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Regulation of the Rat Liver Carnitine Palmitoyltransferase I Gene Transcription by Thyroid Hormone

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L-CPT I isotype is the main locus of control for liver LCFA oxidation. T3 levels have been described as controlling L-CPT I gene expression, and in this paper we demonstrate that rat liver CPT I promoter responds to T3. Using deleted reporter constructs we located the thyroid hormone-responsive element between −2935 and −2918, consisting of a DR4. This response is mediated by the binding of the thyroid to this sequence as a monomer, homodimer, or heterodimer with RXR.

The carnitine shuttle is utilized in mammalian cells for entry of long-chain fatty acids into the mitochondrial matrix, where they undergo β-oxidation. The first reaction, between the acyl-CoA molecule and carnitine, is catalyzed by carnitine palmitoyltransferase I (CPT I) and takes place on the outer side of the mitochondrial membrane, generating free CoA and acylcarnitine. The second reaction, between acylcarnitine and CoA, takes place in the matrix and is catalyzed by CPT II on the inner side of the inner membrane. The acyl-CoA generated is the substrate for the β-oxidation pathway. Two isoforms of CPT I have been described and are referred to as L-CPT I and M-CPT I, since they are mainly expressed in liver and muscle, respectively (1).

It is generally accepted that the L-CPT I isotype is the main locus of control for liver LCFA oxidation through three different mechanisms: (i) changes in gene expression; (ii) variations in the concentration of malonyl-CoA, a lipogenic intermediate and a potent inhibitor of the CPT I isotypes; (iii) modification in the sensitivity of L-CPT I to malonyl-CoA inhibition [reviewed in (1)].

Rat liver CPT I activity is known to increase during the fetal to neonatal life transition and also during the fed to starved state in the adult animal (2, 3). In cultured fetal rat hepatocytes AMPc and LCFA stimulated the transcription of the L-CPT I gene and these effects were antagonized by insulin (4). Thus, the increase of L-CPT I mRNA levels during the fetal to neonatal transition is probably triggered by changes in the insulin/glucagon rates and the increase in LCFA concentration. It is also known that hypothyroidism causes a decrease in L-CPT I mRNA abundance, and hyperthyroidism increases the mRNA levels in rats (5). Thyroid hormone differs from insulin and other hormones in the coordinate regulation of fatty acid oxidation and synthesis. These two pathways are regulated in a reciprocal manner by insulin (6). However, the activity of both pathways is increased by thyroid hormone, and both pathways decrease in hypothyroidism (7).

The thyroid hormones influence a variety of physiological processes, including cell growth and metabolism in mammals, initiation of metamorphosis in amphibia, and development of the vertebrate nervous system (8). The actions of the thyroid hormones are mostly mediated by thyroid hormone nuclear receptors (TRs). The TRs are encoded by two genes (α and β) and are expressed as several isoforms (TRα1, TRβ1, and TRβ2) (9). The TRs belong to the same subgroup of nuclear receptor family as the retinoic acid receptors (RARs), the retinoid X receptors (RXRs), the vitamin D receptor (VDR), and the peroxisome proliferator-activated receptors (PPARs). The TR binds to a target DNA sequence known as TRE, composed of two half site core motifs (AGGTCA) with specific nucleotide spacing and orientation (10, 11). TR binds to a TRE as a monomer, homodimer or heterodimer, particularly

Abbreviations used: CPT, carnitine palmitoyltransferase; LCFA, long-chain fatty acids; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; hRXRα, human 9-*cis*-retinoic acid receptor; T3, triiodothyronine; TRE, thyroid hormone-responsive element; TR, thyroid hormone receptor; DR4, direct repeat separated by four nucleotides; EMSA, electrophoretic mobility shift assay; MCAD, medium-chain acyl-CoA dehydrogenase.

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with retinoic X receptors (RXR) (12). These complexes then activate the transcription of target genes in a ligand-dependent manner. The control of positively regulated genes is dictated by receptor binding to DNA regulatory elements and the recruitment of corepresors in the absence of ligand, leading to a transcriptional repression. These correpresors, like SMRT (13, 14) and NcoR (15), in turn assemble a repression complex made up mainly of histone deacetylases (HDACs) (16) which promote histone deacetylation and chromatin remodeling. In the presence of ligand, correpresors are dissociated and coactivators bind to promote histone acetylation and stimulation of gene transcription (17).

In this study, we report that T3 is able to regulate the gene expression of the rat L-CPT I through the TRs. We have characterized a TRE in the promoter of the L-CPT I responsible for the transactivation caused by T3. This element, in the absence of T3, binds TR and represses gene transcription. Ligand binding of the receptor causes transcriptional activation of the L-CPT I gene. Consequently, thyroid hormone is an important modulator of the long chain fatty acids mithochondrial β-oxidation.

