Expression of the rat liver carnitine palmitoyltransferase I (*CPT-Iα*) gene is regulated by Sp1 and nuclear factor Y: chromosomal localization and promoter characterization

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Carnitine palmitoyltransferase (CPT)-I catalyses the transfer of long-chain fatty acids from CoA to carnitine for translocation across the mitochondrial inner membrane. Expression of the 'liver' isoform of the *CPT-I* gene (*CPT-Iα*) is subject to developmental, hormonal and tissue-specific regulation. To understand the basis for control of *CPT-Iα* gene expression, we have characterized the proximal promoter of the *CPT-Iα* gene. Here, we report the sequence of 6839 base pairs of the promoter and the localization of the rat *CPT-Iα* gene to region q43 on chromosome 1. Our studies show that the first 200 base pairs of the promoter are sufficient to drive transcription of the *CPT-Iα* gene. Within this region are two sites that bind both Sp1 and Sp3 transcription factors. In addition, nuclear factor Y (NF-Y) binds the proximal promoter. Mutation at the Sp1 or NF-Y sites severely decreases transcription from the CPT-Iα promoter. Other protein binding sites were identified within the first 200 base pairs of the promoter by DNase I footprinting, and these elements contribute to *CPT-Iα* gene expression. Our studies demonstrate that *CPT-Iα* is a TATA-less gene which utilizes NF-Y and Sp proteins to drive basal expression.

Key words: fatty acid transport, hepatic genes, transcriptional regulation.

INTRODUCTION

The reaction catalysed by carnitine palmitoyltransferase (CPT)-I is a rate-controlling step in the pathway of long-chain fatty acid β-oxidation [1]. CPT-I is located on the mitochondrial outer membrane and catalyses the conversion of acyl-CoA to acylcarnitine. The acylcarnitine molecule is transported into the mitochondria by the carnitine acylcarnitine translocase in exchange for carnitine. CPT-II is located on the mitochondrial inner membrane and reverses the reaction of CPT-I to regenerate acyl-CoA from acylcarnitine.

Two isoforms of CPT-I have been cloned [2,3]. The 'liver' isoform (CPT-Iα) is expressed in most tissues, including liver, kidney, lung and heart [4]. The 'muscle' isoform (CPT-Iβ) is expressed in the heart, skeletal muscle and adipose tissue [2]. CPT-Iα is not expressed in skeletal muscle or brown adipose tissue [1]. In the heart, CPT-Iα is the main isoform expressed before birth, whereas CPT-Iβ is the predominant isoform in the adult heart [5]. Although these isoforms of CPT-I catalyse the same reaction, they have slightly different properties. Both isoforms are inhibited by malonyl-CoA, which is a precursor for fatty-acid synthesis [6]. CPT-Iβ is inherently more sensitive to malonyl-CoA than CPT-Iα. However, the sensitivity of CPT-Iα to malonyl-CoA inhibition is increased by insulin but the sensitivity of CPT-Iβ is not altered [6].

Expression of the *CPT-Iα* gene is regulated by developmental, dietary and hormonal factors. The *CPT-Iα* gene is not expressed in the liver before birth and expression rises following birth [7]. *CPT-Iα* expression is elevated in starved rats or in rats on high fat diets [6,7]. In addition to dietary regulation, *CPT-Iα* transcription is elevated in hyperthyroid animals and streptozotocin-treated diabetic rats [6,8,9]. Long-chain fatty acids and lipid-lowering agents such as clofibrate enhance transcription of the *CPT-Iα* gene [10]. These observations indicate a prominent role for thyroid hormone, fatty acids and insulin in regulating *CPT-Iα* expression.

To further analyse the mechanisms by which *CPT-Iα* expression is regulated, we cloned the CPT-Iα promoter from a rat genomic library [4]. The *CPT-Iα* gene contains two exons before the exon containing the initiating methionine codon [3,4]. Examination of the sequence of the proximal promoter suggested that the *CPT-Iα* gene has a TATA-less promoter. In the present work, characterization of the proximal promoter of the *CPT-Iα* gene is reported. A region spanning 200 nt in the CPT-Iα promoter is responsible for the regulation of basal gene expression. The transcription factors Sp1, Sp3 and nuclear factor Y (NF-Y) bind to sites in the CPT proximal promoter and stimulate transcription. Other binding sites for nuclear proteins were also identified. Our studies demonstrate that *CPT-Iα* is a TATA-less gene and provide a foundation for further analysis of the regulation of *CPT-Iα* expression.

