A Thyroid Hormone Response Unit Formed between the Promoter and First Intron of the Carnitine Palmitoyltransferase-Io Gene Mediates the Liver-specific Induction by Thyroid Hormone

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Carnitine palmitoyltransferase-I (CPT-I) catalyzes the rate-controlling step of fatty acid oxidation. CPT-I converts long-chain fatty acyl-CoAs to acylcarnitines for translocation across the mitochondrial membrane. The mRNA levels and enzyme activity of the liver isoform, CPT-Io, are greatly increased in the liver of hyperthyroid animals. Thyroid hormone (T3) stimulates CPT-Io transcription far more robustly in the liver than in nonhepatic tissues. We have shown that the thyroid hormone receptor (TR) binds to a thyroid hormone response element (TRE) located in the CPT-Io promoter. In addition, elements in the first intron participate in the T3 induction of CPT-Io gene expression, but the CPT-Io intron alone cannot confer a T3 response. We found that deletion of sequences in the first intron between +653 and +744 decreased the T3 induction of CPT-Io. Upstream stimulatory factor (USF) and CCAAT enhancer binding proteins (C/EBPs) bind to elements within this region, and these factors are required for the T3 response. The binding of TR and C/EBP to the CPT-Io gene in vivo was shown by the chromatin immunoprecipitation assay. We determined that TR can physically interact with USF-1, USF-2, and C/EBPα. Transgenic mice were created that carry CPT-Io-luciferase transgenes with or without the first intron of the CPT-Io gene. In these mouse lines, the first intron is required for T3 induction as well as high levels of hepatic expression. Our data indicate that the T3 stimulates CPT-Io gene expression in the liver through a T3 response unit consisting of the TRE in the promoter and additional factors, C/EBP and USF, bound in the first intron.

Thyroid hormone (T3) has profound effects on various aspects of metabolism and development (1). The effects of T3 are mediated through the thyroid hormone receptor (TR). TR belongs to a class of nuclear receptors that includes the retinoic acid receptor, retinoid X receptor (RXR), vitamin D receptor, and peroxisomal proliferator-activated receptors (2). TR isoforms, TRα and TRβ, are encoded by two separate genes (3). Generally, TR binds as a heterodimer with RXR and regulates transcription by binding to thyroid hormone response elements (TREs) located in the promoters of target genes. The T3 stimulation of gene expression involves the interaction of TR with other transcription factors bound to the promoter as well as the recruitment of coactivators such as SRC-1/p160, CBP/p300, and TRAP220/DRIP205/PBP to the liganded receptor (3).

Carnitine palmitoyltransferase-I (CPT-I) is a rate-controlling enzyme in the fatty acid oxidation pathway (4). CPT-I, which is located on the outer mitochondrial membrane, transfers the fatty-acyl moiety from acyl-CoA to carnitine (5). The acylcarnitine is transported across the mitochondrial inner membrane by carnitine acylcarnitine translocase and re-esterified to acyl-CoA by CPT-II (4, 5). Two isoforms of CPT-I have been identified: the “liver” isoform (CPT-Io) and the “muscle” isoform (CPT-Im). We have shown that expression of the CPT-Io gene in the liver is elevated in hyperthyroidism, fasting, and diabetes (6, 7). T3 stimulates fatty acid oxidation in the liver. T3 up-regulates CPT-I α mRNA levels 40-fold in the livers of hyperthyroid rats compared with hypothyroid rats (6). This increase in mRNA is accompanied by an elevation of CPT-Io enzyme activity (6). The α-isoform is induced in the hearts of fasted or diabetic rats. However, CPT-Io gene expression is increased far more in the liver than in the heart of hyperthyroid animals (8).

