Thyroid hormones directly activate the expression of the human and mouse uncoupling protein-3 genes through a thyroid response element in the proximal promoter region.

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SYNOPSIS

The transcription of the human UCP3 gene in skeletal muscle is tightly regulated by metabolic signals related to fatty acid availability. However, changes in thyroid status also modulate UCP3 gene expression, albeit by unknown mechanisms. We created transgenic mice bearing the entire human UCP3 gene to investigate the effect of thyroid hormones on human UCP3 gene expression. Treatment of human UCP3 transgenic mice with thyroid hormones induced the expression of the human gene in skeletal muscle. In addition, transient transfection experiments demonstrate that thyroid hormones activate the transcription of the human UCP3 gene promoter when MyoD and the thyroid hormone receptor were co-transfected. The action of thyroid hormones on UCP3 gene transcription is mediated by the binding of the thyroid hormone receptor to a proximal region in the UCP3 gene promoter that contains a direct repeat structure. An intact DNA sequence of this site is required for thyroid hormone responsiveness and thyroid hormone receptor binding. Chromatin immunoprecipitation assays revealed that the thyroid hormone receptor binds this element “in vivo”. The murine UCP3 gene promoter was also dependent on MyoD and responsive to thyroid hormone in transient transfection assays. However, it was much less sensitive to thyroid hormone than the human UCP3 promoter. In summary, UCP3 gene transcription is activated by thyroid hormone treatment “in vivo” and this activation is mediated by a thyroid hormone response element in the proximal promoter region. Such regulation suggests a link between UCP3 gene expression and the effects of thyroid hormone on mitochondrial function in skeletal muscle.

INTRODUCTION

Thyroid hormones regulate energy metabolism by increasing respiration and energy expenditure and by lowering metabolic efficiency. Although this has been known for years, the molecular mechanisms for these effects of thyroid hormones remain to be established. In rodents, thyroid hormones affect the energy metabolism by altering the proton leak across the inner mitochondrial membrane through unknown mechanisms (1;2). Thyroid hormones exert most of their known biological effects through the stimulus of gene transcription but the key target genes of thyroid hormones involved in energy metabolism and metabolic efficiency changes remain unidentified.

The discovery of mitochondrial uncoupling proteins 2 and 3, which are closely similar to the thermogenic brown fat uncoupling protein-1 suggested that these proteins are potential targets of thyroid hormone effects and mediators of the thyroid action promoting energy expenditure (3). This research has been especially active after the finding that UCP3 gene expression in muscle is extremely sensitive to the thyroid hormone status in rodents. Thus, early after the discovery of UCP3, it was reported that the administration of thyroid hormones to rodents enhances the expression of UCP3 in skeletal muscle, which is paralleled by a rise in resting metabolic rate (4;5). Experiments using rodents at extreme thyroid status situations (profound hypo- or hyperthyroid) support the major effect of thyroid status on UCP3 gene expression (6;7).

Human UCP3 gene transcription is regulated by fatty acids and retinoic acid, as reported elsewhere (8;9) but the action of thyroid hormone has not been established. Although individual variations in UCP3 mRNA levels in skeletal muscle do not correlate with changes in circulating thyroid hormones in euthyroid healthy subjects (10) a doubling of T3 levels induced in volunteers led to an up-regulation of UCP3 mRNA abundance in skeletal muscle (11). Moreover, UCP3 has been recently reported as a thyroid-sensitive gene in a microarray study of cDNAs from human muscle (12). However, the mechanisms by which thyroid status modulates UCP3 gene expression are unclear.

Results from cultured rodent myogenic cell lines indicate a positive effect of T3 on UCP3 mRNA levels, thus supporting a direct action of thyroid hormones in UCP3 gene expression (13). In human myotubes, differentiated in culture, UCP3 mRNA expression is moderately increased after exposure to thyroid hormones (11). The involvement of transcriptional and/or post-transcriptional mechanisms in the effects on

steady-state UCP3 mRNA levels has not been elucidated and the corresponding experimental approaches have been dampened by the extremely low levels of expression of the human UCP3 gene in cultured myotubes compared with skeletal muscle “in vivo” (8;11;14).

Here, we created a human UCP3 transgenic mouse and showed that thyroid hormones induce the tissue-specific expression of the human UCP3 gene. Moreover, T3 directly induced the expression of the human UCP3 gene through a multi-hormonal response element in the proximal promoter region of UCP3.

EXPERIMENTAL PROCEDURES

Human UCP3 transgenic mice. HUCP3 transgenic mice were generated using standard procedures described elsewhere (15). A genomic P1 clone (plate 324 and well H6) containing human UCP3 (16) was mapped by restriction enzyme analysis and microinjected into pronuclei of fertilized FVB mouse oocytes. Transgenic offspring were identified using standard Southern Blot analysis. Genomic DNA was isolated from the tail of mice and restriction analysis was performed using *Xba I*. Membranes were hybridised with a probe corresponding to human UCP3 exon 1 (200bp, from –181 bp 5' of the start codon to 18 bp 3' of the start codon, accession number AF001787). Transgenic offspring were examined for the presence of a 2.9 kb band which was absent in the non-transgenic littermates. Two lines (TgA and TgB) from two different founders were analysed and used in the present study.

Analysis of human UCP3 mRNA transcripts by RNAse protection assay. RNAse assay was performed as previously described, using an *in vitro* transcribed [32P]-labeled RNA antisense probe corresponding to human UCP3 long form (17), spanning exons 6 and 7 (+631 to +925 relative to ATG). Analysis was performed using 10 and 20 μg of RNA and protected bands for $UCP3_L$ and $UCP3_S$ (16) were visualized by autoradiography.

Northern blot analysis of human UCP3 mRNA. Mice were housed with free access to standard rodent chow and water. For starvation experiments food was removed for 18 hours. For thyroid hormone treatment, control and transgenic mice were treated either with saline or T3 (100 μg/Kg) i.p. and studied 18 hours later. In both cases, animals were sacrificed and tissues were harvested. RNA was prepared with RNAzol (Cinna/Biotecx Laboratory, Houston, TX) except for human skeletal muscle RNA which was obtained from Clontech and used for comparative purposes. 20 μg RNA was loaded for Northern blot analysis. Hybridization was performed by standard methods and ethidium bromide staining was used to confirm equal loading of RNA. Hybridization probe was generated by random priming from cDNA template of human UCP3 (described above). To quantify mRNA expression, Northern blots were analysed using Phosphoimager (Molecular Dynamics, Image Quant software).