EXPERIMENTAL

Fluorescence *in situ* hybridization (FISH)

Fibroblasts from neonatal rats were cultured and harvested following conventional cytogenic procedures and air-dried slides

Abbreviations used: CPT, carnitine palmitoyltransferase; NF-Y, nuclear factor Y; FISH, fluorescence *in situ* hybridization; CRE, cAMP-responsive element; C/EBP, CCAAT-enhancer binding protein; SV, simian virus.

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The nucleotide sequence data for the promoter of the *CPT-Iα* gene has been submitted to GenBank with accession number AF020776.

were made for FISH analysis. The fibroblasts were denatured for 2 min in 70% (w/v) formamide/2 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium citrate) at 70 °C and dehydrated in ethanol. A plasmid clone containing a 10 kb insert of the *CPT-Iα* gene was labelled with digoxigenin dUTP (Boehringer-Mannheim) by nick translation and used as a probe for FISH. Hybridization conditions were described previously [11]. Hybridization signals were detected with anti-digoxigenin–horseradish peroxidase (Boehringer-Mannheim), cyanine-3 tryamide (NEN Life Science Products, Boston, MA, U.S.A.), and the chromosomes were counterstained with 0.4 ug/ml of DAPI (Sigma) in an anti-fade buffer. The preparations were viewed with an Olympus BX60 epifluorescence microscope and photographed.

DNase-I-footprint analysis

DNase-I footprint probes were prepared by PCR amplification of a region in the CPT-Iα promoter. Forward primer: 5′-CACTCGAGCAGCCAAACAACCTAAAGT-3′, and reverse primer: 5′-TGGGATCCCGAGTCTGTGGACGGCGGCG-3′ were used to generate a fragment from nt −383 to +27. The PCR fragment was cloned into the *Xho*I and *Bam*HI sites of pBluescript II K/S$^{+/-}$. The CPT-Iα–pBS vector was digested with *Bam*HI to generate a probe labelled on the top strand or with *Xho*I to label the bottom strand. The resulting overhang was treated with alkaline phosphatase and labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After digestion with the second restriction enzyme, the probe was purified from a 1% (w/v) agarose gel.

Proteins were isolated from rat liver nuclei as described by Gorski et al. [12]. The radiolabelled probes were incubated with rat liver nuclear proteins and treated with DNase I as described by Park et al. [13]. The reactions were resolved on a denaturing (5% w/v) acrylamide gel in 1 × TBE (89 mM Tris/89 mM borate/2 mM EDTA) running buffer.

Electrophoretic mobility-shift assays

Probes for gel-shift assays were created by labelling double stranded oligonucleotides with Klenow enzyme and [α-^{32}P]dCTP [14]. The upstream Sp1 site will be referred to as Sp1(#2) and the downstream Sp1 site as Sp1(#1). The oligonucleotides corresponded to protein binding sites in the CPT-Iα promoter as follows: Sp1(#1), 5′-CTAGAGCCTCGCCCGCCCCTGCTT-3′; Sp1(#2), 5′-CTAGACAGGCCCCGCCCCGTCCTT-3′; CCAAT box, 5′-CTAGAGCTCAGCCAATCCCCGT-3′; region I, 5′-CTAGACCGGCTGGGCTCAGCCAT-3′; region II, 5′-CTAGATCTTTACTGAACTCCT-3′; region III, 5′-CTAGACTAGGTCTGTAGTTCACAAGCTCGGT-3′. Mutated oligonucleotides of these protein-binding regions were generated as follows (the mutated nucleotides are underlined): Sp1(#1) Mut, 5′-CTAGAGCCTCGCATGCATCTGCTT-3′; CCAAT box Mut, 5′-CTAGAGCTCAGCTGGCCACCCGT-3′; region I Mut, 5′-CTAGACCGGCGAATTCCAGCCAT-3′; region II Mut, 5′-CTAGATCTCAGTACGACTCCT-3′; region III(1) Mut, 5′-CTAGACTGAACGATTAGTTCACAAGCTC-GGT-3′; region III(2) Mut, 5′-CTAGACTAGGTCTGTAG-ACATGGTGCTCGGT-3′. Oligomers for the Sp1 and cAMP-responsive-element (CRE) consensus sites contain the following sequences: Sp1, 5′-ATTCGATCGGGGCGGGGCGAGC-3′ and CRE, 5′- CCCCTTACGTCAGAGG-3′.