We have cloned and characterized the promoter of the CPT-Io gene (9, 10). The transcriptional start site of the CPT-Io gene is denoted +1, and the 5′-flanking DNA has been analyzed to nucleotide −6839. Exon 1 contains nucleotides +1 through +27, and exon 2 begins at nucleotide +1201. The CPT-Io TRE consists of a direct repeat separated by four nucleotides (DR4) (11). The TRE is located in the promoter of the CPT-Io gene between nucleotides −2938 and −2923. Mutation of this DR4 motif results in the complete loss of T3 responsive-
ness (11, 12). Interestingly, the first intron of the CPT-Ia gene is also necessary for full T3 induction (11). Removal of the first intron reduces the T3 induction by 80%. Induction of CPT-I-luciferase by T3 is more robust in HepG2 hepatoma cells compared with L6 myoblasts and cardiac myocytes. The first intron is required for the induction in transfected HepG2 hepatoma cells and not in L6 myoblasts and cardiac myocytes, suggesting that the intron contributes to the enhanced T3 induction in the liver (11). The goal of the present study was to examine more extensively the role of the first intron in liver-specific T3 induction of CPT-Ia. We identified regions within the first intron that are necessary to achieve the full T3 induction. Elements within these regions bind proteins that participate in the T3 response, including CCAAT enhancer-binding proteins (C/EBP) and upstream stimulatory factor (USF-1 and USF-2). TR physiologically interacts with USF-1, USF-2, and C/EBP (C/EBP) and upstream stimulatory factor (USF-1 and USF-2). TR response, including CCAAT enhancer-binding proteins (C/EBP) and upstream stimulatory factor (USF-1 and USF-2). We identified regions within the first intron that are necessary to achieve the full T3 induction. Elements within these regions bind proteins that participate in the T3 response, including CCAAT enhancer-binding proteins (C/EBP) and upstream stimulatory factor (USF-1 and USF-2). TR physically interacts with USF-1, USF-2, and C/EBP. Our data suggest that C/EBP is responsible for the liver-specific component of the induction of CPT-Ia by thyroid hormone. Our findings show that the TR in the promoter and C/EBP and USF bound in the first intron comprise a T3 response unit that mediates the liver-selective T3 induction of CPT-Ia.

**EXPERIMENTAL PROCEDURES**

**Electrophoretic Mobility Shift Assays—**CPT-Ia probes for electrophoretic mobility shift assays were created by labeling double-stranded oligonucleotides using Klenow enzyme and [32P]dCTP. The oligonucleotides contained sequences from the first intron and XbaI or MluI overhangs. Double-stranded unlabeled wild-type and mutant oligonucleotides were used as competitors (See Table I for oligonucleotides). Rat liver nuclear extract was prepared as described (13). The protein-DNA binding mixtures contained labeled probe (30,000 cpm) and proteins isolated from rat liver nuclei in 80 mM KCI, 25 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (11). Polyethylene glycol (deoxyxynucleotide) (double-stranded homopolymer) was added to each binding reaction as a nonspecific competitor. Antibodies for TRα1, USF-1, USF-2, Sp1, C/EBPα, C/EBPβ, Oct-1, COUP-TF, and CREB (Santa Cruz) were added to binding reactions for supershift assays. Binding reactions were incubated at room temperature for 20 min and resolved on 5% non-denaturing acrylamide gels (80:1, acrylamide/bisacrylamide) in Tris-glycine running buffer (22 mM Tris and 190 mM glycine). Electrophoresis was carried out at 180 volts for 80 min at 4 °C (11). Sequence analysis of the intron for potential transcription factor binding sites was performed using the TESS transcription factor search.

**Construction of Luciferase Vectors—**Regions of the CPT-Ia promoter and intron 1 were ligated into the pGL3-basic luciferase vector (Promega). Construction of −4495/+19 CPT-Ia-Luc, −4495/+1240 CPT-Ia-Luc, and Δ500a −4495/+1240 has been described previously (11). Internal deletions within intron 1 were constructed by digesting −1653/+1240 Luc−19 and +107 with Smal, followed by ligation of PCR products that contained a Smal restriction site at each end and decreasing lengths of intron 1 sequence. The forward primer corresponded to nucleotides −16/6 (TGGACTAGCTGCTGTACCG). The reverse primers contained BglII restriction sites and corresponded to nucleotides 11653/1166 (5′-GTTCAATGCTCTAGGAAATGGTG/−11652/−999) or (5′-GAGTTTCGACATACAAATCTGTC-3′), +864/+839 (5′-GCGAGGATTCACTGTAAGTGTG-3′), and +803/+778 (5′-GGCATGT- TAGGCGACCTTCGGGTGC-3′). Each PCR reaction contained 1.0 mM MgCl2, 200 μM dNTPs, 1 μg of forward and reverse primers, 2.5 units of AmpliTaq Gold polymerase (Roche Molecular Biochemicals), and 10 ng of −1653/+1240 CPT-Ia-luciferase template. PCR reactions were conducted in a Thermo Cycler 2400 (PerkinElmer Life Sciences). PCR products were digested with MluI and BglII and ligated into the Smal site at +130 in CPT-Ia and BglII at the 3′ end of CPT-Ia in the pGL3-basic luciferase multicloning vector.

