Carnitine palmitoyltransferase I (CPT-I) catalyzes the transfer of long chain fatty acyl groups from CoA to carnitine for translocation across the mitochondrial inner membrane. CPT-Ia is a key regulatory enzyme in the oxidation of fatty acids in the liver. CPT-Ia is expressed in all tissues except skeletal muscle and adipose tissue, which express CPT-Iβ. Expression of CPT-Ia mRNA and enzyme activity are elevated in the liver in hyperthyroidism, fasting, and diabetes. CPT-Ia mRNA abundance is increased 40-fold in the liver of hyperthyroid compared with hypothyroid rats. Here, we examine the mechanisms by which thyroid hormone (T3) stimulates CPT-Ia gene expression. Four potential T3 response elements (TRE), which contain direct repeats separated by four nucleotides, are located 3000–4000 base pairs 5′ to the start site of transcription in the CPT-Ia gene. However, only one of these elements functions as a TRE. This TRE binds the T3 receptor as well as other nuclear proteins. Surprisingly, the first intron of the CPT-Ia gene is required for the T3 induction of CPT-Ia expression, but this region of the gene does not contain a TRE. In addition, we show that CPT-Ia is induced by T3 in cell lines of hepatic origin but not in nonhepatic cell lines.

A rate-controlling step in the pathway of mitochondrial fatty acid oxidation is the formation of acylcarnitines that are required for the transport of long chain fatty acids into the mitochondria (1). This reaction is catalyzed by carnitine palmitoyltransferase I (CPT-I)1 located in the outer mitochondrial membrane. CPT-I transfers the acyl moiety from acyl-CoA to carnitine (1). Acylcarnitine is translocated across the inner mitochondrial membrane in exchange for carnitine by carnitine acylcarnitine translocase (2) and then re-esterified with CoA by the inner mitochondrial membrane CPT (1). CPT-Ia is inhibited by malonyl-CoA, which is a substrate for fatty acid synthesis (3). Insulin increases the sensitivity to inhibition by malonyl-CoA (4). Two isoforms of CPT-I have been identified (5, 6). The cDNA for the “liver” isoform (CPT-Ia) was cloned first from a rat liver cDNA library (5), and the “muscle” isoform (CPT-Iβ) was subsequently cloned from rat brown adipose tissue and rat heart libraries (6, 7). The cDNAs for the human isoforms of CPT-Ia and CPT-Iβ have been described (8, 9). The rat α and β isoforms share 63% identity at the amino acid level (1). The liver isoform is present in all tissues except skeletal muscle and adipose tissue (1).

Thyroid hormone (T3) has profound effects on metabolism and fatty acid oxidation in the liver (10). The oxidation of long chain fatty acids is increased in the hyperthyroid state. We found that CPT-Ia activity, sensitivity to malonyl-CoA, and mRNA levels were altered by T3 (11). In hyperthyroid rats, there was a 5-fold increase in CPT-Ia mRNA abundance in the liver over euthyroid rats. CPT-Ia mRNA levels in the liver decreased 80% in hypothyroidism (11). CPT-Ia enzyme activity was increased in hyperthyroid and decreased in hypothyroid rats (11). The sensitivity of CPT-Ia to malonyl-CoA inhibition was greatly increased in hypothyroidism. CPT-Ia expression in the liver is altered by diet and during development. Oxidation of long chain fatty acids is increased in response to high fat diets and fasting, as well as in disease states such as diabetes (1, 12). Glucagon and long chain fatty acids stimulate whereas insulin inhibits CPT-Ia transcription (4, 13). During fetal development, CPT-Ia is not expressed in the liver, but transcription of the gene is induced at birth (14). Therefore, CPT-Ia gene expression is controlled by hormonal and nutritional factors. The effects of T3 are mediated through the binding of the liganded thyroid hormone receptor (TR) to T3 response elements (TRE) in the promoters of genes (15, 16). The consensus TRE contains a direct repeat of the AGGTCA motif separated by 4 nucleotides (DR4). Small variations in the AGGTCA motifs will affect the affinity of the TR for the DR4 motif. In addition, the TR can bind to single repeats and repeated motifs separated by various spacing (17, 18). The TR binds to DNA primarily as a heterodimer with the retinoid X receptor (RXR) (19). On DR4 motifs, RXR binds to the 5′ motif, whereas the TR binds to the 3′ repeat (19). There are two isoforms of the TR: TRα and β (15). The β isoform of the TR appears to mediate many of the effects of T3 in the liver. The T3 induction of several hepatic genes including S14 and malic enzyme is blunted in TRβ knockout mice (20).