Construction of transfection plasmids. Fragments from –4511 to +47 and from –2903 to +47 of the human UCP3 promoter were amplified by PCR and were cloned

into pGL3 basic to generate –4511hUCP3-luc and –2903hUCP3-luc respectively. From –2903hUCP3-Luc, a *Sac*I restriction and re-ligation generates the –1588hUCP3-Luc construct, and the rest of the constructs were generated according to a previous report (8). A fragment of the mouse UCP3 gene was amplified using 200ng of mouse genomic DNA. The complementary 3' primer corresponded to bases from +60 to +35 downstream to the transcription initiation site, according to GenBank/EMBL data (Accession AB011070). The complementary 5' primer corresponded to –1946 to –1924. The 3' and 5' complementary primers generated included 6 bp non-complementary extensions capable of generating *Kpn*I and *Hind*III restriction sites. Reaction was performed using the Expand Long Term PCR System (Roche) in 50 µl final volume containing 15 pm of each primer, 350 µM each dNTP, 1,75 mM $MgCl_2$ and 2,5 units of Taq DNA polymerase. Ten cycles were performed at 94ºC for 10 sec, 60ºC for 30 sec, and 68ºC for 2 min and twenty cycles were performed at 94ºC for 10 sec, 60ºC for 30 sec, and 68ºC for 2 min 30 sec. The resulting DNA product of ~2 kb was digested with *Kpn*I and *Hind* III, purified and ligated into pGL3-basic (Promega), which contains the cDNA for firefly (*Photinus pyralis*) luciferase as a reporter gene. The whole fragment was sequenced by the dideoxy method. The insertion of the fragment from –1946 to +60 of the mouse UCP3 gene into pGL3-basic generated -1946mUCP3-Luc. Point mutation constructs were generated using a Quick-change site-directed mutagenesis kit (Stratagene). In summary, two complementary oligonucleotides containing the desired mutations, flanked by unmodified nucleotide sequence, were synthesized. Of each oligonucleotide, 50 ng of double stranded –1588hUCP3-Luc or –1946mUCP3-Luc were incubated with 2.5 units of *Pfu*Turbo DNA polymerase. PCR reaction was performed for 17 cycles at 95ºC for 30 sec, 55ºC for 1 min, and 68ºC for 14 min. Ten units of *Dpn*I restriction enzyme was then added to each PCR reaction and incubated at 37ºC for at least 1 h to digest parental DNA. One microliter of the *Dpn*I-treated DNA was then transformed. To generate point mutation of the complete TRE1 (both half sites) of the –165hUCP3-Luc and –1588hUCP3-Luc to create –165mutTRE1hUCP3-Luc and –1588mutTRE1hUCP3-Luc, the oligonucleotide used was: GTCAACCAACTTCTCTAGGATAtcGTTTCAaaTCAGCCTGTGTG. To generate point mutation of the mouse TRE in –1946mUCP3-Luc, the oligonucleotide used was GCTTCTCAGAATTCCGTTTGATATCAGCTGGTGCACAGGGCC to create –1946m*mut*TREUCP3-Luc. Point-mutated constructs were checked by direct DNA sequencing.

Cell culture and transient transfection assays. Rat myoblastic L6 cells were obtained from American Type Culture Collection (Rockville, MD) and grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS). Transfection experiments were carried out in L6 cells at 50% confluence using FuGene6 Transfection Reagent (Roche) following the manufacturer instructions. For L6 transfection, each point was assayed (unless otherwise indicated) in triplicate in a 6-well plate and contained 1.5 μg of luciferase reporter vector, 0.3 μg of the mammalian expression vectors pCMV-MyoD (18), pRSV-human thyroid hormone receptor β1 (TRβ) (19), pRSV-chicken thyroid hormone receptor α (20) and 3 ng of pRL-CMV (Promega), an expression vector for the sea pansy (*Renilla reniformis*) luciferase used as an internal transfection control. When indicated 0.3 μg of pCMV-rat thyroid hormone receptor α and pCMV-rat thyroid hormone receptor β1 expression vectors (21) were included. Cells were incubated for 48 h after transfection and, when indicated, treated for 24 h before harvest with or without T3 treatment was performed for 24 h at 50 nM unless otherwise indicated.

Firefly luciferase and Renilla luciferase activities were measured in a Turner Designs Luminometer (model TD20/20) using the Dual Luciferase Reporter assay system kit (Promega). Homogenates from cells were prepared with 500 μl of PLB (passive lysis buffer, Promega). Luciferase activity elicited by UCP3 promoter constructs was normalized for variation in transfection efficiency using Renilla luciferase as an internal standard.

Electrophoretic mobility shift assays. Nuclear protein extracts from L6 cells were isolated as reported elsewhere (22) and protein concentration was determined by the micro method of Bio-Rad (Richmond, CA) using bovine serum albumin as standard. For gel retardation assays, the double-stranded human UCP3-TRE1 oligonucleotide corresponding to the –79 to –50 sequence of the human UCP3 gene or the mutated ones, UCP3-TRE1*m1* or UCP3-TRE1*m2*, (see Figure 5) or mouse UCP3-TRE corresponding to –59 to –30 of mouse UCP3 gene were end-labeled using [α^{32}P]dCTP and klenow enzyme. The labeled DNA probe (25.000 cpm) was incubated for 30 min at 25ºC even with 5 μg of nuclear protein extract or 200 ng of recombinant chicken TRα (Santa Cruz # sc-4087) plus or minus "in vitro" translated RXR (Promega; TNT Coupled Reticulocyte Lysate System). Reactions were carried out in a final volume of 25 μl containing 20 mM Hepes (pH 7.6), 0.1 mM EDTA, 1 mM dithiothreitol, 50 mM NaCl,

10% glycerol, and 2.5 μg of poly dI.dC (deoxyinosinic-deoxycytidylic acid). Samples were analyzed by electrophoresis at 4ºC for 60–80 min in nondenaturing 5% polyacrylamide gels in 0.5X TBE. In the competition experiments, 20, 50 and 100-fold molar excess of unlabeled double-stranded oligonucleotides was included in each respective binding reaction. When indicated 2 μl of antisera capable to react both with rat TRα and TRβ (Santa Cruz #sc-772x) or C/EBPα (Santa Cruz #sc-9314) was added to the incubation media for 15 min.

Chromatin immunoprecipitation assay. L6 cells were transfected with -1588hUCP3-Luc or –1588*mut*TRE1hUCP3-Luc in the presence of MyoD and TR expression vectors (pCMV-MyoD and pRSV human TRβ1). Protein-DNA cross-linking was achieved by adding formaldehyde (final concentration 1%) to the culture medium for 10 min at 37ºC. The cells were washed twice with cold PBS containing 1 mM phenylmethylsulfonyl fluoride 1 μg/ml aprotinin and 1 μg/ml pepstatin A (protease inhibitors). Cells were then scraped in PBS, centrifuged, and re-suspended in 0.2 ml cell lysis buffer (5mM Pipes (KOH), pH 8.0, 85mM KCl, 0.5% (v/v) Nonidet P-40 plus the protease inhibitors). Samples were incubated on ice for 10 min and centrifuged for 10 min at 4ºC. Pellets were re-suspended in 0.2 ml SDS buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1 plus the protease inhibitors) and incubated on ice for 10 min. The samples were sonicated, centrifuged for 10 min at 4ºC and supernatants were diluted 10-fold with immunoprecipitation dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl plus the protease inhibitors). To reduce non-specific binding, samples were incubated at 4ºC for 1 h, with 80 μl of protein G-sepharose slurry in the form of 50% suspension in TE, which was pretreated with salmon sperm DNA.

Precleared chromatin solutions were incubated overnight at 4ºC with 20 μg of anti-Trα1 antibody (Santa Cruz, #sc-772) or an equal amount of an unrelated immunoglobulin (Santa Cruz #sc-9314). The immunocomplex was then collected by binding to 60 μl protein G-sepharose slurry as described above. After incubation for 1 h at 4ºC, the sepharose beads were collected by centrifugation and washed with 1 ml low-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1mM EDTA, 10 mM tris-HCl, pH 8.1) and twice with TE. The complexes were eluted

by two successive 15-min incubations with 250 µl of elution buffer (1% SDS, 0.1M $NaHCO_3$) at room temperature. The pooled eluates were heated to 65ºC for 4 h in presence of 20 µl NaCl 5M to reverse the formaldehyde crosslinks and then treated with proteinase K for 1 h at 45ºC. After phenol/chloroform extraction, DNA was used for PCR analysis.