Gal4-SV40-luciferase vectors were constructed by digesting Gal4-SV40-luciferase with MluI and BglII and then ligating the Smal (+707) and MluI (+1066) fragments into the Smal and MluI sites within Gal4-SV40-luciferase. Site-directed mutations of protein binding sites were created within the −4495/+1240 CPT-Ia-luciferase vector using the QuikChange site-directed mutagenesis kit (Stratagene). Protein binding sites were mutated to Nhel restriction enzyme sites for identification of mutated clones. The forward and reverse primers used in mutagenesis reactions corresponded to nucleotides +642/+683 (5′-GCCCGAGGTGTTAAAGGCGTT- AGCGGGTGTCAGAGTCTGCTGTC-3′), +656/+700 (5′-GCGGACC- GTGGGTGTCAGAGTCAAAGGCTGGCCTGGAACC-3′), +659/+704 (5′-CAAGTGGTTGTCAGAGTCTGCTGCTACCGCG- CTCGGGACAG-5′) and +650/CAGAAGACACACGGCTGA- AGCACCCGGCTGATGGAA-3′). Mutagenesis reactions were conducted using the +130/+166 Gal4 SV40 vector as a template (11). After introduction of mutations into the +130/+166 Gal4-SV40-luciferase vector, mutated vectors as well as the −4495/+1240 CPT-Ia-luciferase vector were digested with MluI, which digests the CPT-Ia gene at nucleotides +130 and +1066. The mutated +130/+166 fragments were ligated into the +130 and +166 sites within the −4495/+1240 CPT-Ia-luciferase vector. All mutations and confirmed by sequence analysis.

**Transient Transfection of Luciferase Vectors—**CPT-Ia-luciferase constructs were transiently transfected into HepG2 cells by the calcium phosphate method. Transfections were conducted using the luciferase pGL3-basic luciferase multicloning vector along with RSV-TRβ and TK-renilla vectors. Cells were transfected in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% calf serum/5% fetal calf serum and incubated overnight at 37 °C. Following two washes with phosphate-buffered saline, the medium was replaced by DMEM containing no serum. Cells were treated with 100 nM T3 for 24 h. After T3 treatment, cells were washed twice with phosphate-buffered saline and lysed in passive lysis buffer (Promega). Cell lysates were frozen and thawed to facilitate cell lysis. Luciferase assays were conducted on extracts from cells in serum-free media and cells treated with T3. Both luciferase and renilla activity was measured. Protein content in each lysate was determined by Bio-Rad protein assay (Bio-Rad). Luciferase activity was corrected for both protein content and renilla activity to account for cell density and transfection efficiency, respectively. Data are expressed as fold induction of luciferase in cells exposed to T3 as compared with cells that received no hormone (Fig. 3) or relative induction of luciferase in mutant construct as compared with the induction of wild-type −4495/+1240 CPT-Ia-luciferase (Figs. 1, 4, and 6).

GST-pull-down assays GST and GST-C/EBPα were prepared as described by Yin et al. (14). 35S-labeled chicken TRα and human RXRα were expressed using TNT reticulocyte lysates (Promega). GST-pull down assays were conducted as described (14). Bound proteins were eluted and resolved by SDS-PAGE and visualized by storage phosphor imaging. GSTTRα complexes were excised from the gel, digested with trypsin, and sequenced on an Applied Biosystems 470A Protein Sequencer. Protein identity was confirmed by mass spectroscopy (14). Bound proteins were eluted with 2.5 mM glutathione and resolved by SDS-PAGE.