Previously, we cloned the promoter of the CPT-Ia gene (21). The CPT-Ia promoter is TATA-less, and basal expression is driven by Sp1 and NF-Y (22). Here, we wished to examine the mechanisms by which T3 stimulates CPT-Ia gene expression. Our results demonstrate that there is a TRE in the promoter of the CPT-Ia gene. However, T3 induction is dependent on sequences within the first intron.
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**EXPERIMENTAL PROCEDURES**

**DNase I Footprint Analysis**—The probe used for DNase I footprint analysis of the CPT-Ia TRE was generated by PCR amplification of sequence between nucleotides −3143 and −2684 in the CPT-Ia promoter. The sequence of the CPT-Ia promoter, first exon and first intron has been entered into GenBankTM under accession number AF020776 (21). The PCR reaction contained the forward primer 5’-TTGAGCAGCTGCC-3’ and the reverse primer 5’-TGGACCTGCCC-3’. The reaction was performed using 1 μg of M13 single stranded DNA, 1 μCi of [32P]dATP (3000 Ci/mmol; DuPont-New England Nuclear), 100 μM dNTPs, 1.5 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl (pH 8.5), 0.01% gelatin, 10% DMSO, 0.05% (w/v) SDS, and 5 units of Taq DNA polymerase (Promega, Madison, WI). The reactions were performed under standard PCR conditions (22): 1 cycle at 95°C for 1 min, 30 cycles of 1 min at 95°C and 1 min at 72°C, with a final extension of 10 min at 72°C. The labeled PCR product was purified by agarose gel electrophoresis.

**Construction of Luciferase Vectors**—The luciferase reporter vector pGL3-Basic (Promega, Madison, WI) was digested with XhoI and partially digested with SacI to remove the −1653/+1240 CPT-Ia fragment that was ligated into pGL3-basic at XhoI/SacI site to create −1653/+1240 CPT-Ia-Luc. The −4495/+1240 CPT-Ia-Luc vector was constructed by ligating nucleotides −1653/+1240 into EcoRI/BglII of −4495/+19 CPT-Ia-Luc. The additional 5’ region of the promoter (nucleotides −6839/−4495) was initially isolated by PCR and then inserted into the PBS II KS+™ multiple cloning site at SalI/EcoRI. The −6839/+1653 CPT-Ia-Luc vector was made by ligating the −6839/+1653 fragment from PBS and ligating into KpnI/EcoRI of −1653/+1240 CPT-Ia-Luc. The −4384/+1240 CPT-Ia-Luc vector was created by digesting −4495/+1240 CPT-Ia-Luc with Smal and religating the vector. Similarly, the Mlu-Mlu region of the intron was removed (ΔMlu −4495/−19) from ΔMlu digestion and religation. The Smal-Mlu region of the first intron (nucleotides +199/+707) was ligated into the XhoI site of the SV40-Luc vector and the Mlu-Mlu region (+130/+1066) was ligated into SV40-Luc at the Mlu site. A Gal4 DNA-binding site corresponding to the sequence 5’-TCGGAGTACTGCTCCTCG-3’ was ligated into the SV40 enhancerless pGL3-promoter vector (Gal4-SV40). The Smal-Mna region of the CPT-Ia first intron, the Mlu-Mna region, and the Sma-Mna region were ligated into the Gal4-SV40 vector at the SacI and BglII sites. Deletions within the Smal-Mna region were created by PCR with the following reverse primer containing a BglII restriction site: 5’-AATAAGATTTCCGAAAAAACCTTCTAGACTC-3’. The forward primers contained a MluI site as follows: MluI(+983) 5’-CAGACCGGCTGTC-CAAGACAGATTATTTGC-3’ and MluI(+900) 5’-GTGACCGGTCTGATACAACTGACACC-3’. The PCR products of the intron regions and +901/+1066 and +1066 ligated into the MluI and BglII sites of the Gal4-SV40-Luc vector.