EXPERIMENTAL

5′ RACE. A Rapid Amplification cDNA End (RACE) kit from Clontech was used following the manufacturer's instructions. Briefly, 2 μg of poly(A)$^+$ mRNA from the liver of an adult rat was the template of the first-strand cDNA synthesis, using RT5 (5′-CACCACGATAAGCCAGCTGGACG) rat-specific reverse primer. After the RNA degradation an anchor sequence (3′-ggagacttccaaggtct-tagctatca**cttaagcac**; nucleotides designated in lower case indicates non L-CPT I sequences) was ligated to the 3′ end of the cDNA first strand with T4 RNA ligase. The 5′ end of the cDNA was subsequently amplified by PCR with an anchor primer (5′-ctggttcggcccacctctgaaggttccagaatcgatag) and AMP5 (5′-aagctt**gaattc**GATGATGCCAT TCTTGAACG) primer, a L-CPT I specific primer located upstream of RT5. The PCR product was cloned into pBSSK+ taking advantage of an *Eco*RI restriction site introduced in the anchor sequence and AMP5 primers (bold in the respective sequences) and used as a probe for the screening of a rat genomic library.

Screening of rat genomic library. Two independent screenings were carried out with the RACE product probe and the 5′ region probe (described below). Both screenings were carried out using rat genomic library Lambda FIX II (Stratagene) following the instructions supplied by the manufacture. Briefly, 300,000 clones were analyzed in each screening, plated in LB agar plates, transferred to HA nitrocellulose filters from Millipore and hybridized with [α-^{32}P]dCTP random priming labeled probe. Positive plaques were submitted to three rounds of purification and finally lambda DNA was extracted using the Qiagem Lambda Maxi kit.

Genomic Southern blot. Genomic Southern blot was performed as described in Maniatis (20) using 10 μg of digested rat genomic DNA.

Plasmids. Oligonucleotides DH164 (5′-aactgCAGAGAGCTCT-TCCAAGACCAGG, position −969 to −946, nucleotides designated in lowercase were added to provide restriction sites) and DH159 (5′-acgcgtcgacGGACGGCGGCGGCGGAATGAG, position +21 to −3) were used to amplify the proximal promoter of the liver CTP I gene using rat genomic DNA as a template. The PCR product was digested with *Pst*I and *Sal*I and cloned into the *Pst*I/*Sal*I sites of the pCAT Basic (reporter plasmid encoding for chloramphenicol acetyltransferase), generating the plasmid LCPTICAT. This plasmid was used as a template to amplify with oligonucleotides DH234 (5′-ctgcaggctagCAGAGCTCTTCCAAGACCAGG, from −969 to −946) and DH236 (5′-gaattcctcgagGGACGGCGGCGGCGGAATGAG, position +21 to −3) a PCR product that was digested with *Nhe*I and *Xho*I and ligated into the *Nhe*I/*Xho*I sites of the pGL3 Basic (firefly luciferase reporter vector). The new construct was called p965CPTI. Digestion of p965CPTI with *Nhe*I and *Xho*I liberated an insert of approximately 1 kb that was used as a probe for the screening of the rat genomic library. The isolated clone contained exon 2 and 1 and more than 8 kb of the 5′ region of the rat CPT I gene. A 3-kb fragment resulting from the *Eco*RI and *Sal*I digestion of this clone was cloned into *Eco*RI and *Sal*I sites of BluescriptSK+ to generate plasmid p1600BS. This fragment contained exon 2 and 1 and 1642 bp of the proximal 5′ region. A λ *Xho*I/*Eco*RI 3-kb fragment that contained the distal 5′ region of the rat L-CPT I (−4489 to −1641) was cloned into *Xho*I/*Eco*Ri sites of BluescriptSK+ to generate construct p4500BS. p1600BS was digested with *Sac*I to generate a fragment of 690 bp that was cloned into the *Sac*I site of p965CPTI in order to create plasmid p1600CPTI. *Kpn*I/*Eco*RI digestion of p4500BS produced a 3-kb fragment that was subcloned into the *Kpn*I/*Eco*RI sites of p1600CPTI. The resulting plasmid was called p4500CPTI and contained approximately 4500 bp of the 5′ region. Deletions of the promoter were constructed by cloning into *Kpn*I/*Eco*RI sites of p1600CPTI *Kpn*I/*Eco*RI digested PCR products amplified using oligonucleotides DH265 (5′-atggtaccCCAGCACTACACAGAC, position −3975 to −3959) and DH268 (5′-CTTTGCATCCGTCTGGATTGG, from −1519 to −1540) for deletion p4000CPTI, oligonucleotides DH266 (5′-atggtaccCTGTCCTCATGGAACC, position −2935 to −2919) and DH268 for deletion p2950CPTI and oligonucleotides DH323 (5′-ctgcagggtaccCAACATTATTAT GGGATAG, from −2793 to −2774) and DH268 for deletion p2800CPTI. Plasmid −2935/−2793CPTI contained a PCR product amplified with oligonucleotides DH266 and DH330 (5′-ctgcaggctagCTATCCAATAAT AATGTTG, from −2773 to −2792) digested with *Nhe*I and *Kpn*I and cloned into pGL3 Promoter *Nhe*I/*Kpn*I sites. Plasmid 2XTRE was generated by annealing the oligonucleotides DH371 (5′-cCTGTCCTCATGGAACCTGGTACCCTGTCCT CATGGAACC) and DH372 (5′-tcgaGGTTCCATGAGGACAGGGTACCAGGTT CCATGAGGACAGGgtac) and cloning into the *Kpn*I and *Xho*I sites of pGL3 Promoter (which contains the SV40 promoter upstream of the firefly luciferase reporter gene). Mutation of the promoter was assessed by overlap extension PCR (18). First two PCRs were performed using oligonucleotides DH367 (5′-GTACCTTTTACTGCTGAGC, position −3283 to −3264) and DH364 (5′-GCGTCACCAGaaTCCATGAGaACA GCGGCGTTGTC, position −2910 to −2945) and primers DH363 (5′-ACGCCGCTG TtCTCATGGAttCTG GTGACGCTGGC, from −2941 to −2906, the mutated nucleotides are designated in underlined lowercase) and DH368 (5′-TGGAAAGAATAAGATAGGG, position −2452 to −2471), respectively. The two PCR products were combined in a third PCR producing a mutated product of 790 bp that was digested with *Bst*XI and *Mph*11031. The *Bst*XI/*Mph*1103I fragment from p4500CPTI was substituted by the mutated fragment obtained by PCR to generate plasmid p4500CPTIM. Automatic sequencing using the fluorescent terminator kit (Perking–Elmer) was performed to check all constructs.