**Chromatin Immunoprecipitation Assays—**Rat hepatocytes were prepared as described previously (15). Chromatin immunoprecipitation assays were conducted with minor modification as described by Jurado et al. (16). PCR reactions were conducted in the iCycler (Bio-Rad) using Thermocycle DNA polymerase (Invitrogen), 1 μl of template, and 1 μl of each primer. The primers used for PCR correspond to nucleotides −642/−650 (5′-CTCGGCGCTCGTCAAGCCTG-3′), −3078/−3056 (5′-GACGACCGGTGACTTTGCTACCCAC-3′), −2802/−2825 (5′-GGAAGCGC- GTTACCTTCCCTCCTAC-3′), +200/220 (5′-GACCAGACCGGGGAAGAGATGGTT-3′), and +750/730 (5′-CCAAAGTTCACTGAGCCGGCTG). Cycling
Interactions between Promoter and Intron Mediate T3 Responsiveness

Production and Characterization of CPT-Io-Luc Transgenic Mouse Lines—Restriction fragments containing −6938/+1240 or −6839/+19 CPT-Io-Luc genes were isolated and injected into the pronuclei of one-cell C57BL/6J × SJL/JF2 hybrid (B6/SJL) mouse zygotes to produce transgenic mice (17, 18). The founders were bred with B6/SJL mates to obtain a second generation of transgenics. Mice containing the luciferase gene constructs were identified by Southern analysis of genomic DNA obtained from the tail (17). Two transgenic mouse lines of each CPT-Io gene construct were selected for use in the T3 experiments based on initial characterization for transgene expression (data not shown). Mice were injected with 0.33 mg/Kg body weight of triiodothyronine (T3) 24 and 48 h prior to sacrifice (6). The mice were sacrificed, and pieces of the liver and heart were homogenized in luciferase assay buffer (Promega). Luciferase assays and protein determinations were conducted as described above.

RESULTS

Identification of Regions within the +199/+707 Region of the First Intron Required for the Full T3 Response—Fig. 1 illustrates the −4495/+1240 CPT-Io-luciferase vector containing 4495 base pairs of the promoter, exon 1, intron 1, and a segment of exon 2. Deletion of nucleotides between +199/+707 in the first intron reduced the T3 induction of CPT-Io by 50% (Fig. 1). To define smaller regions in the intron that contribute to the T3 stimulation, additional internal deletions were made in the context of the −4495/+1240 CPT-Io vector. Removal of the +515/+707 region decreased the T3 response −50%, as did deletion of the 80-base pair +628/+707 region (Fig. 1). Deletion of the +628/+707 region did not alter the basal expression of the CPT-Io-Luc vector (data not shown). These data indicate that sequences within the +628/+707 region are required for a full response to T3.

Binding of Transcription Factors to the +628/+707 Region of the Intron—Our next studies focused on the +628/+707 region of the first intron. Electrophoretic mobility shift assays were conducted using double-stranded oligonucleotides that corresponded to nucleotides +628/+655, +653/+682, and +674/+707 in the CPT-Io gene. Only the +653/+683 and +674/+707 regions bound proteins isolated from rat liver nuclei (Fig. 2, A and B). A consensus E-box motif (CANNTG) was found at nucleotides +659/+664. The E-box motif binds a family of proteins that contain helix-loop-helix and leucine zipper dimerization domains, including c-Myc, sterol regulatory element binding protein (SREBP), and upstream stimulatory factor (19). Supershift assays were conducted using antibodies that recognize Sp1, C/EBPβ, Oct-1, USF-1, and USF-2. Protein binding to the +653/+682 region was completely disrupted by USF-1 and USF-2 antibodies, whereas the other antibodies did not alter the binding of nuclear proteins (Fig. 2A). Western blot analysis confirmed that USF-1 and USF-2 are present in RLNE as well as in HepG2 cells, which was the cell type used in transient transfection experiments (data not shown). Competition analysis revealed that a 100-fold excess of unlabeled wild type +653/+682 oligomer completely competed for nuclear protein binding to the labeled probe, whereas an oligomer that contained a mutation in the E-box motif (Table I, Mut. #1) was unable to compete for protein binding (Fig. 2A). However, competition with unlabeled oligomer that contained a mutation 3' to the E-box (Mut. #2) reduced protein binding as effectively as the wild type oligomer. Our data show that USF-1 and USF-2 bind within intron 1 of CPT-Io at the E-box motif located at +659/+664.