**Cell Culture, Transfections, and Luciferase Assays**—Cell cultures were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum and 5% calf serum. Cells were trypsinized and resuspended in culture media at 6–7×10^5 cells/ml. 4 h after cell attachment, the calcium phosphate DNA precipitate was added to each plate and incubated overnight. The cells were washed with phosphate-buffered saline and incubated in 10% charcoal stripped fetal calf serum and 100 nM T3.
for 18 h. Rat L6 myoblasts were transfected under identical conditions as were HepG2 cells.

HepG2 and L6 cells were harvested and luciferase activity was determined as described previously (21). Luciferase activity was measured in a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA). Protein concentration of the cell lysate was measured with a colorimetric assay using Bio-Rad protein dye reagent. Luciferase data are expressed as luciferase activity corrected for protein concentration in the cell lysate. Each transfection was conducted in duplicate and repeated three to six times.

Primary culture of Percoll-purified neonatal cardiac ventricular myocytes was conducted as described by Bahouth (26). Ventricle samples from 1-day-old rats were minced in buffer containing 0.075% Viocase. Cardiac myocytes were separated from nonmuscle cells by centrifugation through Percoll gradients and were cultured on collagen-coated plates in 68% Dulbecco’s modified Eagle’s medium, 17% medium-199, 10% horse serum, 5% fetal bovine serum. Beating cardiomyocytes were maintained in culture for 5–7 days before transfection. Transient transfections of cardiac myocytes were carried out using the LipofectAMINE Plus system from Life Technologies, Inc. using the manufacturer’s recommendations. Myocytes were harvested, and luciferase activity was determined for firefly luciferase and Renilla luciferase (transfection control) using the Dual luciferase assay system (Promega).

Sequencing—DNA was sequenced with the Dye terminator cycle sequencing system (PerkinElmer Life Sciences) and analyzed at the Molecular Resource Center of the University of Tennessee. Additional sequencing was conducted at the Center for Biotechnology, St. Jude Children’s Research Hospital (Memphis, TN).

RESULTS

Our initial experiments were designed to define broad regions of the CPT-Iα gene that were involved in the stimulation of expression by T3. To conduct these experiments, the CPT-Iα promoter was ligated in front of the luciferase reporter gene (CPT-Iα-Luc). Previously, we had cloned the promoter of the CPT-Iα gene and characterized the proximal promoter of the gene (21). The −6839/+1240 CPT-Iα-Luc vector contains the full-length promoter, the first exon, the first intron and a portion of the second exon (Fig. 1A). Exon 1 is only 27 nucleotides long. The −6839/+19 CPT-Iα-Luc vector contains the CPT-Iα promoter and a portion of exon 1. The −6839/+1240 CPT-Iα-Luc vector was transiently transfected into HepG2 cells in the presence or absence of T3. Addition of T3 stimulated transcription of −6839/+1240 CPT-Iα-Luc 5.5 ± 0.1-fold (Fig. 1B). The −4495/+1240 CPT-Iα-Luc vector maintained T3 responsiveness with a 5.8 ± 0.6-fold induction by T3. Deletion of the promoter between −4495 and −1653 resulted in a loss of T3 responsiveness. Also, deletion of the first intron as in the −4495/+19 greatly reduced T3 stimulation from a 5.5 ± 0.1-fold to a 2.0 ± 0.1-fold induction. These observations indicate that two widely separated elements are required for the T3 induction of the CPT-Iα gene. As a control, one or two copies of an idealized TRE, which consists of DR4, were ligated in front of the enhancerless SV40 promoter driving the luciferase reporter gene. The vector containing two copies of DR4 was stimulated 5.7 ± 0.7-fold by T3.