Statistical analysis. Where appropriate, statistical analysis was performed by Mann-Whitney non-parametric U test. Significance is indicated in the text.

RESULTS

Human UCP3 gene expression in transgenic mice. Effects of starvation. To study the regulation of human UCP3 "in vivo", transgenic mice bearing 80 kb of human genomic P1 clone containing UCP3 gene sequence were created. The P1 clone includes the entire human UCP3 sequence, as well as 50 kb upstream sequence and 30 kb downstream sequence relative to the start codon. We obtained several transgenic lines and all of them showed similar levels of human UCP3 mRNA expression in skeletal muscle. No apparent phenotype (body weight, growth rate,..) was observed in any of them. This is consistent with the phenotype of transgenic mice bearing the human UCP3 gene, obtained using the same experimental strategy as reported by Horvath et al. (23), which showed a mild reduction in body weight.

Further experiments were performed using two transgenic lines, A and B. The expression of hUCP3 mRNA was predominant in skeletal muscle in transgenic mice and no detectable levels of hUCP3 mRNA were found in white adipose tissue, brown adipose tissue or heart (Fig 1A). The levels of human UCP3 mRNA in transgenic mice were similar to those observed in human skeletal muscle samples (82 ± 15 %). Moreover, both the long and the short form of hUCP3 mRNA transcripts were expressed in hUCP3 transgenic mice, as revealed by the RNAse protection assay (Fig 1A). This finding is in agreement with the observation that human skeletal muscle contains both forms of UCP3 transcripts (16).

Mice UCP3 gene expression is up-regulated in skeletal muscle during starvation (4). Thus, we tested whether the expression of the human UCP3 gene was also regulated during starvation in transgenic mice. For this purpose, food was removed from transgenic mice for 18 h and RNA was isolated from skeletal muscle. Northern blot analysis was performed using a probe specific for human UCP3 mRNA, generated as described in Methods, to avoid cross-hybridization of the human transcripts with mouse UCP3 mRNA (Fig 1B). Northern blot analysis detected the expression of the human UCP3 mRNA transcripts as a single band given the small difference in size between UCP3L and UCP3S mRNAs (100bp). In both lines, A and B, human UCP3 mRNA levels from starved transgenic mice were a 5.8 and 6.5 fold higher, respectively, than human UCP3 mRNA from fed transgenic littermates (Fig 1B). Using a specific probe to detect exclusively the mouse UCP3 transcripts, a similar induction was observed for mouse UCP3 mRNA due to fasting (data not shown). Therefore, the human UCP3

transgene is not only expressed in a tissue-specific manner but is also regulated by changes in the physiological status.

T3 treatment induces human UCP3 gene expression in transgenic mice. We then analyzed the effect of thyroid hormone treatment "in vivo" on the human gene in the transgenic mice. A dose of 100 μg of T3 /kg or saline as a control was injected intraperitoneally to transgenic mice. Eighteen hours later mice were killed, hindlimb skeletal muscle extracted and RNA was analyzed by Northern blot using the human-specific probe as described above. Human UCP3 mRNA in skeletal muscle was significantly induced in both transgenic lines (A by 4.5 fold and B by 3,3 fold respectively) (Fig 2). As performed for starvation experiments, a specific probe was used to detect the mouse UCP3 mRNA. In our conditions, mouse UCP3 mRNA was not significantly induced after the treatment with T3 (1.3 ± 0.25 fold induction by T3 treatment versus saline). Thus, although starvation induced both the human and mouse UCP3 mRNA, the single dose of T3 used selectively induced the expression of the human gene.

Thyroid hormone activates the human UCP3 promoter through a proximal element in the promoter. To determine the molecular basis for T3 responsiveness of the human UCP3 gene, we performed transient transfection experiments using various constructs containing the human UCP3 promoter fused to the luciferase reporter gene (Fig. 3). Because the human UCP3 promoter requires MyoD for basal and fatty acid stimulated activity (9), the effects of T3 were determined in the absence or presence of co-transfected MyoD. As expected, in the absence of MyoD, a construct containing a 4.5 kb fragment (from –4511 to +47) of the 5' non-coding region of the human UCP3 promoter was poorly expressed and not sensitive to T3, either in the absence or presence of TR. In the presence of MyoD, T3 treatment induced several fold the expression of the construct and this effect was completely dependent upon co-transfection with the expression vector for either TRβ1 or TRα.

Data from computer analysis of the 5' non-coding region of the human UCP3 gene reveal four potential thyroid hormone response elements (TREs) in the promoter (24;25) (Fig 4). Thereafter, we assayed serial deletions of the human UCP3 5' region from -4.5 kb to a –165 bp construct, which were activated by T3 with a similar fold-induction respect to the 4.5 kb hUCP3-Luc. When the fragment between –165 to –61 was deleted, responsiveness to T3 was lost (Fig 4). These results indicate that the

element responsible for T3 response is located within the proximal region. This region (from –165 to +47) of the human UCP3 promoter contains TRE1, a DNA element responsive to retinoic acid as well as to PPAR agonists, with an imperfect structure of direct repeat with one base spacing (8;9). Point-mutant analysis of this TRE1 element was undertaken to establish whether it is also responsible for mediating the effect of T3. The wild type construct -165hUCP3Luc responded to T3, whereas the mutant –165hUCP3*mutTRE1*hUCP3-Luc was not significantly induced by the hormone (Fig 4). The same result was obtained when the point mutation was introduced into the –1588hUCP3 plasmid, thus indicating that the TRE1 element located at –71 to -59 is essential for the human UCP3 gene to respond to thyroid hormone.

Thyroid hormone receptor binds to the multi-hormonal response element TRE1 in human UCP3 promoter. To test whether TR binds to the TRE1 element, electrophoretic mobility shift analysis was performed. An oligonucleotide corresponding to nucleotides from –79 to –57 named UCP3-TRE1 was labeled together with those containing mutations in one or the other half site of the direct repeat structure of the TRE1 (UCP3-TRE1*m*1 and UCP3-TRE1*m*2) (see Fig 5A for sequence). The presence of RXR from a transcription-translation reaction alone did not result in the formation of any protein-DNA retarded band, indicating that the direct repeat structure of the TRE1 element is not compatible with binding RXR monomers or homodimers (Fig 5B). When recombinant TR was present in the reaction mixture, two bands (I and II) were formed, in agreement with the known binding of TR to TREs as a monomer and homodimer (20). The addition of the RXR in the reaction mixture containing the recombinant TR resulted in the formation of another two retarded complexes (bands III and IV). Therefore, bands III and IV contain protein–DNA complexes formed by the presence of RXR plus TR, likely to include the RXR-TR heterodimer, which is thought to mediate most of the effects of T3 on gene transcription (26). When UCP3-TRE1*m*1 labeled probe was incubated, either with TR plus or minus RXR, none of the bands was formed, indicating that this half site is crucial to allow the binding of TR or TR-RXR complexes. The results obtained using UCP3-TRE1*m*2 were similar to those obtained with the wild type oligonucleotide, suggesting that although the site 2 was mutated, binding of the TR alone or TR-RXR still occurred. In addition, we performed competition experiments using labeled UCP3-TRE1 always in presence of RXR and TR (Fig 5C). When the non-labeled UCP3-TRE1 oligonucleotide was used for competition, the amount of the DNA-protein complex decreased as increasing amounts of non-

labeled oligonucleotide were added. The bands disappeared completely at 100-fold excess of cold oligonucleotide, pointing to the specificity of the binding of the TR and TR-RXR complexes to the TRE1 element. When the competition assay was performed using UCP3-TRE1*m*1, even at 100-fold excess all retarded DNA-protein complexes were still formed. Thus, there was no competition between the oligonucleotides by the nuclear proteins bound at the specific site when the AGGTCA half site was mutated. As expected from the previous results, competition with UCP3-TRE1*m*2 showed the same results as the wild type.