Cell culture and transfections. HepG2 cells were cultured in minimal essential medium supplemented with nonessential amino acids and 10% fetal bovine serum. Cells were co-transfected by the calcium phosphate method (19, 20) with 2 μg of the reporter CPT I-Luciferase gene construct and 500 ng of eukaryotic expression vectors encoding for TRα (pSG5-CEA) or an equal amount of salmon sperm DNA. In all experiments 40 ng of pRL (cytomegalovirus promoter-renilla luciferase) was included as internal control in transfections. Cells were treated with 1 μM T3 for 24–42 h. Cell extracts were prepared by passive lysis and luciferase assays were performed following the instructions of the Dual Luciferase Reporter Assay System (Pro-

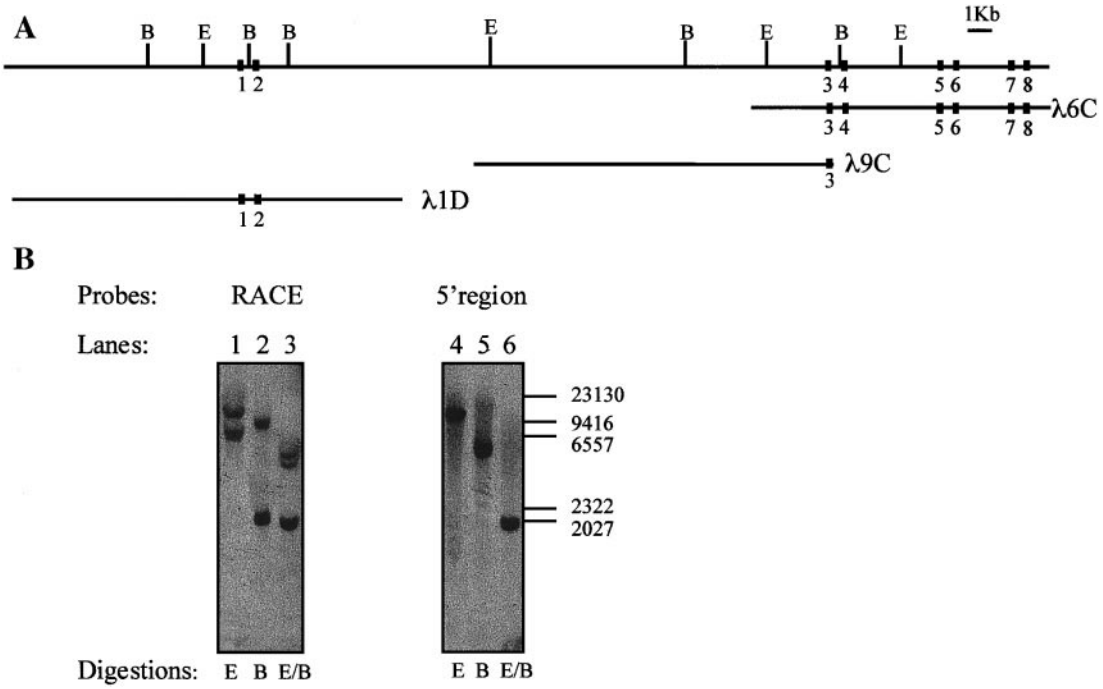

FIG. 1. Genomic organisation of rat liver CPT I gene. (A) Scheme showing the new proposed genomic organisation. Exons are represented by black boxes. Genomic sequences obtained through rat lambda library screening are designated as λ9C, λ6C and λ1D. Location of exons 1, 2, 3, 4, and 8 was determined by Southern blot and DNA sequencing. Location of exons 5, 6, and 7 was deduced from previously reported data (21). Restriction sites are showed as follows: B, *Bam*HI; E, *Eco*RI. (B) Southern blot analysis of rat genomic DNA confirms the proposed organisation. First blot (lanes 1 to 3) was hybridised with RACE probe and second blot (lanes 4 to 6) was hybridised with 5′ flanking region probe. Restriction enzymes are indicated under each lane.

mega). Luciferase activity was measured using the TD-20/20 luminometer (Turner Designs).