To further analyze the T3 induction of CPT-Iα gene expression, we identified the TRE in the CPT-Iα gene. Initially, serial deletions were created in the promoter of the CPT-Iα gene

![Diagram](Image)
between −4495 and −1653 to define a narrow region required for T3 responsiveness. The serial deletions removed approximately 50–400-base pair fragments from the 5’ end of the promoter in the −4495/+1240 CPT-Iα-Luc vector (Fig. 2). The average fold induction by T3 was lost with removal of the nucleotides between −2971 and −2912, suggesting that this region contained a TRE.

Examination of the sequences between −4495 and −1653 for potential TREs revealed the presence of four DR4-like motifs that are shown in Table I. Each of these elements appeared to be good candidates for TRβ-RXRα binding and raised the possibility that multiple TREs were involved in T3 action. However, deletion of the three most 5’ motifs had no effect on T3 responsiveness. The fourth DR4 fell between nucleotides −2971 and −2912 in the T3-responsive region, suggesting that this element was most likely the functional TRE.

To specifically determine which of these elements was involved in the T3 induction of CPT-Iα transcription, each of the DR4 motifs was altered by site-directed mutagenesis in the context of the −4495/+1240 CPT-Iα-Luc vector (Fig. 3). The specific mutations that were introduced are shown in Table I. In agreement with the data from our serial deletions, disruption of the three most 5’ elements did not diminish the 5.8 ± 0.6-fold T3 induction seen with the wild type vector. However, mutation of the most 3’ DR4 (−2938 Mut) resulted in a complete loss of T3 responsiveness (Fig. 3). These data demonstrate that T3 stimulates CPT-Iα gene expression through a single TRE and not by synergy between several weak TREs. It is not clear why this DR4 motif serves as the TRE as opposed to the other DR4-like sequences.

Next, we determined whether TRβ could bind to the TRE in the CPT-Iα promoter. Because the TR binds with higher affinity as a heterodimer with the RXR, TRβ and RXRα were prepared by overexpression in an E. coli expression system. These proteins contained a histidine tag on the amino terminus and were purified by nickel affinity chromatography (24). Both DNase I footprinting and gel shift mobility assays were used to examine binding to the TRE. A DNase I footprinting probe was generated from nucleotides −3143 to −2684. The probe was incubated with recombinant TRβ and RXRα as described under “Experimental Procedures.” Nucleotides −2973 to −2923, which contain the most 3’ DR4 motif in the CPT-Iα promoter, bound the TRβ-RXRα heterodimer and were protected from DNase I digestion (Fig. 4). To further analyze the binding of the TRβ to the CPT-Iα TRE, electrophoretic mobility shift assays were conducted with a labeled double-stranded oligonucleotide containing the TRE. As shown in Fig. 5A, the probe was incubated with TRβ and RXRα. Double-stranded oligonucleotides containing various mutations in the TRE were used in competition studies. The sequences of these oligomers are shown in Table II. The shifted DNA-protein complex was competed with the unlabeled CPT-Iα TRE oligomer. An oligomer with a mutation in both half-sites of the DR4 (Mut1) was unable to compete for TRβ-RXRα binding. This mutation was identical to the mutation that was introduced into −4495/+1240 CPT-Iα-Luc and eliminated T3 responsiveness. Similarly, an oligomer containing only a 3’ half-site disruption (Mut2) was unable to compete. A mutation in the 5’-flanking sequence of the CPT-Iα TRE (Mut3) and an oligomer consisting of an idealized TRE (Cons) were both able to compete for protein binding. Additional assays showed no binding of TRβ monomers or homodimers to the CPT-Iα TRE probe (data not shown). These data indicate that TRβ, as a heterodimer with RXRα, can bind to the TRE in the CPT-Iα promoter.

