (for a schematic representation, see Fig. 6). This would result, *in vivo*, in a fatty acid-dependent stimulatory effect of T_3 only in the case of the rat UCP3 gene. In a previous study (19), a specific role of MyoD acetylation in UCP3 promoter activity was indicated by the reduced transactivation capacity of a nonacetylable mutant form of MyoD. The finding that the nonacetylable form of MyoD remained partially sensitive to PPAR-dependent activation may indicate that other factors or histones are acetylated by p300 (19).

The present data have highlighted important differences between the T_3-mediated regulation of UCP3 in different species, which may help to shed light in the interpretation of data on the effect of T_3 on both UCP3 expression/activity and on its role in energy metabolism. In contrast to the situation in the rat, single-dose T_3 administration (10 μg/100 g BW) does not significantly increase UCP3 mRNA expression in mouse skeletal muscle, although a trend toward a weak increase was observed (16), and administration of T_3 (2.5 μg/100 g BW, daily for 6 d) to euthyroid or thyroidectomized mice induced UCP3 mRNA expression only to a maximum of 1.5-fold (15) (in both studies, mitochondrial UCP3 protein levels have not been measured). Taking our recent and previous (12) data into consideration, it may be that a relatively higher dose of T_3 has to be applied to achieve a pronounced effect on UCP3 transcription in mouse skeletal muscle. In a study comparing the effect of T_3 on wild-type and UCP3 knockout mice (16), a higher dose of T_3 was used (100 μg/100 g BW, daily for 4 d), but unfortunately UCP3 expression data (mRNA and mitochondrial protein levels) in wild-type littermates were not reported. In these supraphysiological hyperthyroid conditions, the UCP3 knockout mice showed a lower RMR value than their wild-type littermates (+72 and +89%, respectively), but the differences were not significant. These data may be interpreted in the sense that UCP3 does not play any role in the effects elicited by T_3 on RMR even if in a previous study we showed that UCP3 has the potential to be a molecular determinant of T_3-induced increase in RMR. These discrepancies, however, may be apparent rather than real. Indeed, apart from the differences in the promoter behavior, other aspects should be considered and, in particular, 1) the doses used by the previous authors (16) were very high and, in these conditions, other thermogenic mechanisms may be overactivated, and 2) injecting T_3 in condition in which the deiodinase enzymes are active a deiodinated product of T_3 may be effective in stimulating RMR, such as

3,5-diiodothyroinine, which is able to increase RMR and whose effects may overrule those of UCP3 (33). For this reason, in our previous (12) and present studies in the rat, we chose to generate hypothyroid animals by simultaneous administration of P and I. This combined treatment produces hypothyroid animals and at the same time inhibits all three known types of deiodinase enzymes, which should permit us to attribute the observed effects to T_3 rather than to any of its deiodinated products (34).

Together, the results of this study clearly indicate a differential regulation of UCP3 by relatively low doses of T_3 among mice, rats, and humans, and we suggest that this should be taken into account when hypotheses are put forward regarding the putative role of this protein related to T_3-mediated effects on energy metabolism in physiological situations.

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Address all correspondence and requests for reprints to: Antonia Lanni, Dipartimento di Scienze della Vita, Seconda Università degli Studi di Napoli, Via Vivaldi 43, 81100 Caserta, Italy. E-mail: antonia.lanni@unina2.it.

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