In vitro transcription and translation. cDNAs for the human hRXRα receptor and pSG5 as an unprogrammed lysate, were transcribed and translated using a commercially available kit according to the instructions of the manufacturer (Promega).

Electrophoretic mobility shift assay. Four hundred nanograms of cTRα (purchased from Santa Cruz Biotechnology) with or without unprogrammed lysate or hRxRα synthesized *in vitro* was preincubated on ice for 10 min in 10 mM Hepes, pH 7.9, 80 mM KCl, 1 mM DTT, 5% glycerol, and 2 μg of poly(dI–dC). When indicated 1 μM T3 was added. For competition experiments the unlabeled probe was included during preincubation. The −2935/−2793 probe was generated by PCR using oligonuleotides DH 266 and DH330 and p4500CPTI as a template. The CPTI TRE probe resulted from the annealing of oligonucleotides DH266 and DH357 (5′-tcgAGGTTCCATGAGGACAGggtac, position −2918 to −2935). The Mut TRE probe resulted from the annealing of oligonucleotides DH363 and DH364. The unrelated probe consisted on the annealing of oligonucleotides DH262 (5′-CTAGCGTACAGGAGCTCAAAGTTCAAGTTCAC) and DH263 (5′-TCGAT GAACTTGAACTTTGAGCTCCTGTACG). The second half site mutated probe corresponds to the annealing of oligonucleotides DH375 (5′-ATGGTACCCTGTt CTCATGGAACC) and DH 376 (5′-TCGAGGTTCCATGAGaACAGGGTAC). The first half site mutated probe corresponds to the annealing of oligonucleotides DH377 (5′-ATGGTACCTTGTCCTCATGGttCC) and DH 378 (5′-TCGAGGaaCCATGAGG ACAGGGTAC). Two nanograms of ^{32}P-labeled probe was then added and the incubation was continued for 15 min at room temperature. The final volume for all reactions was 20 μl. Samples were electrophoresed at 4°C on a 5% polyacrylamide gel in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.0).

RESULTS

Genomic Organization of Rat L-CPT I Gene

Figure 1A shows the organization for the 5′ end of rat L-CPT I gene. This organization differs from that previously reported by Park *et al.* (21). We proposed this new organization of the L-CPT I gene following the analysis of λ genomic clones and Southern blot analysis of rat genomic DNA. Two different probes were used in this study: a 5′ RACE product and the 5′ flanking region. The 5′ RACE probe corresponds to the rat L-CPT I cDNA, from position +1 to +255 (using the transcription star site as +1), and extends 57 nucleotides from the 5′ end of a previously isolated cDNA clone (22). These data were in agreement with a previously reported L-CPT I 5′ RACE experiment (21). The 5′ flanking region probe (from position −969 to +21) was amplified by PCR from rat genomic DNA using primers designated from previously reported L-CPT I gene sequence (21).

Figure 1B shows the organization of two genomic clones, λ6C and λ9C, obtained with the 5′ RACE probe. The identification of exon 3 in λ9C clone and exons 3 and 4 in λ6C clone was performed by restriction fragment analysis (data not shown) and DNA sequence alignment, which allowed the partial characterization of those clones. Thus, both clones contained exon 3 but

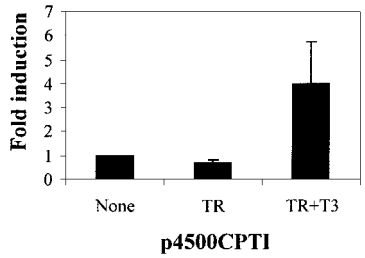

FIG. 2. Rat liver CPT I promoter responds to T3. Construct p4500CPTII was cotransfected with or without pSG5-CEA into HepG2 cells and when indicated treated with 1 μM T3 for 42 h. Average values of renilla normalized luciferase activity (means ± SD), from three independent transfections with two plates each, are expressed as "fold induction" with the activity in the absence of expression vector and treatment defined as 1.

not exons 1 and 2. This result is in disagreement with the published genomic organization of rat L-CPT I, since the size of λ9C clone was large enough to contain exon 1 and 2. Figure 1B also shows the organization of a third genomic clone, λ1D, obtained with the 5′ flanking region probe. This clone contained more than 8 kb of DNA upstream of exon 1, and more than 8 kb downstream of exon 2 but did not contain exon 3. This result was also in disagreement with previously published data describing that intron 2 was 5.5 kb long (21).