To determine whether the TR in a nuclear extract could bind the CPT-Iα TRE, the binding of proteins isolated from rat liver nuclei (RLNE) to this element was examined using gel shift mobility assays. The CPT-Iα TRE probe was incubated with RLNE, and three DNA-protein complexes were observed (Fig. 5B). Competition analysis was conducted with the same oligomers that were used in Fig. 5A. All three complexes were competed away by a 100-fold excess of wild type probe. The most slowly migrating (top) complex was not competed by the consensus DR4 or the Mut3 oligomer, indicating that this complex does not contain the TR. The competition pattern of the middle complex was similar to that seen with the TRE-TRβ-RXRα complex in Fig. 5A, suggesting that this complex might contain the TR. Binding of the most rapidly migrating (bottom) complex was eliminated by unlabeled oligomers containing mutations in the DR4. To determine whether the middle complex contains the TR, supershift analyses with the TRβ antibody were conducted (Fig. 5C). Addition of the TRβ antibody to the binding reaction disrupted the middle DNA-RLNE complex. Antibody alone produced no shifted band. These data indicate that TRβ-RXRα binds the TRE in the CPT-Iα promoter. In addition, other proteins present in rat liver nuclei can bind this element.

Our data in Fig. 1 indicated that sequences in the first intron were required for the full T3 induction of CPT-Iα gene expression. These data raised the possibility either that there was a second TRE in the first intron or that an accessory factor site in the first intron was required for the T3 stimulation. Our next experiments examined the role of the first intron in the T3 induction of CPT-Iα gene expression. Internal deletions in the first intron were introduced in the context of the −4495/+1240 CPT-Iα-Luc vector. The −4495/+1240 CPT-Iα-Luc vector containing the Sma to Sma (ΔSma) and Mlu to Mlu (ΔMlu) deletions within the first intron were tested for T3 responsiveness (Fig. 6A). The ΔSma deletion that removes nucleotides (+199/+707) reduced the T3 induction to 2.5-fold, and the ΔMlu vector, which eliminates nucleotides (+130/+1066), was stimulated only 1.4-fold by T3. These data suggest that at least two elements in the intron contribute to the T3 response.

To determine whether the intron contained a TRE, the Sma to Sma (+199/+707) or Mlu to Mlu (+130/+1066) regions were...
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Nucleotides containing the DR4 motifs in the CPT-Iα promoter are indicated in the left column. In the central column, the AGGTCA-like motifs of the DR4 are in capital letters and are underlined. The intervening letters are in lowercase. The sequences are from the top strand. In the right column, mutated nucleotides are indicated by bold type. These mutations were introduced into the CPT-Iα-Luc vectors shown in Fig. 3.

| Promoter location | Potential TREs (DR4) | Mutations introduced |
|-------------------|---------------------|---------------------|
| −4251/−4228 (−4251 Mut) | TGCCCTcctcTGAACcggggga | TGGACATcctcTGAACcggggga |
| −3925/−3902 (−3925 Mut) | AGGTAGTggcAGGACAcAGGAGT | AGGTAGTggcAGGACAcAGGAGT |
| −3225/−3204 (−3225 Mut) | AGGTTTgcacAGGACAcAGGAGG | TGATTcgcacAGGACAcAGGAGG |
| −2938/−2923 (−2923 Mut) | gcgTGTCCTcgcGGACcgtg | gcgTGTCCTcgcGGACcgtg |

Fig. 3. Identification of a T3-responsive element in the CPT-Iα promoter. Specific mutations shown in Table I were introduced into the promoter of the −4495/+1240 CPT-Iα-Luc vector. The positions of four potential TREs corresponding to DR4 motifs are indicated with a shaded box. The most 5’ nucleotide of the mutated DR4 motif is delineated by the nucleotide number followed by Mut. The × indicates a disrupted DR4 motif. The luciferase vectors were transiently transfected into HepG2 cells and exposed to T3 as described in the legend to Fig. 1. Each transfection was conducted in duplicate and repeated four to six times. The data are presented as fold induction by T3 ± S.E.