Gel shift mobility assays were conducted with an oligomer representing the +674/+707 region. Several complexes were formed indicating that either multiple proteins or a family of proteins bind at this site (Fig. 2B). We analyzed the binding of nuclear factors to this site by the addition of antibodies to the gel shift assays. Antibodies to C/EBPα and C/EBPβ disrupted the binding of nuclear factors (Fig. 2B). COUP-TF and TR antibodies did not alter protein binding. The +677/+689 region contains an AGGTCA-like motif that might interact with nuclear receptors. However, antibodies to hepatocyte nuclear factor-4, RXR, and peroximal proliferator-activated receptor α did not alter the binding of nuclear proteins (data not shown). Our results indicated that C/EBP proteins could bind to this site. Competition analyses were conducted using unlabeled oligomers that corresponded to the wild type +674/+707 sequence as well as oligomers that contained mutations across the +674/+707 region. A 100-fold excess of wild type oligomer or an oligomer that contained a mutation in nucleotides +695/+700 (Mut. #7) competed effectively for protein binding to the labeled oligomer. However, oligomers that contained mutations in the +677/+682 (Mut. #3 and #5) and +684/+689 (Mut. #4 and #6) sites did not compete for protein binding. Therefore, we conclude that nucleotides within the +677/+689 element are necessary for protein binding within the +674/+707 region.

Contribution of the +628/+707 USF and C/EBP Binding Regions to the T3 Response—To investigate the importance of elements within the +628/+707 region, we removed these sites from the −4495/+1240 CPT-Io-luciferase vector by making Δ+680/+707 and Δ+653/+707 deletions within the intron.
These vectors were transiently transfected into HepG2 cells along with RSV-TRβ. Deletion of the +680/+707 and the +653/+707 regions caused a 35% reduction in the T3 induction (Fig. 3), which was identical to the Δ+628/+707 vector in this set of experiments (data not shown). These data demonstrated that the elements contributing to the T3 response are located in the +680/+707 region.

Role of the +707/+1066 Region in T3 Induction of CPT-Iα—In addition to the +653/+707 region, sequences between +707 and +1066 also participated in the T3 induction (Fig. 4). To assess the contribution of the 3′-end of the intron to the T3 induction of CPT-Iα, serial deletions were created from the second exon in the −4495/+1240 CPT-Iα-luciferase vector. These vectors were cotransfected with RSV-TRβ into HepG2 cells and tested for T3 responsiveness (Fig. 4). The full T3 effect was maintained with deletion of nucleotides +803 to +1240. However, the T3 response decreased upon deletion of the additional nucleotides between +803 and +707. Deletion of the +707/+1240 region modestly decreased basal expression of the gene (data not shown). This stimulation was reduced further upon deletion of the intron to +199. An internal deletion of the +707/+1066 region also diminished the T3 response by 40%. These findings show that sequences between +707 and +803 of intron 1 are involved in the enhancement of T3 induction.

We ligated the +707/+1066 sequences in front of the SV40 promoter driving the luciferase reporter gene. The reporter vector contained a Gal4 binding site. The Gal4-SV40-luciferase vectors were transfected with an expression vector for Gal4-TRβ in which the DNA binding domain of the TRβ was replaced with the DNA binding domain of Gal4 (20). Inclusion of the +707/+1066 region enhanced the T3 response 2.7-fold compared with Gal4-SV40-luciferase (Fig. 5A). Addition of the +707/+810 sequences allowed an additional 6.8-fold induction of the Gal4-SV40-luciferase vector. These results further demonstrate that factors binding in the +707/+810 region enhance the T3 induction of CPT-Iα.