Sequence analysis and DNA walking experiments indicated that clone λ9C was not overlapping with clone λ1D. Therefore, to confirm L-CPT I 5′ genomic organization Southern Blot experiments were carried out with rat genomic DNA using the 5′ RACE product or the 5′ flanking region as probes. Figure 1B shows that intron 2 is more than 15 kb long since an *Eco*RI digestion produced a fragment of 15 kb which hybridized with the 5′ RACE product or the 5′ flanking region probes. A second fragment of 6.5 kb (see lane 1 of Fig. 1B) was also detected with the 5′ RACE probe, as expected from the genomic organization of exons 3 and 4 in clone λ6C. The proposed genomic organization was confirmed by single or double enzymatic restriction digestion (Fig. 1B). Thus, these data support the genomic organization presented here and indicate that the previous genomic organization, in which exon 2 is 5.5 kb long, is mistaken.

Rat Liver CPT I Promoter Responds to T3

To determine whether the promoter of the Rat L-CPT I responds to T3 in HepG2, we performed transient transfection assays with plasmid p4500CPTI. It has been reported that most of the cultured cell lines do not respond to T3 because they lack TR (23). To achieve such a response, the cells were also cotransfected with the expression plasmid of TRα (pSG5-CEA). As shown in Fig. 2, in the absence of T3, reporter gene expression was reduced more in the cells transfected with p4500CPTI and the TR-expression plasmid than in the cells transfected with p4500CPTI alone. This reduced expression probably results from the repression by TR of the basal promoter activity. When p4500CPTI cotransfected with pSG5-CEA was treated with T3, the reporter gene response increased more than 5-fold. These results indicate that rat L-CPT I promoter is regulated by T3.

Localization of the TRE Element in the Rat L-CPT I Promoter

To identify the location of the TRE in the rat L-CPT I promoter, several 5′-deletion constructs of the promoter, using luciferase activity as a reporter, were tested for their expression in HepG2 cells. All the reporter gene plasmids were cotransfected with pSG5-CEA in absence or presence of T3 and luciferase activity was measured. As shown in Fig. 3 all the reporter gene plasmids except p2800CPTI responded to T3, indicating that nucleotides up to position −2935 were deleted without loss of the response to T3, which was

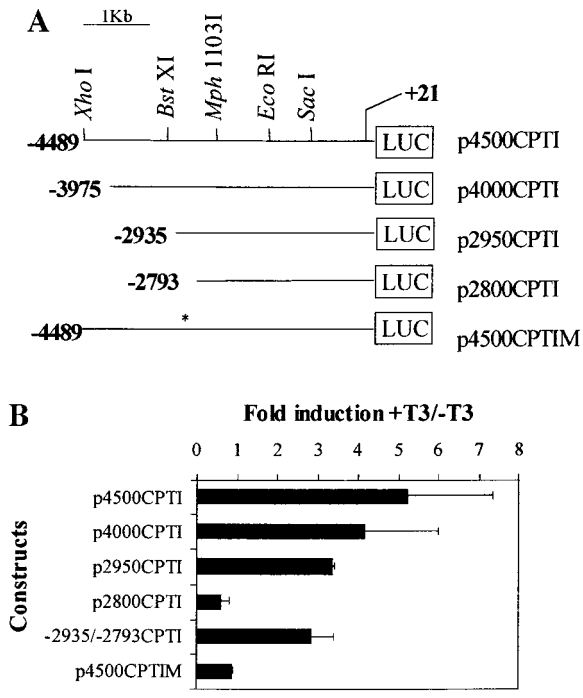

FIG. 3. Localization of the T3 response element of the CPT I gene by progressive deletion analysis. (A) Deleted constructs of the human CPT I promoter. The number at the left denotes the coordinate of the 5′ deletion end point of the construct. The asterisk denotes the position of the three point mutation in the mutated construct. (B) Luciferase reporter constructs containing the indicated position of the 5′-flanking region of the CPT I gene were cotransfected with pSG5-CEA into HepG2 cells and with or without T3 treatment. Average values of renilla normalized luciferase activity (means ± SD), from three independent transfections with two plates each, are expressed as "fold induction" relative to the activity of each construct in the absence of T3.