The labelled probe (30000 c.p.m.) was combined with proteins isolated from rat liver nuclei in 25 mM Tris/HCl, pH 7.4/80 mM KCl/0.1 mM EDTA/1 mM dithiothreitol and 10% (v/v) glycerol [15]. The non-specific competitor added to each binding reaction was either 1 μg of poly[dI.dC] or 1 μg of a 1:1 (w/w) mixture of poly[dI.dC]/poly[dA.dT] for region 3. In the supershift assays, antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and added to the binding reaction before the addition of the nuclear protein extract. Preimmune serum was added in a control binding reaction. Binding reaction mixtures were incubated at room temperature for 20 min and resolved on a non-denaturing [5% w/v) acrylamide gel (80:1, w/w) acrylamide/bisacrylamide] in 0.5 × Tris/glycine running buffer (22 mM Tris/190 mM glycine) at 4 °C [15].

Construction of luciferase vectors

Nucleotides from −1653 in the promoter to +64 in exon 2, including the first intron, were removed from the original P1 clone by *Eco*RI–*Sal*I digestion. This fragment (−1653/+64 CPT-Iα) was ligated into the pGL3 basic luciferase vector (Promega). The −1653/+19 CPT-Iα–Luc vector was prepared by digesting −1653/+64 CPT-Iα–Luc with *Bss*HI–*Bgl*II and removing the first intron. A 70-bp double-stranded oligomer from nt −52 to +19 of the *CPT-Iα* gene, with appropriate overhangs, was ligated into the *Bss*HII–*Bgl*II sites. The additional 5′ region of the promoter (nt −6839/−1653) was initially isolated from the P1 clone and inserted into the multiple cloning site of pBluescript K/S$^{+/-}$ at *Sal*I–*Eco*RI. The upstream promoter region was then removed from pBS by a *Kpn*I–*Eco*RI digest and ligated into the −1653/+19 CPT-Iα–Luc vector, resulting in −6839/+19 CPT-Iα–Luc. The −4495/+19 CPT-Iα–Luc vector was created by ligating −6839/+19 CPT-Iα–Luc, which was digested at *Xho*I sites in the multiple cloning site and at nucleotide −4495.

Serial deletions from the 5′ end of −1653/+19 CPT-Iα–Luc were created with the Erase a Base kit (Promega). The −1653/+19 CPT-Iα–Luc vector was digested with *Spe*I and filled in with α-phosphorothioate dNTPs. Exonuclease III digestion began from an *Eco*RI overhang at nt −1653 and aliquots were collected at various times from a 30 °C incubation mixture. CPT-Iα–Luc vectors were generated through ligation of the serial deletion constructs. A −52/+19 CPT-Iα–Luc vector was constructed by ligating an *Eco*RI–*Bss*HII −1653/+19 CPT–Luc digest. The −965/+19 and −193/+19 CPT-Iα–Luc vectors were prepared as described previously [4].