Fig. 4. Binding of thyroid hormone receptor to the T3 response element. A DNase I footprint probe was constructed from nucleotides −3143 to −2697. The probe contains the most 3’ DR4 motif in the CPT-Iα promoter. The probe was labeled on the top strand with [γ-32P]ATP and incubated with TRβ and RXRα before being subjected to DNase I digestion. The G-ladder is shown in the left lane. The protected sequence is indicated by a box to the right of the footprint. The sequence of the footprinted region is indicated.

The CPT-TRE is placed in the context of the CPT-Iα gene, elements within the first intron are essential for the T3 effect.

We tested whether elements within the intron could enhance T3 responsiveness outside the context of the CPT-Iα gene. A Gal4 site was ligated in front of the SV40-luciferase (Gal4-SV40-Luc) vectors containing portions of the first intron. These vectors were cotransfected with a mammalian expression vector for TRβ in which the TRβ DNA-binding domain was replaced by the Gal4 DNA-binding domain (Gal4-TRβ) (27). The Gal4-TRβ vector was used to eliminate the possibility that the TRβ was binding to a TRE in the intron.

T3 stimulated the Gal4-SV40-Luc vector 7.8 ± 0.8-fold (Fig. 7A). The T3 induction of the intron vectors were compared with Gal4-SV40-Luc, which was normalized to 1.0. Addition of the Sma to Sma (−707/+1066) region to Gal4-SV40-Luc did not further stimulate the T3 responsiveness. Gal4-SV40-Luc vectors containing the Mlu to Mlu (−130/+1066) and the Sma to Mlu (−707/+1066) regions were stimulated an additional 3.1 ± 1.0 and 3.4 ± 0.3-fold by T3, respectively (Fig. 7A). The Mlu to Mlu vector without the Gal4 DNA-binding site was not stimulated by T3 when cotransfected with the Gal4-TRβ expression vector (data not shown). These data indicate that an accessory factor site between the +707 and +1066 region of the first intron enhances the T3 induction.

To further characterize the involvement of this 360-base pair intron region, we first conducted DNase I footprint analysis. A probe containing nucleotides +707 through +1066 (Sma to Mlu) was labeled on the sense strand and incubated with proteins isolated from rat liver nuclei. Four protein-binding regions were protected from DNase I digestion (Fig. 8). Next, we further defined regions within the Sma to Mlu portion of the intron that were involved in the T3 induction. The positions of the DNase I protected sites within the Sma to Mlu region are indicated by shaded ovals in Fig. 7B. Regions containing each protein-binding site were deleted in the Gal4-SV40 Luc vector as described previously for Fig. 7A. The 3.4-fold enhancement of T3 responsiveness was completely lost upon deletion of nucleotides +707 to +901 (Fig. 7B). However, the +707 to +901 region alone was not able to enhance the T3
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The regulation of gene expression by T3 has profound effects on hepatic metabolism. We show here that T3 regulates the transcription of a gene encoding a key enzyme in long chain fatty acid β-oxidation. The promoter of the CPT-Iα gene contains a TRE located 3000 base pairs upstream of the transcriptional start site. DNA-protein binding studies reveal that TRβ binds this element as a heterodimer with RXRα. To achieve the maximum stimulation by T3, the first intron of the gene is required. Thus, our results indicate that TRβ binds a TRE in the CPT-Iα 5′-flanking region and works in concert with accessory factor(s) bound to sequences in the first intron.