Binding of USF within the +707/+810 Region of the Intron—Our next experiments characterized the binding of nuclear proteins within the +707/+803 region of the first intron. We designed three oligomers that spanned this region of the gene. Using these oligomers in gel shift mobility assays, we found that only the oligomer corresponding to the +700/+744 region bound proteins from rat liver nuclear extract (Fig. 5B). This region contains an E-box element. Antibodies to USF-1 and USF-2 were able to supershift the binding to this site, indicating that USF proteins can bind to this element. Competition analysis using a 100-fold excess of unlabeled wild-type and mutant oligomer that contained an altered USF binding site showed that USF proteins can bind to this element.
+724/+729 confirmed that this E-box motif is necessary for the binding of nuclear proteins (data not shown). Previously, we had identified three sites in the +800/+1066 region that bound nuclear proteins by DNase footprint analysis (11). The +824/+842 element was analyzed in gel shift mobility assays. Several factors were able to bind to this site (Fig. 5B). Addition of antibodies to C/EBPα and -β to the binding reaction disrupted the binding of proteins to this element.

To determine which sites in the +653/+850 region are important for the T3 induction, we disrupted each by site-directed mutagenesis. The sites were altered in the context of +4495/+1240 CPT-I-Luc by introducing the mutations that had been...
shown to disrupt binding in gel shift mobility assays. Mutation of either USF binding site and the +677/+689 C/EBP site decreased the T3 induction, strongly suggesting that these factors contributed to the T3 induction (Fig. 6). Disruption of the +827/+842 C/EBP binding site did not alter the T3 induction, indicating that not all C/EBP binding sites contribute to the response of the CPT-I gene to T3. Our data show that USF and C/EBP are accessory factors in the T3 induction of the CPT-I gene.

Thyroid Receptor and C/EBPα/β Interact in Vitro—Using glutathione S-transferase-linked TRα and C/EBPα immobilized on glutathione-conjugated-Sepharose and 35S-labeled USF-1, USF-2, RXRα, and TRα, we determined that TRα can interact with USF-1, USF-2, and C/EBPα (Fig. 7). These interactions occurred in the presence and absence of ligand (Fig. 7, A and B).

To determine the TR motif through which the physical interaction with C/EBPα occurs, we conducted pull-down assays using truncated 35S-labeled TRα proteins and GST-C/EBPα. Removal of the first 50 amino acids had no effect on the interaction between TRα and C/EBPα (Fig. 7C). Further deletion of amino acids through 120 completely abolished binding to C/EBPα. Isolated polypeptides corresponding to amino acids 1–118 and 1–157 interacted with C/EBPα. However, removal of the residues 1–50 diminished its ability to interact with C/EBPα. Our results indicate that the interaction between TRα and C/EBPα occurs through a region of the TR that encompasses the DNA binding domain and is independent of T3. We have also found that TRβ can interact with C/EBPα (data not shown).

Physical interactions between TR and the accessory factors, USF and C/EBP, may contribute to the T3 induction of the CPT-Ia gene.

In Vivo Binding of TR and C/EBP to the CPT-Ia Gene—Previously, we showed that TR binds to the CPT-Ia TRE in vitro (11). To investigate if such binding occurs in vivo we conducted chromatim immunoprecipitation (ChIP) assays. Rat hepatocytes were treated with 1% formaldehyde to cross-link DNA and proteins. Immunoprecipitations were performed using an antibody that recognized both the TRα and -β isoforms as well as antibodies to C/EBPα and C/EBPβ. IgG was used as a control in these experiments. PCR reactions were conducted using primer sets that corresponded to nucleotides −6473/−6450 and −6076/−6099, −3079/−3056 and −2802/−2825 within the CPT-Ia promoter as well as +200/+220 and +750/+730 within intron 1. The promoter primers −3079/−3056 and −2802/−2825 encompassed the CPT-Ia TRE, which is located at nucleotides −2938/−2923. Antibodies to the TR, C/EBPα, and C/EBPβ immunoprecipitated sequences in the promoter and intron 1 (Fig. 8). IgG failed to pull down promoter or intron sequence. The −6473/−6450 and −6076/−6099 primers, which were our upstream controls, produced no PCR product in our experiments. Our results show that TR and C/EBP interact with sequences within intron 1 of the CPT-Ia gene and the promoter at the TRE region.