Site-directed mutagenesis was conducted using the pALTER system (Promega). The DpnI QuickChange method (Stratagene, La Jolla, CA, U.S.A.) was used to generate mutations in footprinted region III. The −1653/+19 region of the promoter was ligated into the pALTER vector using the *Kpn*I and *Hin*dIII sites. Phosphorylated oligonucleotides were generated based on the mutations made in the gel-shift oligomers that disrupted protein binding at specific sites in the promoter, and are given below: Sp1(#1) Mut, 5′-GCCTAGCCTCGCATGCATCTGC-TCGGACTC-3′; Sp1(#2) Mut, 5′-CCTGGTCCAGGCCAT GCATCGTCCT GCCCGC-3′; NF-Y Mut, 5′-GGCTGGGCT-CAGCTGGCCACCCGCGGCGC-3′; region I Mut, 5′-TCC-TGCCCGCGCCGCAGCCCGGCGAATTCCAGCCAATCA-CCCGCGGCG-3′; region II Mut, 5′-GGGTCCTAGCC-TCAGTTTCTCAGTACTACTCCTCCCCGCAGCCCCG-3′; region III Mut, 5′-TAAGGAATGCTGTCCTAGGTCTGTA-GCCATGGTGCTCGGGTCCTAGCCTCAGTTTCTTTA-3′.

Mutations introduced into the CPT-Iα promoter were confirmed by sequence analysis. The mutated −1653/+19 CPT-Iα fragments were removed from pALTER-1 and ligated into the *Kpn*I and *Bgl*II sites of the pGL3–basic-luciferase vector. The mutation of the most 5′ Sp1 site was removed from the CPT-

Iα–Luc vector by *Eco*RI–*Bss*HII digestion. This fragment was ligated into the *Eco*RI–*Bss*HII sites of the Sp1(#1) mutant CPT-Iα–Luc vector, creating the Sp1(#1)–Sp1(#2) double mutant. Similarly, the Sp1(#1)–NF-Y double mutant was created by utilizing the *Eco*RI and *Bss*HII restriction sites.

Cell culture, transfections and luciferase assays

Transient transfections were conducted in HepG2 cells using calcium phosphate precipitation as described previously [16]. The calcium phosphate precipitate contained equimolar concentrations of CPT-Iα–Luc vector and 1 μg of simian virus (SV)40–β-galactosidase as a transfection control. Sufficient pBluescript was added for a final concentration of 4 μg of DNA per plate to account for the variation in sizes of the CPT-Iα–Luc vectors. HepG2 cells were maintained in Dulbecco's modified Eagle's medium containing 5% (v/v) fetal-bovine serum and 5% (v/v) calf serum. Cells were treated with trypsin and replated at 60–70% confluence. After 4 h, to allow cell reattachment, the calcium phosphate–DNA precipitate was added to each plate and incubated overnight. The cells were washed twice with PBS and incubated for 24 h in serum-free medium.

Luciferase activity was measured as described previously [4]. Transiently transfected HepG2 cells were harvested in reporter lysis buffer (Promega). Cells were subjected to one round of freeze–thawing to enhance lysis. The lysed cells were centrifuged to remove cell debris and the supernatant was collected. Luciferase assays were conducted with 10 μl of cell extract and 100 μl of luciferase assay reagent (Promega) and luciferase activity was measured in a TD 20/20 luminometer. β-Galactosidase activity was determined with 200 μl of cell extract and *O*-nitrophenyl-β-D-galactopyranoside in a colorimetric assay [4]. The protein concentration of the cell lysate was measured using the Bio-Rad protein assay reagent (Bio-Rad). Luciferase data are expressed as luciferase activity corrected for β-galactosidase activity and for protein concentration in the cell lysate. Each transfection was conducted in duplicate and repeated four to six times.

Expression of site-directed mutant CPT-Iα–Luc vectors was analysed using the dual-luciferase reporter assay system (Promega). Transient transfections of HepG2 cells were conducted as described above but included the co-transfection of pRL-cytomegalovirus *Renilla*. The *Renilla* luciferase activity replaced β-galactosidase activity as the transfection control.

Sequencing

DNA was sequenced with the Dye terminator cycle sequencing system (Perkin-Elmer) and analysed at the Molecular Resource Center of the University of Tennessee.