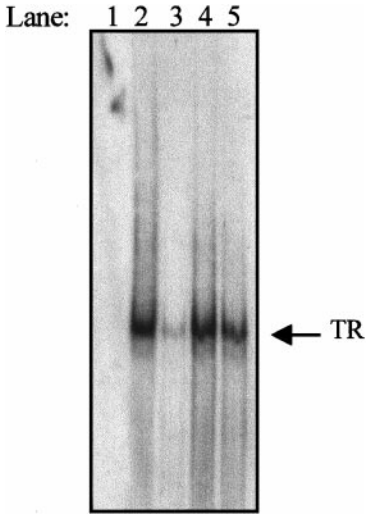

FIG. 4. Electrophoretic mobility shift assay of the −2935/−2793 probe. The −2935/−2793 probe was amplified by PCR and labeled with [γ-^{32}P]dATP and the T4 polynucleotide kinase. The probe was incubated as described under Materials and Methods with 2 μl of unprogrammed lysate (lane 1), 500 ng of TR (lane 2), 500 ng of TR and 21-fold excess of unlabeled probe (lane 3), 500 ng of TR and 50-fold excess of an unlabeled unrelated probe (lane 4) and 500 ng of TR and 100-fold excess of the unlabeled unrelated probe (lane 5).

eliminated by further deletion up to nucleotide −2793. To confirm the existence of the TRE, we cloned the segment between −2935 and −2793 into the pGL3 Promoter vector (−2935/−2793CPTI), a plasmid containing the luciferase gene under the control of the SV40 promoter, showing that this sequence conferred T3 responsiveness to the SV40 promoter (Fig. 3).

Figure 4 shows that −2935 through −2793 labeled fragment was retarded in the presence of cTRα (lane 2). A 21-fold molar excess of unlabeled probe competed for the binding (lane 3), but a 50- or 100-fold molar excess of an unrelated 30-bp-long probe did not compete for the binding (lanes 4 and 5), showing that the binding of TR to the −2935/−2793 probe is specific.

To determine the location of the TRE, we used several shorter probes that covered the entire nucleotide sequence of −2935 through −2793, as unlabeled competitors for the −2935/−2793 ^{32}P-labeled probe in an EMSA (data not shown). We found that the annealed oligonucleotides DH266 and DH357 competed with the radioactive probe. This probe was called CPTI TRE and contains a TRE motif (Fig. 5) similar to the consensus TRE sequence (A/GGGTCANNNNA/GGGTCA) (24) composed by a direct repeat separated by four nucleotides (DR4). The ^{32}P-labeled CPTI TRE probe was able to bind to TR in EMSA experiment. This binding was competed by 12.5- to 50-fold molar excess of unlabeled probe while a mutated probe (Mut TRE), obtained by the annealing of oligonucleotides DH363 and DH364, did not compete the binding.

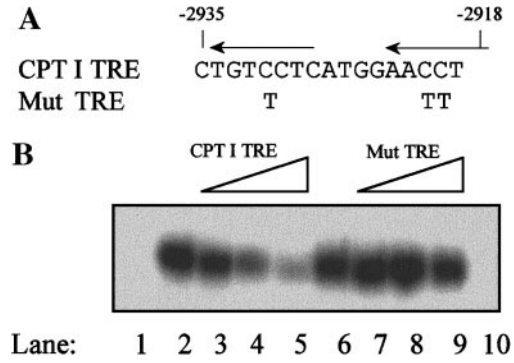

FIG. 5. Electrophoretic mobility shift assay of the TRE probe. (A) The nucleotide sequence between −2935 and −2918 is shown. Arrows over the sequence indicate the motifs and their orientation. The TRE probe (−2935 to −2918) was labeled with [α-^{32}P]dATP using the Klenow fragment of DNA polymerase I and incubated with lysate (lane 1) or 500 ng of TR (lanes 2–9). Lanes 3, 4, and 5 contain, respectively, 12.5-, 25-, and 50-fold molar excess of unlabeled CPT I TRE probe. Lanes 7, 8, and 9 contain, respectively, 12.5-, 25-, and 50-fold molar excess of unlabeled mutated CPT I TRE (Mut TRE) probe (the mutated bases are shown in A). Lane 10 contains Mut TRE probe incubated with 500 ng of TR.

Two copies of the rat L-CPT I TRE (−2935 to −2918) were cloned upstream of the SV-40 promoter of plasmid pGL3 Promoter and this construct, called 2XTRE, was activated by 2.5-fold in the presence of T3 when cotransfected with TR (Fig. 6). Therefore, the sequence located from −2935 to −2918 was able to confer T3 positive responsiveness to the SV-40 promoter. When we tested the plasmid pGL3 promoter for T3 responsiveness we observed that the SV-40 promoter behaved as a typical T3 negatively regulated promoter (17). The reporter activity was stimulated in the presence of TR (data not shown) and repressed by the addition of the

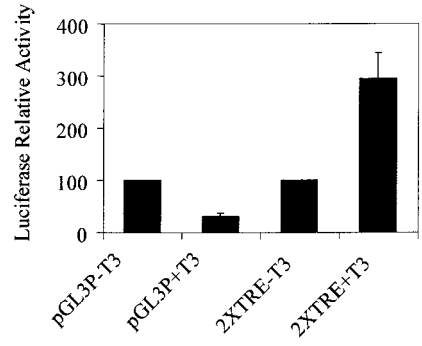

FIG. 6. The CPTI I TRE confers T3 positive responsiveness to SV40 promoter. HepG2 cells were cotransfected with the expression vector for TRα and the reporter plasmid pGL3 Promoter that contains the luciferase gene under the control of the SV40 promoter or the plasmid 2XTRE that contains two copies of the CPT I TRE cloned into pGL3 Promoter. Cells were incubated with or without 1 μM T3 for 42 h. Average values of renilla normalized luciferase activity (means ± SD), from three independent transfections with two plates each, are expressed as relative luciferase activity in which the activity of each construct in the absence of T3 was calculated as 100%.