To further support these results, we checked whether the thyroid receptor present in L6 nuclear extracts binds to this site. Briefly, the UCP3-TRE1 labeled oligonucleotide was incubated with 5 μg of protein extracts from L6. Upon incubation with nuclear extracts, one major band and three other retarded bands were formed. When a specific antibody against TR was added to the incubation media, the intensity of *i* and *ii* bands decreased, and a new band of lower mobility, marked as S, was formed. (Fig 5D). Of note, only the reduction in the *i* band was specific, since the *ii* band also disappeared when CEBPα antibody was added as a negative control. Therefore, the *i* band is formed by a DNA-protein complex containing TR, thus indicating that TR binds the hUCP3 TRE1 site.

In vivo **binding of TR to the TRE1 element by Chromatin Immunoprecipitation assay**. We next examined the interaction of TR with the human UCP3 promoter "in vivo". We performed Chromatin Immunoprecipitation (ChIP) assays on transfected L6 cells with two human UCP3 reporter constructs, the wild type (-1588hUCP3-Luc) and the one containing the mutation in the TRE1 site (-1588*mut*TRE1hUCP3-Luc) always in the presence of MyoD and TRβ1. Immunoprecipitation of protein-DNA complexes with the TR antibody caused a specific enrichment of the 191 bp PCR product corresponding to the –166 to +26 region of the hUCP3 gene in the wild type construct when compared with the control with IgG antibody (Fig 6). Correspondingly, when the TR antibody was added to the protein-DNA complexes from cells transfected with the mutated TRE1 construct, the specific band was not enriched. These results confirm that TR binds "in vivo" to TRE1 in the human UCP3 promoter.

The mouse UCP3 promoter is also activated by thyroid hormone but it is less sensitive than the human promoter. In order to explore the basis of the higher

effects of T3 observed in the human UCP3 transgene respect to the endogenous mouse UCP3 gene, we cloned a 2 kb fragment of the 5' non-coding region of the mouse UCP3 gene upstream the transcription initiation site. When transiently transfected into L6 cells, the mouse UCP3 promoter showed low basal expression levels but it was strongly induced by co-transfected MyoD, to a similar extent to that observed in the human UCP3 promoter. When TR was co-transfected, T3 induced the mouse UCP3 promoter activity by 2 fold. This induction is significantly lower than the induction observed in the human UCP3 promoter (Fig 7A). This result was also confirmed in dose-response curves where induction of the mouse UCP3 promoter by T3 was lower than in the human UCP3 promoter at all T3 concentrations tested (Fig 7B). This lower sensitivity did not depend on the subtype of TR receptor co-transfected as it was equally observed when co-transfecting chicken TRα (3.8 $\pm$ 0.2 fold induction by T3 in mouse versus 14.6 $\pm$ 1.8 induction in human), rat TRα (2.5 $\pm$ 0.1 fold induction by T3 in mouse versus 13.0 $\pm$ 1.0 in human) and rat TRβ1 (2.1 $\pm$ 0.1 fold induction by T3 in mouse versus 5.7 $\pm$ 0.1 in human) expression vectors. Computer assisted analysis (Matinspector program) of the 5' non-coding region of the mouse UCP3 gene did not reveal any consensus site for a TRE, in agreement with previous observations (27). However, comparing human and mouse UCP3 promoter sequence, a site was detected in the mouse proximal UCP3 promoter region with a similar sequence to the TRE1 previously characterized in the human gene (see Fig 7C). Point mutation of this region abolished the responsiveness of the mouse UCP3 promoter to T3 (Fig 7D) (as also happened with the human UCP3 promoter, Fig 4), thus indicating that this site behaves as a TRE in the mouse UCP3 promoter. Band shift analysis of this site in comparison with the human TRE1 indicated that the binding observed in the mouse TRE was weaker, especially the bands *III* and *IV* corresponding to TR/RXR heterodimers which were almost undetectable (Fig 7E).

DISCUSSION

We have determined the effect of thyroid hormones on the human UCP3 gene. The study of the regulation of the UCP3 gene in humans is particularly complex owing to the extremely low levels of expression in human myotubes in culture. Therefore, we created transgenic mice bearing a human P1 clone that contains the entire UCP3 gene. The expressed transgene in mice retained the tissue-specific regulation of the UCP3 gene in humans, i.e. highly preferential expression in skeletal muscle. It is noteworthy that the human UCP3 transgene expression was almost absent in heart and brown fat, in which the rodent UCP3 gene is substantially expressed (28). This finding, together with the lack of UCP3 mRNA detection in human brown fat and extremely low levels in human heart (28;29), indicates a more strict tissue-specificity of the UCP3 gene expression in skeletal muscle in humans respect to rodents. Moreover, human UCP3 transgene expression is up-regulated by starvation, similarly to the murine UCP3 gene, which may be due to the fatty acid-mediated stimulation of human UCP3 gene transcription, as already reported (9). The results obtained after T3 administration in transgenic mice demonstrate that the human UCP3 gene is up-regulated by thyroid hormones "in vivo", in agreement with previous findings in muscle biopsies from human volunteers (11). Most of the reported experiments in which UCP3 mRNA levels are induced by a single T3 injection were carried out in hypothyroid rats (7). When we applied T3 to euthyroid mice, only the human UCP3 transgene was induced, but not the endogenous murine UCP3 mRNA. This suggested that the human UCP3 gene is more sensitive to thyroid-dependent stimulation than the mouse gene. The construct of the mouse UCP3 gene promoter transiently transfected to the same cells in identical conditions to those used for the human UCP3 promoter, was responsive to T3 stimulation but to a lesser extent than the human UCP3 promoter construct. Both the human and mouse UCP3 gene promoters required a similar TRE in the proximal promoter region, but the mouse TRE appeared to be weaker than the human TRE. By contrast, other features of regulation such as dependency on MyoD were equally shared by both promoters.

Thyroid hormones can induce lipolysis and fatty acid oxidation (30) and hyperthyroidism increases the levels of free fatty acids. Most of the physio-pathological situations reported to date in which UCP3 expression is modified in skeletal muscle are associated to parallel changes in fatty acid availability to skeletal muscle. The effects of

fatty acids on UCP3 gene transcription are mediated by PPARα and PPARδ receptors which interact with the promoter region of the human UCP3 gene (9) . Therefore, it could not be excluded that the effects of T3 on UCP3 gene expression “in vivo” may be due to the action of fatty acids. Moreover, thyroid hormones induce master transcription factors for mitochondrial gene expression, such as nuclear respiratory factors, which can affect UCP3 gene expression (31). However, our present results establish that the action of thyroid hormones is direct and mediated by TR binding to the proximal region of the UCP3 gene promoter. In the human promoter, the TRE element required for responsiveness is coincident with the reported site responsible for responsiveness to PPAR and retinoids, thus indicating that it behaves as a multihormonal responsive element. Moreover, the action of T3 on the human UCP3 promoter requires MyoD, as does the PPAR and RAR-dependent activation (8;9). This confirms that MyoD acts as permissive factor for basal and hormone-dependent transcriptional activity of the human UCP3 promoter.