RESULTS AND DISCUSSION

The goals of this study were to identify promoter elements and trans-acting factors that regulate expression of the *CPT-Iα* gene. Previously, we had reported the cloning of the CPT-Iα promoter from nt −965 to −1 [4]. To further characterize the *CPT-Iα* gene, we first cloned and sequenced the upstream promoter region from nt −6839 to −965 (results not shown). Secondly, the chromosomal location of the rat *CPT-Iα* gene was determined (Figure 1). Complete metaphase spreads of rat chromosomes were examined and consistent fluorescent signals were observed on chromosome 1 in the q43 region. The human *CPT-Iα* gene maps to chromosome 11q13-ter [17,18] and this region is syntentic with the rat 1q4 [19]. The human CPT-Iβ gene is found on chromosome 22q13.3 [18,20], and the rat CPT-Iβ gene maps to the syntentic region on chromosome 7q34 [11,19]. These data show that, like their human counterparts, rat *CPT-Iα* and *CPT-Iβ* genes are present at different chromosomal loci.

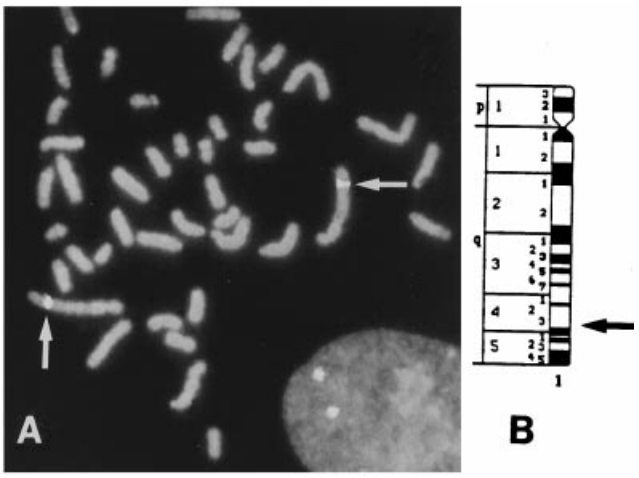

Figure 1 Chromosomal localization of the *CPT-Iα* gene

(**A**) FISH was used to conduct chromosomal mapping of the rat *CPT-Iα* gene. Preparation of rat metaphase chromosomes and hybridization conditions are given in the Experimental section. White arrows indicate hybridization signals of the *CPT-Iα* probe to chromosome 1. (**B**) Idiogram of rat chromosome 1 [19], with an arrow indicating the region of hybridization on region q43.

The next experiments were designed to identify regions in the CPT-Iα promoter that controlled basal expression of the *CPT-Iα* gene. A series of serial deletions from the 5′ end of the CPT-Iα promoter were constructed and ligated to the luciferase reporter gene. These deletions were created using exonuclease III digestion from the 5′ end of the −1653/+19 CPT-Iα promoter fragment. Approx. 200 bp were removed from the 5′ end of each deletion. Two longer CPT-Iα–Luc constructs (−6839/+19 and −4495/+19) were created to evaluate the contribution of the entire promoter to transcriptional activity. These CPT-Iα–Luc constructs were transiently transfected into HepG2 cells and luciferase assays were performed. Luciferase activity for each serial deletion was compared with the activity of the full length −1653/+19 CPT-Iα –Luc vector, which was given a relative value of 100% (Figure 2). The −1653/+19 CPT- Iα–Luc vector was chosen as the baseline because the site-directed mutagenesis of specific promoter elements was conducted in this vector.

The longer promoter regions drove expression of the luciferase reporter gene with a similar efficiency to the −1653/+19 promoter fragment (Figure 2). The relative activity of the −6839/+19 vector was 70±13% and that of the −4495/+19 vector was 123±25% of that of the −1653/+19 vector. A modest increase in basal expression was observed with progressive deletion of the CPT-Iα promoter beyond the −1653 point. This 2-fold increase in luciferase activity was constant for the shorter constructs until there was truncation beyond the −193 nucleotide. The −193/+19 CPT-Iα–Luc construct had a relative activity of 230±30%, whereas the relative activity of the −52/+19 CPT-Iα–Luc construct was 7±2%. Based on these data, the sequences between nt −193 and −52 had a major influence on the basal activity of the CPT-Iα promoter. Therefore the remaining experiments focused on characterizing this region of the promoter.