Regulation of CPT-Ia-Luciferase Genes in Transgenic Mice—To test whether the CPT-Ia intron was important for the T3 response in vivo, we created transgenic mice that expressed either the −6839/+1240 or −6839/+19 CPT-Ia-Luc transgenes. We initially characterized five independent transgenic lines with the 6839/+1240 CPT-Ia-Luc transgenes and two lines with the −6839/+19 CPT-Ia-Luc transgenes for liver-
specific expression of luciferase (data not shown). From the initial seven transgenic lines, we tested two independent transgenic mouse lines expressing each luciferase reporter gene for the present studies. Several important observations were made regarding the regulation of the CPT-Iα gene using these mice. First, the expression of the −6839/+1240 CPT-Iα-Luc gene is at least 100-fold higher than the expression of −6839/+19 CPT-Iα-Luc transgenes that do not contain the intron. Second, both of the lines expressing CPT-Iα-Luc genes containing the intron responded to T3. Line one was induced 3-fold, whereas line two was increased 9-fold (Fig. 9). However, the expression of the −6839/+19 gene was so low in lines 3 and 4 that the extent of the T3 induction was difficult to evaluate. These data are consistent with the concept that sequences within the intron are vital for hepatic expression of the CPT-Iα gene in vivo.

DISCUSSION

In this study, we have examined the mechanisms by which T3 induces the CPT-Iα gene. We show that the first intron is required for the basal expression and T3 induction of CPT-Iα in the liver. We determined that the CPT-Iα gene contains a T3 response unit consisting of a TRE at nucleotides −2938/−2923 in the promoter, USF binding sites at +659/+664 and +724/+729, and a C/EBP binding site at 677/+689. Each of these binding sites is independently required to obtain a full T3 response. The importance of accessory factors in the hormonal regulation of gene expression is becoming increasingly apparent (21). Accessory factors may contribute to the control of gene expression by modulating the actions of liganded receptors, recruiting coactivators to the promoter, and in the case of the CPT-Iα gene enhancing the T3 induction of CPT-Iα in the liver.

The first component of the T3 response unit is the CPT-Iα-TRE. We have found that this TRE contains a DR4 motif which binds purified TR-RXR heterodimers (11). In the current studies, we have used the ChIP assay to show that TRβ is associated with the CPT-Iα gene in vivo. Utilizing the ChIP assay, Fondell and coworkers have demonstrated that the TR is associated with the TREs of genes in the presence and absence of T3 (22). The addition of ligand leads to association of coactivators with the TR (22). The CPT-Iα-TRE is contained within a DNase I hypersensitive site in the CPT-Iα promoter, indicating that the TRE is in a transcriptionally active region (23). Louet and et al. (23) identified a CREB binding site immediately adjacent to the TRE. In addition, there is a DR1 element located within 100 base pairs of the TRE that binds both peroxisomal proliferator-activated receptor α and HNF-4 (23, 24). Also, they identified a C/EBP binding site within the hypersensitive region (24). In the liver, the TRβ is more highly expressed than TRα (25). To determine whether the TRβ was more effective than TRα in mediating a T3 induction, we cotransfected CPT-Iα-Luc with expression vectors for both TRα and TRβ. Both TR isoforms induced CPT-Iα-Luc to a similar extent, indicating that the greater induction of CPT-Iα by T3 in the liver than in the heart is not due to the preponderance of the TRβ isoform (data not shown).