The CPT-Iα TRE loosely conforms to the consensus DR4 motif (AGGTCAxxxxAGGTCA), which is found in several genes including the myosin heavy chain α and malic enzyme genes (28, 29). The bottom strand DNA sequence of the CPT-Iα TRE is AGGTTCatgAGGACA, differing from the consensus DR4 by three nucleotides. Three additional DR4-like motifs are located upstream of the TRE and contain sequences suggesting that TRβ would bind. Interestingly, these elements did not contribute to the T3 responsiveness. Although it is unclear as to why these elements do not function as TREs, subtle sequence alterations may modulate their affinity for TR. Another possibility is that other factors may bind these elements and preclude the binding of TR. Katz and Koenig (17) reported that the optimal DNA-binding half-site for the TR is the octameric half-site TAAAGGCTA. The 3′-half-site of the CPT-Iα TRE conforms to the optimal half-site by 6 of 8 nucleotides. Spacing of half-sites and variations of the nucleotides within the sequence of this octomer can alter TR binding as well as change ligand responsiveness of the receptor and its ability to transactivate (17, 18, 30).

Variability of the TRE sequence plays a role in transcriptional regulation through several mechanisms. Alterations in the flanking sequence of the AGGTCA half-site can increase the requirement for RXRα heterodimerization with TR for T3-mediated gene expression to occur (31). Using gel shift assays, we did not observe monomeric or homodimeric binding of TRβ to the CPT-Iα TRE (data not shown). Coactivator involvement in TR-mediated responses may also be dictated by the sequence of the TRE. For instance, SRC-1 greatly stimulates TR-mediated gene expression from optimal TREs but plays a lesser role at suboptimal sites where TR/RXRα heterodimers bind (31). The architecture of the TRE can affect the release of TR-associated corepressors (32). In addition, divergence from the consensus DR4 may allow other proteins to bind these sites. Our studies reveal that proteins from rat liver nuclei other than the TRβ can bind the CPT-Iα TRE. Supershift assays conducted with the CPT-Iα TRE, nuclear proteins from rat liver and an antibody to CUP-TF indicated that CUP-TF did not bind this element (data not shown).

Although the regulation of gene expression by T3 requires the binding of TR to the gene, other transcription factors, called accessory factors, modulate the effect of T3. The participation of accessory factors in hormone responsiveness occurs in many genes and with numerous hormones. We have shown that the T3 stimulation of the PEPCK gene requires the TR and the CCAAT enhancer-binding protein (C/EBP) (24). Cotransfection of the −4495/+1240 CPT-Iα-Luc vector with a dominant negative expression vector for C/EBP did not affect the T3 induction, although basal expression was reduced (data not shown). These results indicate C/EBP is not involved in the T3 regula-

2. G. Cook, unpublished observations.
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Table II
Oligonucleotide sequences of competitors used in gel mobility assays
Abbreviations of the oligomers used in Fig. 5 are listed in the left column in parentheses. The top strand oligonucleotide of the CPT-Iα promoter TRE is provided in the right column. The DR4 motif is underlined, and mutated nucleotides are indicated in bold type. The wild type (WT) competitor corresponds to the TRE present in the CPT-Iα promoter. Mutation 1 (Mut1) contains mutated nucleotides in both the 5' and 3' half-sites. Mutation 2 (Mut2) contains a mutation in the 3' half-site. Nucleotides in the 5' flanking sequence of the DR4 are mutated in mutation 3 (Mut3). The sequence of a consensus DR4 (Cons) is shown. All oligonucleotides were generated with XbaI restriction enzyme overhangs.

| Competitors          | Oligonucleotide sequence (top strand 5'-3') |
|----------------------|--------------------------------------------|
| Wild type CPT-Iα TRE (WT) | CTAGACCTCGCGCTCCTCATGGAACCTGGTGACGT |
| Mutation 1 (Mut1)    | CTAGACCGccccgcgtcctcatggaacctgggacgt |
| Mutation 2 (Mut2)    | CTAGACCcgcgtcctcatggaacctgggacgt |
| Mutation 3 (Mut3)    | CTAGACC CGCGCTCCTCATTGGAACCTGGTGACGT |
| DR4 Consensus (Cons) | CTAGACC CGCGCTCCTCATTGGAACCTGGTGACGT |

Fig. 6. Participation of the first intron in the induction of CPT-Iα by T3. A, deletions were introduced into the first intron in the context of the −4495/+1240 CPT-Iα-Luc vector. The ΔSma deletion removes nucleotides +199 to +707, and the ΔMlu deletion removes nucleotides +130 to +1066. These vectors were transfected into HepG2 cells and assessed for T3 responsiveness as described in Fig. 1. B, regions from the first intron of the CPT-Iα gene were ligated in front of the enhancerless SV40 promoter. These vectors were transfected along with RSV-TRβ into HepG2 cells as described above. C, one or two copies of the TRE were ligated in front of the SV40-Luc vector. These vectors were transfected with 1 μg of RSV-TRβ as described in Fig. 1.