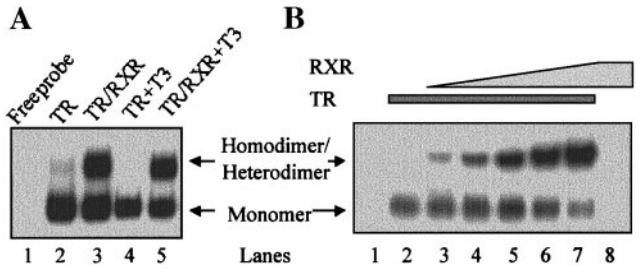

FIG. 7. Electrophoretic mobility shift assay of the TRE probe with TR and RXR. (A) The labeled TRE probe was incubated with lysate (lane 1), 500 ng of TR (lane 2) or 500 ng of TR and 2 μl of *in vitro* synthesized RXR (lane 3). Lanes 4 and 5 are identical to lanes 2 and 3 but in presence of 1 μM T3. (B) Competition experiments with labeled TRE incubated with lysate (lane 1), 500 ng of TR (lane 2) or 500 ng of TR and 0.5, 1, 2, 4, or 8 μl of *in vitro* synthesized RXR (lanes 3–7). Lane 8 contains labeled TRE probe and 8 μl of RXR. Protein concentration was normalized in all lanes by the addition of unprogrammed lysate.

hormone. The presence of the CPT I TRE not only reverts the negative regulation of the SV40 promoter but also is able to confer positive regulation of the SV40 promoter by T3.

To confirm the relevance of the −2935/−2918 sequence in the T3 response we introduced in p4500CPTI three point mutations in the located TRE. Figure 4 shows the point mutations generating the plasmid p4500CPTIM. Figure 3 shows that this reporter construct was not activated by T3.

Characterization of the L-CPTI TRE

Figure 7A shows that cTRα binds to the ^{32}P-labeled CPTI TRE probe, in the absence of RXR, basically as a monomer. A less intense second further retarded complex that corresponds to the homodimer form also appears. The intensity of this retarded band varied through different experiments but was always less intense than the one corresponding to the monomeric form. The presence of T3 avoids the formation of the homodimeric complex. In the presence of RXR the heterodimeric complex appears and shows a mobility similar to the homodimeric complex. We confirmed that this new complex corresponded to the heterodimeric form performing competition assays with increasing amounts of RXR (Fig. 7B). In these conditions the intensity of the lower mobility band increases and the intensity of the binding of the monomeric form decreases, confirming that the lower mobility band corresponds to the heterodimeric complex TR/RXR.

To characterize the TRE we performed EMSA with independent 5′ or 3′ half site-mutated probes. Figure 8 shows that when 3′ half site was mutated TR did not bind the element. Only when RXR was added did a small fraction of TR–RXR complex bind the element. When the 5′ half site was mutated the binding of TR decreased, especially the homodimer binding. The heterodimeric form with RXR still bound to the element but more weakly than to the wild type element. These results indicate that this element is a typical TR in which RXR binds to the 5′ half site and TR to the 3′ one.

DISCUSSION

The L-CPTI promoter contains a typical TRE located from −2935 to −2918 consisting on a DR4. In the absence of a ligand, TR strongly represses transcription and in the presence of a ligand T3 transcription is stimulated. Consequently, a very large dynamic range in the level of transcriptional activity is achieved. TR binds this element as monomer, homodimer and heterodimer with RXR. The TR homodimer seems relatively unstable and T3 prevents its formation probably due to ligand-induced conformational changes that impair double TR occupancy of the response element, whereas such an event does not prevent TR/RXR heterodimers from binding to the TRE. Independent mutations of both half sites indicate that TR binds to the 3′ half site and RXR binds to the 5′ half site. When the 3′ half site is mutated no TR and minor heterodimer is able to bind, indicating that the binding of RXR and the homodimeric form depends on the binding of TR to the