To identify potential transcription factor binding sites, DNase I footprint analysis of the proximal promoter region was

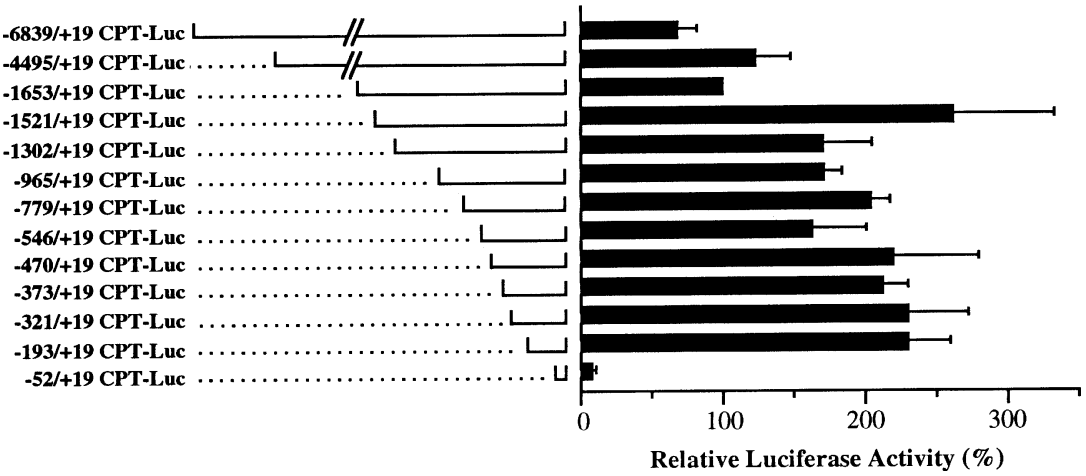

Figure 2 Identification of regions in the CPT-Iα promoter that regulate basal expression

Serial deletions of the −6839/+19 CPT-Iα (CPT) promoter were ligated to the luciferase (Luc) reporter gene. HepG2 cells were transiently transfected with 3.0 μg of CPT-Iα–Luc and 1.0 μg of SV40–β-galactosidase reporter gene. The SV40–β-galactosidase vector served as a control for transfection efficiency. Following transfection, the cells were left in serum-free DMEM for 24 h. Luciferase activity was normalized for protein content of the cells and β-galactosidase activity. The data are presented as a percentage of the −1653/+19 CPT-Iα–Luc vector, which was given a relative value of 100%. All transfections were conducted in duplicate and repeated four to six times and the results are the means ± S.E.

conducted. A DNA probe was created from base pair −383 to +27 and labelled with [γ-^{32}P]ATP on either the top or bottom strand with T4 polynucleotide kinase. The radiolabelled probe was incubated with proteins isolated from rat liver nuclei. Several regions in the CPT-Iα proximal promoter were protected from DNase I digestion on both the top (Figure 3A) and the bottom (Figure 3B) strands. Within this region, there are consensus sequences for two Sp1 sites and a CCAAT box. The 5′-most Sp1 site, located between nt −108/−99, and the CCAAT box (−66/−62) were protected from DNase I digestion. The binding of nuclear proteins to the downstream Sp1 site (−28/−21) was not evaluated by DNase I footprinting because this element was too close to the end of the probe. Four additional regions of the promoter were protected by protein binding, and these regions were labelled I to IV. Region I contained protected nt −71/−76 and region II consisted of nt −144/−138; region III covered nt −187/−165. Region IV (−215/−244) was strongly protected in the DNase I footprint. The sequences of the protected regions can be seen in Figure 6. We focused our studies on the Sp1 sites, the CCAAT box element and footprinted regions I–III, contained within the −193/+19 CPT Iα–Luc vector. The deletion of the promoter region from nt −193 to −52 greatly decreased basal expression, suggesting that the factors binding to the sites within this sequence regulate basal transcription. Based on data shown in Figure 2, the deletion of sequences in the region between nt −320 and −193 did not reduce the basal promoter activity. Therefore no further analysis of footprinted region IV was conducted.