Fig. 7. Contribution of specific intron regions in the T3 induction of CPT-Iα. A, a Gal4 DNA-binding site was introduced in front of the enhancerless SV40-Luc. Either the Sma to Sma (+199/+707), Mlu to Mlu (+130/+1066), or the Sma to Mlu (+707/+1066) regions of the first intron were included in these vectors. These constructs were cotransfected into HepG2 cells with 100 ng of a mammalian expression vector for Gal4-TRβ into HepG2 cells and exposed to T3 as described for Fig. 1. The Gal4-SV40-Luc vector was induced 7.8-fold by T3. The T3 induction of the intron vectors were compared with Gal4-SV40-Luc, which was normalized to 1.0. Each transfection was conducted in duplicate and repeated four to six times. The data are presented as relative induction by T3 ± S.E. B, the positions of the protein-binding sites within the Sma to Mlu region of the first intron are indicated by shaded ovals. Deletions within this region were introduced into the Gal4-SV40-Luc vector and analyzed for T3 induction as described for A. Each transfection was conducted in duplicate and repeated four to six times. The data are presented as relative induction by T3 ± S.E. normalized to the Gal4-SV40-Luc vector.

The function of CPT-Iα gene expression. In the heart, myocyte-specific enhancer factor 2 is involved in the T3 induction of the α-cardiac myosin heavy chain gene (33). In the liver, NF-Y is required for the stimulation of S14 gene transcription by T3 (34). The S14 TREs are located between −2700 and −2500, while the NF-Y binding site is near the start site of transcription (34, 35). The induction of human placental lactogen B (hCS-B) and the NF-Y binding site is near the start site of transcription (34, 36). The induction of rat liver-specific very low density apolipoprotein II (apoVLDLII) gene contributes to a 4-fold increase in estrogen responsiveness (37). T3-responsive elements have been described that are >2,000 base pairs upstream from the transcriptional start site in several genes. Transcription of the β3-adrenergic receptor gene is stimulated by T3 in cardiac ventricular myocytes (41). We have demonstrated that although the TRE of the β3-adrenergic receptor gene is located 3' to the transcriptional start site, the induction by T3 is dependent on promoter elements located more than 2,000 base pairs upstream (41). The NRGN gene in the human brain is regulated by T3 through a TRE located in the first intron of the gene (42). The rat growth hormone gene contains a complex TRE in
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The first intron of the CPT-Iα gene does not contain a TRE, and homology searches have not identified any consensus sequences for TR binding. Experiments with vectors containing internal deletions of the intron have localized two broad regions that are important in T3 responsiveness. At least one of these regions (nucleotides +707 to +1066) can stimulate T3-mediated transcription outside the context of the CPT-Iα promoter. Nucleotides +707 to +1066 were characterized by DNase I footprint analysis, and four protected regions were identified (Fig. 8). Whether any of these elements are involved in T3 responsiveness of the CPT-Iα gene is not known.

CPT-Iα-Luc constructs containing the first intron stimulate T3-mediated gene expression in hepatoma cell lines but not in other cell lines tested. Because the accessory factor appears to enhance T3 responsiveness only in hepatoma cells, it is possible that the accessory factor may be predominantly expressed in the liver. Future studies will not only identify the intron factor involved in T3 induction of CPT-Iα but will also characterize its role in cell type-specific regulation. These experiments will provide further insight into the mechanisms underlying how metabolic disease states such as hyperthyroidism affect fatty acid oxidation.

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