The finding that responsiveness to thyroid hormone shares a common DNA region with the retinoic acid and PPAR-dependent pathways suggests a cross-talk between these regulatory pathways in the human UCP3 promoter, as reported for other genes (32;33). Despite a recent report showing that a single injection of T3 to fasted rats can further induce UCP3 mRNA expression (34), the interaction between the thyroid hormone and fatty acid-dependent regulation of the UCP3 gene "in vivo" has been poorly explored. Current research is under way to determine the mutual influence of PPAR and thyroid-dependent regulation of UCP3 gene transcription and the involvement of the common multi-hormonal responsive site in their cross-talk.

The analysis of the regulation of the UCP3 gene in humans does not provide direct information on the function of the UCP3 protein. However, the high sensitivity of human UCP3 transcription to thyroid hormones is consistent with a potential role of UCP3 in the response of skeletal muscle mitochondrial functions to the action of thyroid hormones. Although thyroid hormones increase body temperature in rodents and humans by unknown mechanisms, current information on the function of UCP3 is poorly supportive of a role for this protein in thermogenesis. It is possible that thyroid stimulation of UCP3 gene expression was related to the regulation of production of reactive oxygen species, a major current hypothesis for UCP3 protein function. Activation of mitochondrial respiration by thyroid hormones results in increased reactive oxygen species production, at least in liver cells (35), and it has been also

proposed that a high production of reactive oxygen species is on the basis of muscular injury caused by hyperthyrodism (36). In this context, induction of UCP3 gene expression by thyroid hormones could be viewed as a protective mechanism for excessive ROS production when cell respiration is enhanced by a physiological increase in these hormones.

In summary, we conclude that thyroid hormones directly activate the human and mouse UCP3 genes through interaction of thyroid receptors with the proximal promoter regions. This suggests that UCP3 up-regulation is a relevant component of the regulation of mitochondrial function in human skeletal muscle in response to thyroid hormones.

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FIGURE LEGENDS

Figure 1. Expression of the human UCP3 gene in transgenic mice. A) Tissue distribution of hUCP3 expression in transgenic mice. 10 and 20 μg of total RNA were analyzed by RNAse protection assay in white fat (WAT), brown fat (BAT), heart and skeletal muscle (sk ms). Short ($UCP3_S$) and long ($UCP3_L$) forms of human UCP3 mRNA (16) were detected as described in Experimental procedures. B) Induction of human UCP3 mRNA expression by starvation in transgenic mice. Northern blot was performed with 20 μg of total RNA from skeletal muscle and hybridized with a specific human DNA probe that hybridizes exclusively with human but not mouse UCP3 mRNA. Control mice were used to avoid cross-hybridization of the human probe with mouse UCP3 mRNA. Control and hUCP3 transgenic mice were either fed *ad libitum* or starved for 24 h (Stv).

Figure 2. Thyroid hormones induce human UCP3 gene expression in transgenic mice. Two different lines of transgenic mice (Tg A and Tg B) were injected i.p. with either saline or T3 (100 μg/Kg) and studied after 18 hours. Northern blot was performed as in Fig 1B. Results are the mean ± SEM of 6 different mice for each transgenic line. Differences were considered significant where* $p< 0.05$.

Figure 3. Effects of T3 on human UCP3 promoter activity in L6 cells. 1,5 μg of a construct containing 4511 bp of human UCP3 promoter region (4511hUCP3-Luc) was co-transfected when indicated with 0,3 μg of MyoD, TRβ1 or TRα expression vectors. Treatment with T3 at dose of 50 nM was performed for 24 h. Results are expressed as fold-induction of luciferase activity compared with transfection of 4511hUCP3-Luc alone and are the mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences due to the co-transfection with MyoD expression vector were shown as * and those due to the addition of T3 as # ($p< 0.05$).

Figure 4. Deletion and point mutation analysis of the T3-responsiveness of the 5' non-coding region of the human UCP3 gene. Potential thyroid hormone response elements (TRE) on the basis of computer assisted analysis (24) are shown. TRE1 is located in between –71/-59, TRE2 in –1506/-1492, TRE3 in –2143/-2136 and TRE4 in

–3358/-3343. Crossed box represents a point mutation performed in the TRE1 (see Methods section). Results are expressed as the percent induction of the constructs by T3 compared with the induction observed in -4511hUCP3-Luc (100%). Transfections were performed in the presence of co-transfected MyoD and TRβ expression vectors. Results are the mean ± SEM of at least 3 independent experiments performed in triplicate. Statistical significance respect to -4511hUCP3-Luc is shown as ** ($p < 0.01$).

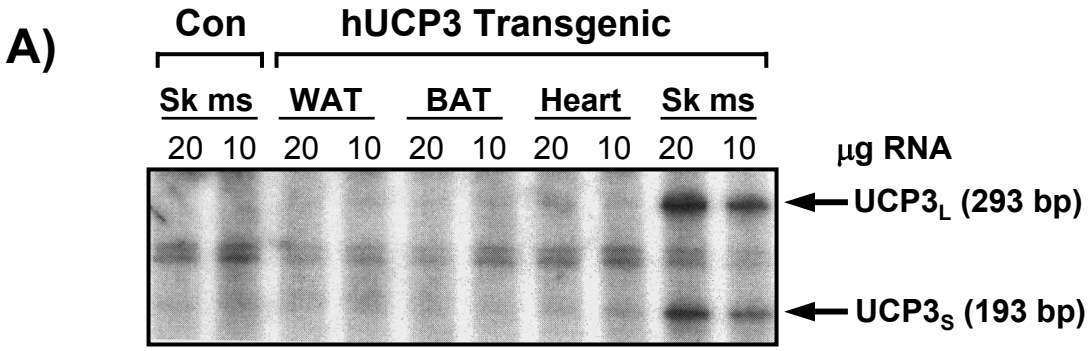
Figure 5. Thyroid receptor binds to the TRE1 in the proximal region of the human UCP3 gene promoter. A) Sequence of the three oligonucleotides corresponding to –79 to –50 of the promoter region, the wild type (UCP3-TRE1) and the ones with two point mutations, (UCP3-TRE1m1 and UCP3-TRE1m2). B) Electrophoretic mobility shift analysis of the three labeled-oligonucleotides depicted above. When indicated, 5 μl of the RXRα product from a transcription-translation "in vitro" reaction and/or 200 ng of recombinant TR was added to the assay. Four retarded complexes named as *I*, *II*, *III* and *IV* were formed. C) Competition analysis. Electrophoretic mobility shift assay was performed using UCP3-TRE1 as labeled probe and in the presence of RXRα plus TR as described above. Increasing concentrations of non-labeled UCP3-TRE1, UCP3-TRE1*m*1 and UCP3-TRE1*m*2 (25, 50 and 100 ng, respectively) were added to the incubation mixture. D) Electrophoretic mobility shift assay using nuclear extract. UCP3-TRE1 was incubated with 5 μg of L6 nuclear extracts and when indicated an antibody against TRα or CEBPα was added. *i* and *ii* are two of the bands modified by the addition of TR antibody and *S* represents the new band that appears owing to the addition of the TR antibody.