The second component of the CPT-Iα T3 response unit is C/EBP. Two isoforms of C/EBP (α and β) are expressed in a variety of tissues including liver, lung, adipose, and intestine (26, 27). C/EBPα is not expressed in the heart, and C/EBPβ is expressed only at low levels (26). Both C/EBP isoforms contribute to the regulation of gene expression by a variety of hormones including T3, glucocorticoids, cAMP, and insulin (16, 21, 28, 29). Previously, we have found that both C/EBPα and β participate in the T3 induction of the PEPCK gene (16). It has been shown that C/EBPα is important for the T3 induction of malic enzyme (30). These results raise the possibility that C/EBP proteins are accessory factors for multiple hepatic genes that are stimulated by T3. T3 induces C/EBPα gene expression, and there is a TRE in the promoter of the C/EBPα gene (31). C/EBP is essential for the tissue-selective induction of CPT-Iα in the liver. However, we believe that T3 stimulates CPT-Iα in
association with C/EBP proteins that are already bound to the intron of the CPT-Iα gene.

C/EBP proteins have a crucial role in the regulation of gluconeogenesis. C/EBPα null mice die at birth from hypoglycemia and other complications (32). The C/EBPβ null mice have a complicated phenotype in which 50% die shortly after birth and the remaining mice survive to adulthood (33). Our data indicates that C/EBP proteins regulate some aspects of CPT-Iα gene expression and suggest that as in gluconeogenesis C/EBPs may contribute to the regulation of hepatic ketogenesis because CPT-Iα is a rate-controlling step in this process.

The third component of the CPT-Iα T3 response unit is USF-1 and USF-2. These factors are expressed ubiquitously (35). F. B. Hilkert and associates (34) reported that CUP-1 and E-box-binding proteins enhance the T3 responsiveness of the malic enzyme gene in avian hepatocytes. However, the authors did not find that USF proteins were binding to the E-box in the malic enzyme gene. Previous groups have identified USF as an important component of glucose response complexes in the liver, together with the DNA-binding domain of TR. We also show by ChIP assay that TR interacts with sequences within intron 1, which suggests that the DNA loops so that the TR can interact with USF and C/EBP in the intron. Previously, we have ligated a Gal4 site in front of several C/EBP binding sites. The inclusion of C/EBP binding sites enhanced the induction by T3 and a Gal4-TRβ protein that does not have the TR DNA binding domain (16). However, these observations do not rule out direct physical interactions between the TR and C/EBP. We do not know if multiple USF sites can potentiate T3-mediated transcriptional induction out of the context of the CPT-Iα gene.

It is possible that coactivator proteins are involved in the interactions between the accessory factors and the TRβ. Recent studies have shown that there is a sequential order of coactivator recruitment to the liganded TR (21). First SRC-1 and CBP/p300 are recruited to the liganded receptor followed by the recruitment of the TRAP/mediator complex. The accessory factors may help to recruit or stabilize the coactivators that are associated with the CPT-Iα gene. C/EBPβ interacts with p300 through its amino terminus and the E1A binding region of p300 (45). We have found that overexpression of CBP, the functional homologue of p300, modestly increases the basal expression of the CPT-Iα gene (data not shown). We have recently reported that CBP enhances the T3 induction of the PEPCK gene (16). In addition, we have observed weak physical interactions between C/EBPα and CBP, although these interactions might be stabilized in the context of the CPT-Iα gene (16). Furthermore, hepatocyte nuclear factor-4 can interact with SRC-1, so other proteins adjacent to the CPT-Iα-TRE as well as those in the intron may assist in the recruitment of coactivators (46).

Our studies have established the necessity of intron 1 in the transcriptional regulation of the CPT-Iα gene. The present
work has defined a unique regulatory arrangement for thyroid hormone involving cooperation between transcription factors in the intron and promoter. The interaction of TR with sequences in the intron and its ability to physically interact with C/EBP and USF present a novel model for cooperation in gene induction between nuclear receptors and accessory factors. Coactivators may also participate by providing a physical link between the TR in the promoter and accessory factors in intron 1. Future studies will investigate the role of coactivators in this regulation.

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A Thyroid Hormone Response Unit Formed between the Promoter and First Intron of the Carnitine Palmitoyltransferase-I α Gene Mediates the Liver-specific Induction by Thyroid Hormone

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