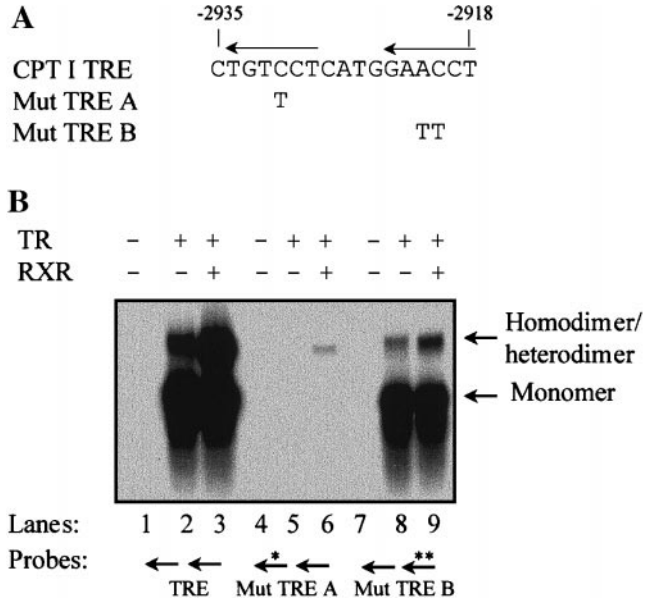

FIG. 8. Characterisation of both half sites by mutagenesis and EMSA assays. (A) The nucleotide sequence between −2935 and −2918 is indicated as CPT I TRE. Mutated probes are shown below. Probe Mut TRE A includes a point mutation in the 3′ half site of the DR4. Probe Mut TRE B includes two point mutations in the 5′ half site of the TRE. (B) EMSA with the CPT I TRE (lanes 1 to 3), Mut TRE A (lanes 4 to 6) and the Mut TRE B (lanes 7 to 9). Lanes 1, 4, and 7 correspond to free probe. In lanes 2, 5, and 8 probes were incubated with 500 ng of TR and in lanes 3, 6, and 9 probes were incubated with 500 ng of TR and 2 μl of *in vitro* synthesised RXR.

3′ half site. Mutation of the 5′ half site makes homodimer and heterodimer formation difficult, indicating that the 5′ half site is recognized by RXR in the heterodimeric complex or by TR in the homodimeric complex.

During development the L-CPT I gene expression is highly regulated in accordance with cellular fatty acid oxidation rates. Immediately after birth the rat is fed with a high-fat low-carbohydrate diet and the capacity for fatty acid oxidation develops rapidly in many peripheral tissues including the liver. The activation of the L-CPT I is a postnatal event that allows ATP production by mitochondrial β-oxidation and ketone bodies formation (25). It is known that the regulation of the L-CPT I expression is dependent upon hormonal and/or nutritional factors rather than on the precise stage of development (4). An important increase of expression of the L-CPT-I is also observed in the fed to starved state in adult rats (2, 3). Both activity and mRNA abundance of L-CPT I are known to increase in hyperthyroidism (5).

Recently, the static view of thyroid hormones being maintained at a basal level just to keep the metabolic machinery working in the proper rate has changed. Thyroid hormones have an important role in postnatal mitochondrial differentiation and are able to promote a differential response in gene activation for those nuclear genes coding for mitochondrial proteins (26). Several genes, such as the β-F1-ATPase (27) or the MCAD (28), which are implicated in the fatty acid oxidation or ATP production and increase markedly after birth are regulated transcriptionally by T3. This data, in conjunction with our study, is consistent with recent results supporting a role for thyroid hormone in the postnatal regulation of oxidative enzymes expression in various developing tissues.

In addition to changes in thyroid hormone that occur in development, thyroid hormone levels are subject to major physiologic regulation during the transition from the fed to the starved state. Starvation rapidly suppresses T4 and T3 levels in order to reduce the obligatory use of energy stores (reviewed in 29). As thyroid hormones paradoxically increase both the lipogenic and the lipolytic pathways, the falling in thyroid hormones produced by starvation would probably cause a decrease in both pathways. The suppression of the lipogenic pathway should provide a way of energetical saving. However, in this special situation the expression of L-CPT I is required to obtain energy from fatty acids which are the main energetic substrates and also to allow the production of ketone bodies that will be used by peripheral tissues like the brain. Consequently, during starvation the levels of L-CPT I should not only be maintained but increased. This induction is probably produced by changes in the insulin/glucagon rates and the increase in circulating fatty acids. In fetal hepatocytes peroxisome proliferators increase the mRNA levels of the L-CPT I (4) suggesting that the induction of this gene by fatty acids occurs through PPAR. In the PPARα-null mouse the peroxisome proliferators response is lost (30) but the gene is strongly induced by starvation (31). This induction can be explained by the confluence of other hormones signaling pathways like glucocorticoids or glucagon. All these findings reveal the multihormonal complexity of L-CPT I regulation.

We postulate that the postnatal induction of the L-CPT I is regulated by T3, probably in coordination with other hormones. This activation would be produced to initiate a program of mitochondria maturation and to satisfy the energy requirements of the newborn. In adults the basal level of thyroid hormone should set and maintain the basal metabolic rate and control the L-CPT I basal level of expression. During the transition from fed to starved state the T3 levels are suppressed in order to slow down the energetical rate, but the expression of the L-CPT I is induced. This expression is probably caused by other hormones and/or metabolites than T3. How these hormones or metabolites promote the L-CPT I gene transcription remains unknown.

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