Figure 6. Chromatin Immunoprecipitation (ChIP) analysis of TR binding to the human UCP3 promoter in L6 cells. ChiP was performed on -1588hUCP3-Luc construct and on -1588hUCP3*mut*TRE1-Luc, a construct containing a point mutation in TRE1 (see Fig 2). The experiment was performed in presence co-transfected MyoD (pCMV-MyoD) and TRβ1 (pRSV-human TRβ1) expression vectors. Immunoprecipitation and PCR were performed as described in Experimental procedures**.** The arrow indicates the 191 bp PCR product from hUCP3 gene. H_2O and

Con represent negative (no DNA) and positive (plasmid amplification) PCR controls, respectively**.** Results are representative of two independent experiments**.**

Figure 7. Effects of thyroid hormones on the mouse UCP3 gene promoter. A) Transient transfection assays included 1,5 μg of the constructs containing 1588 bp of the human UCP3 (1588hUCP3-Luc) and 1946 bp of the mouse UCP3 (1946mUCP3-Luc) promoter regions. When indicated 0.3 μg of MyoD and TRβ1 (TR) expression vectors were co-transfected. T3 treatment was performed at a dose of 50 nM for 24 h. Results are expressed as fold-induction of luciferase activity respect to basal promoter values and are the mean ± SEM of at least 3 independent experiments performed in triplicate. Statistically significant differences due to the co-transfection with the MyoD expression vector were shown as * $p< 0.01$ and those due to the addition of T3 as # $p< 0.05$ and ## $p< 0.01$. B) 1,5 μg of human UCP3 (1588hUCP3-Luc) and mouse UCP3 (1946mUCP3-Luc) promoter regions were co-transfected with 0,3 μg of MyoD and TR expression vectors and treated with T3 at the indicated concentrations for 24 h. Results are expressed as fold-induction of luciferase activity achieved by the addition of T3 and are the mean ± SEM of at least 3 independent experiments performed in triplicate. **C)**, Comparison of the human TRE1 and mouse TRE sites in the proximal promoter regions. D) Functional analysis of the TRE present in the mouse UCP3 promoter in comparison with the human TRE1 was performed by point mutation of the site, as shown by italics in C, and assayed in transient transfections. Bars represent the percent induction by T3 of each construct when co-transfected with MyoD and TR. Maximal induction (100%) was assigned to the non-mutated human UCP3-Luc and mouse UCP3-Luc constructs. Statistically significant differences due to mutation in the TRE site was shown as * $p< 0.01$ E) Electrophoretic mobility shift analysis of the human UCP3 TRE1 and the TRE region in the mouse UCP3 gene. Assay conditions and indications of the four retarded complexes *I*, *II*, *III* and *IV* were as in Fig 5.

Figure 1


A)
Con
hUCP3 Transgenic
Sk ms
WAT
BAT
Heart
Sk ms
20 10 20 10 20 10 20 10 20 10
µg RNA
UCP3L (293 bp)
UCP3S (193 bp)

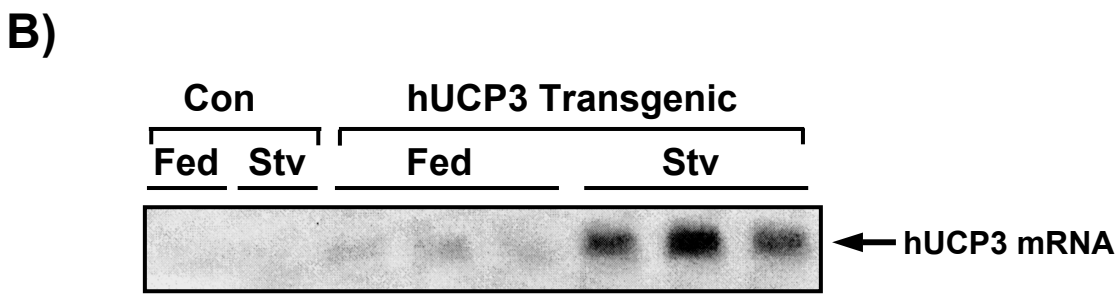

B)
Con
hUCP3 Transgenic
Fed
Stv
Fed
Stv
hUCP3 mRNA

Figure 2

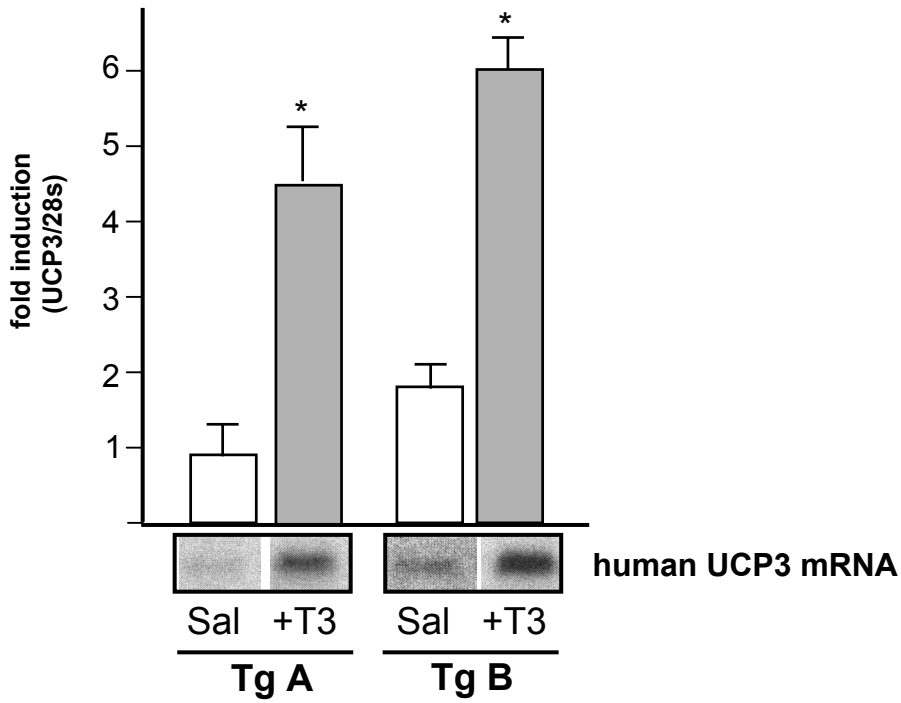

fold induction
(UCP3/28s)
6
5
4
3
2
1
*
*
human UCP3 mRNA
Sal
+T3
Sal
+T3
Tg A
Tg B

Figure 3

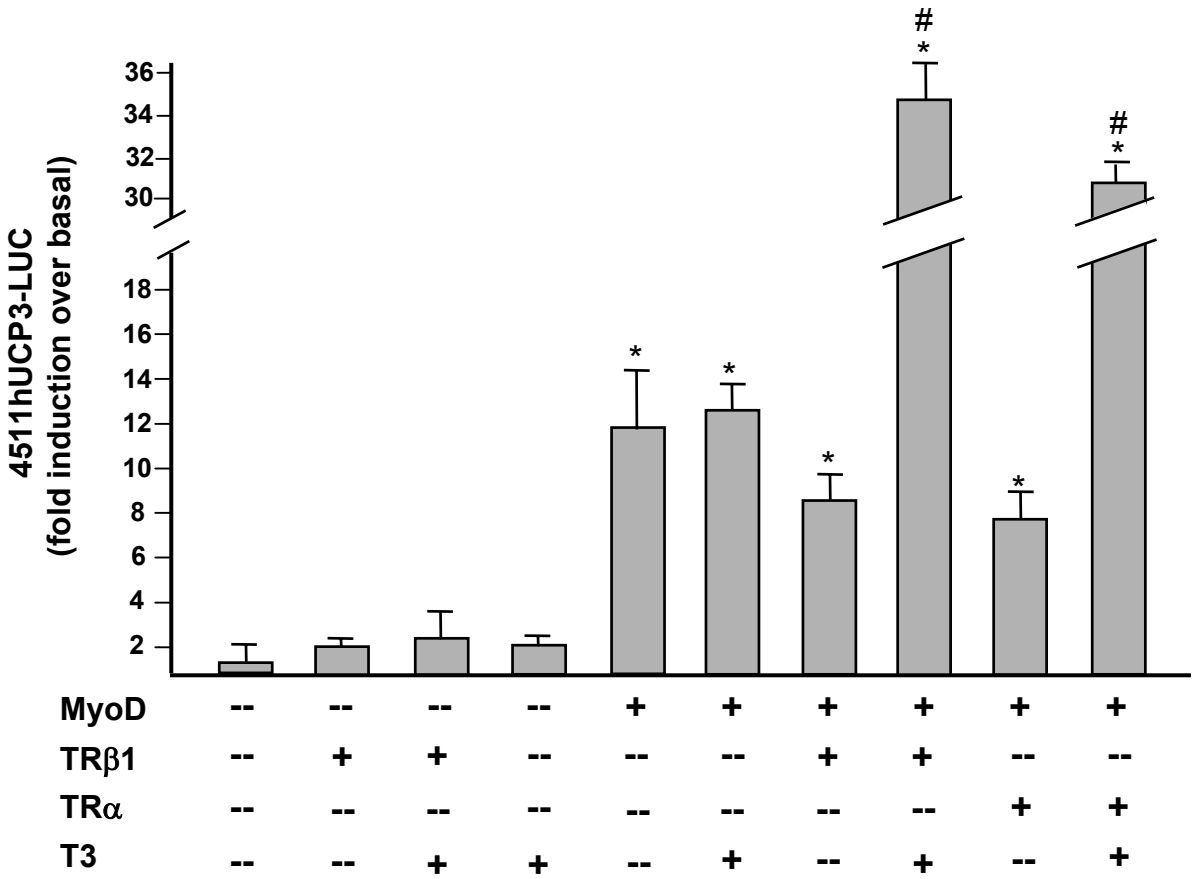

4511hUCP3-LUC
(fold induction over basal)
36
34
32
30
18
16
14
12
10
8
6
4
2
*
#
MyoD -- -- -- -- + + + + + +
TRβ1 -- + + -- -- -- + + -- --
TRα -- -- -- -- -- -- -- -- + +
T3 -- -- + + -- + -- + -- +

Figure 4

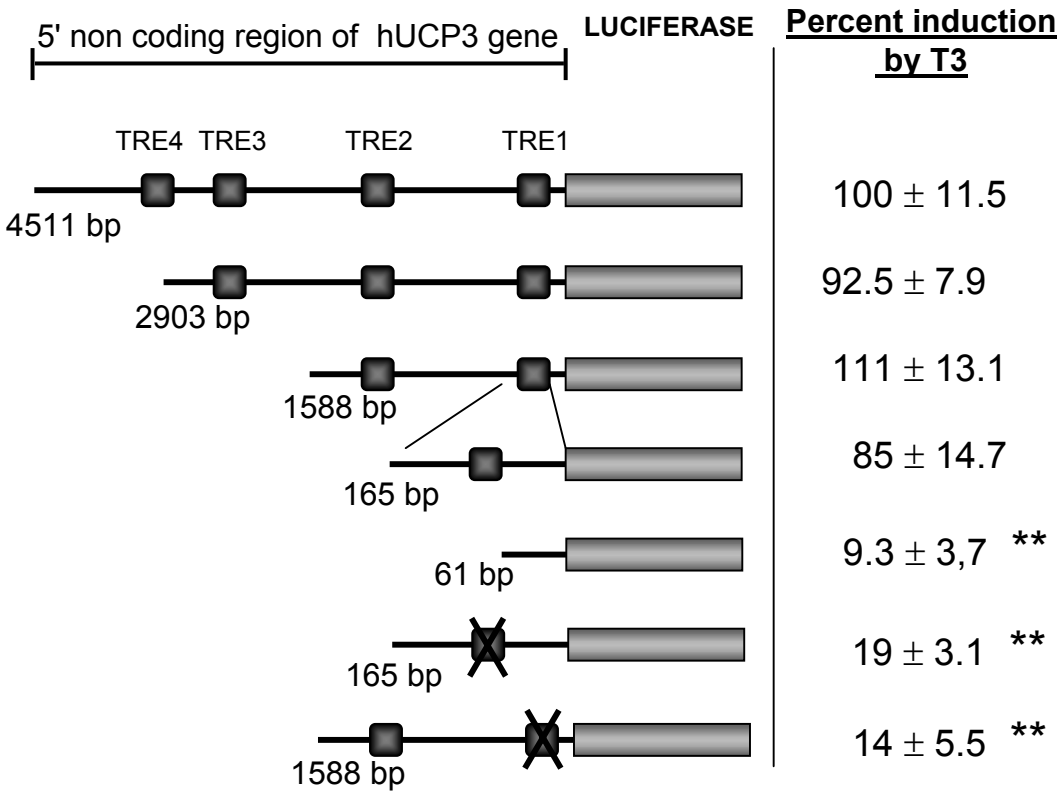
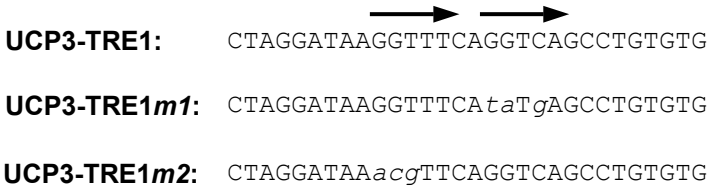
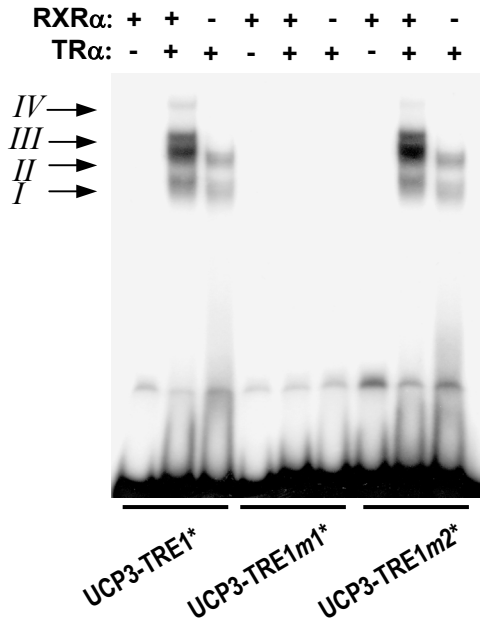

5' non coding region of hUCP3 gene
LUCIFERASE
Percent induction by T3
TRE4 TRE3 TRE2 TRE1
4511 bp
100 ± 11.5
2903 bp
92.5 ± 7.9
1588 bp
111 ± 13.1
165 bp
85 ± 14.7
61 bp
9.3 ± 3,7 **
165 bp
19 ± 3.1 **
1588 bp
14 ± 5.5 **

Figure 5

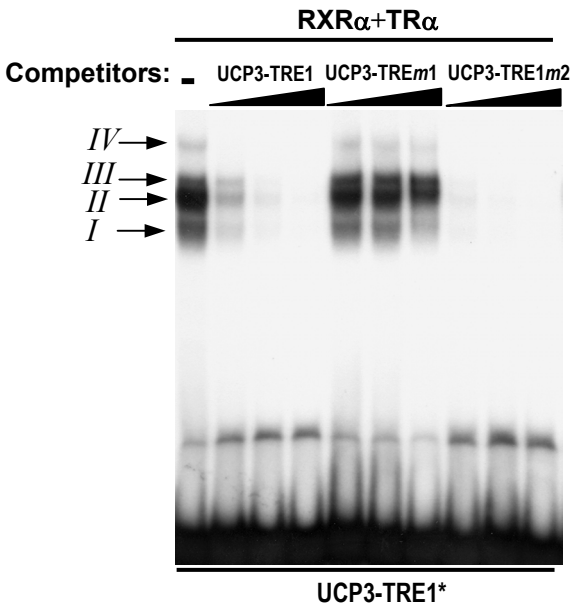
A)



B)



C)



D)

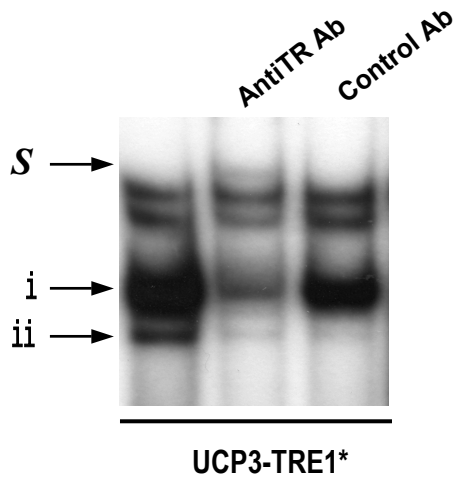


Figure 6

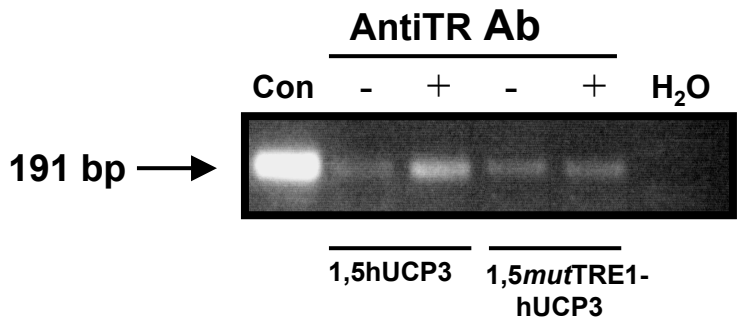
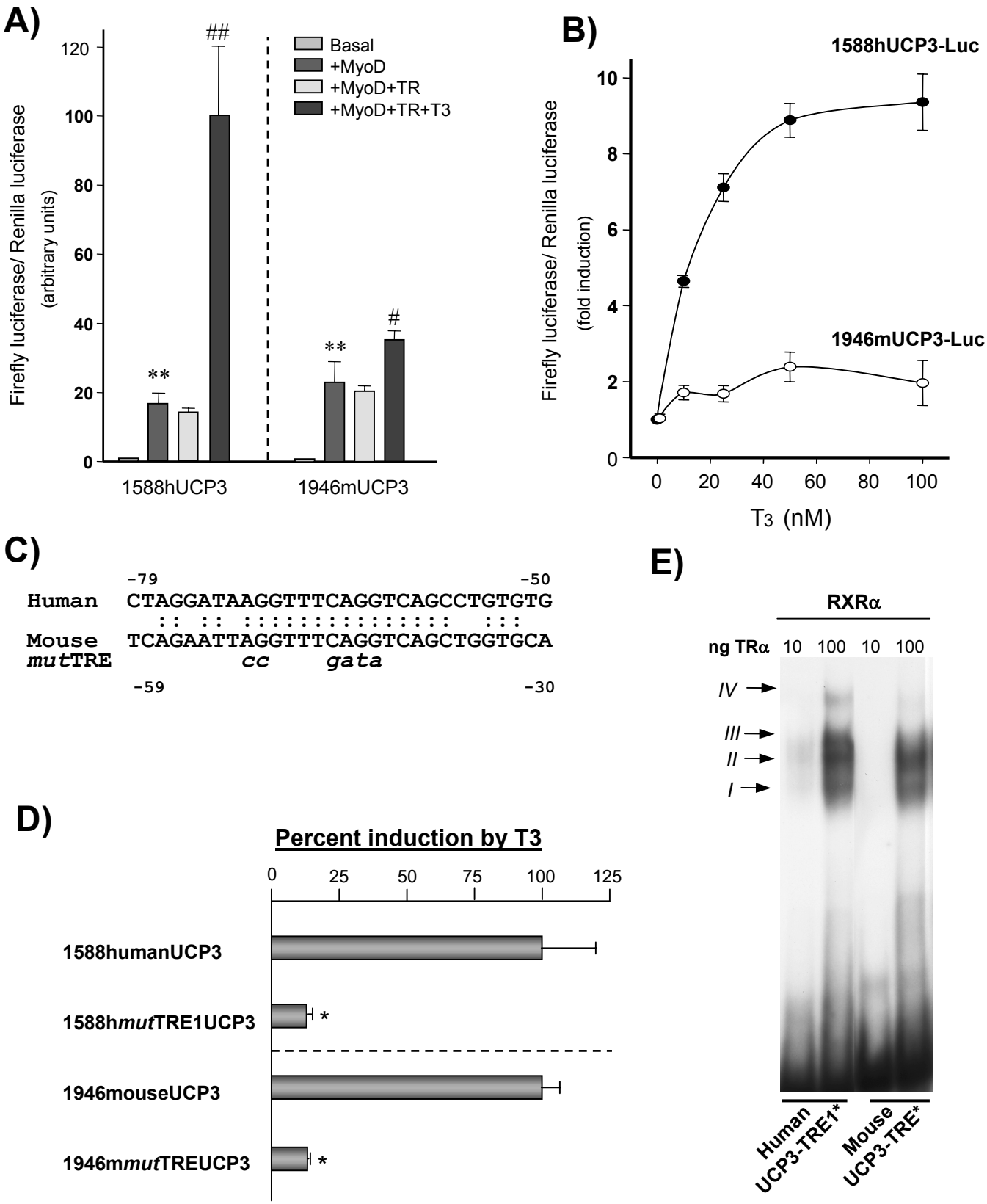


Figure 7


A)
Firefly luciferase/ Renilla luciferase
(arbitrary units)
Basal
+MyoD
+MyoD+TR
+MyoD+TR+T3
1588hUCP3
1946mUCP3
B)
1588hUCP3-Luc
1946mUCP3-Luc
Firefly luciferase/ Renilla luciferase
(fold induction)
T3 (nM)
C)
Human CTAGGATAAGGTTTCAGGTCAGCCTGTGTG
Mouse TCAGAATTAGGTTTCAGGTCAGCTGGTGCA
mutTRE cc gata
D)
Percent induction by T3
1588humanUCP3
1588hmutTRE1UCP3
1946mouseUCP3
1946mmutTREUCP3
E)
RXRα
ng TRα 10 100 10 100
IV
III
II
I
Human UCP3-TRE1*
Mouse UCP3-TRE*