Gel mobility-shift assays were conducted to further characterize the binding of nuclear proteins to elements in the CPT-Iα promoter that were protected by DNase I footprinting. Double-stranded oligonucleotides were generated which corresponded to the two Sp1 sites, the CCAAT box and footprinted regions I–III. Initially, competition studies were conducted with excess of unlabelled probe and rat liver nuclear extract (Figure 4). As shown in Figures 4(A) and 4(B), oligomers representing sites Sp1(#1) and Sp1(#2) formed one primary complex with the nuclear proteins. This binding activity could be competitively removed by adding an excess of either unlabelled wild-type *CPT-Iα* Sp1-site oligomer or unlabelled consensus Sp1 oligomer. However, an oligomer representing the cAMP responsive element (CRE) did not compete for binding of the nuclear extract. These observations indicated that Sp proteins specifically bound to these sites. Figure 4(C) shows that oligomers with mutations in the Sp1(#1) binding site were unable to compete for binding of nuclear proteins. Identical results were seen in a competition-binding study with a mutant Sp(#2) oligomer (results not shown). Similar experiments were conducted with the CCAAT-box element. The binding of nuclear proteins was competitively prevented by the wild-type CCAAT box oligomer, and excess mutant CCAAT box oligomer was unable to compete for the nuclear protein binding.

In Figures 4(D) and 4(E), gel-shift assays were conducted with oligomers representing regions II and III. One major complex was formed between each of these oligomers and proteins from rat liver nuclei. These studies revealed a specific protein–DNA interaction at each of these sites. To further confirm that the protein interactions at each site were specific, gel-shift probes were generated with 3–4 bp mutations in the protein binding site (described in the Experimental section). An excess of unlabelled oligomers containing the mutated sites was added to the gel-shift competition studies and it was found that the mutant oligomers did not compete for protein binding. Because region III was so broad, two mutant oligonucleotides were generated. Neither mutation was an effective competitor of nuclear protein binding. Similar observations were made with region I (results not shown). We can conclude from these competition experiments that each of the six CPT promoter protein binding sites shows specificity for nuclear protein binding, and that mutations within these sites specifically disrupt protein binding.

To identify specific proteins that bind to various elements in the promoter, supershift studies were conducted by adding specific antibodies to each of the DNA–protein binding reactions. Multiple members of the Sp transcription factor family have been identified [21]. As is shown in Figure 5(A), addition of either Sp1 antibody or Sp3 antibody was able to form a supershifted complex with the Sp1(#1) oligomer. When both antibodies were added simultaneously, the entire Sp protein binding activity was

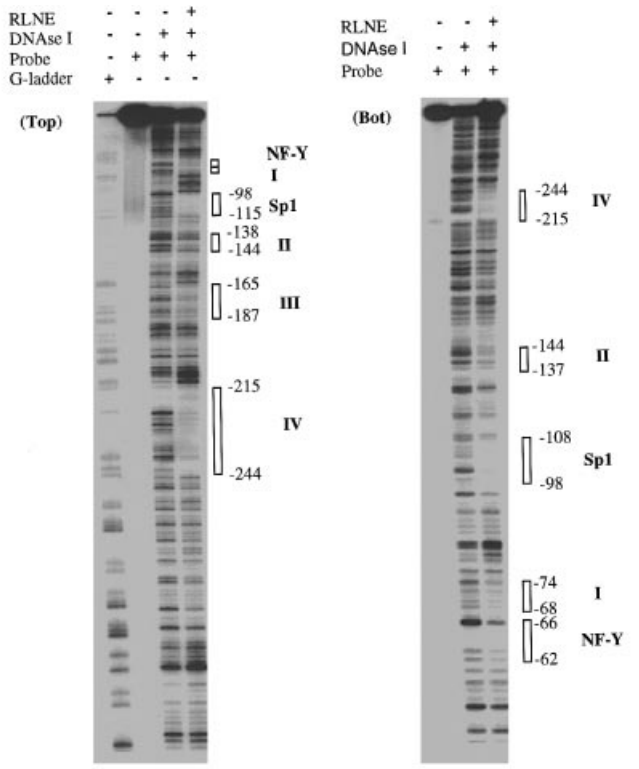

Figure 3 DNase-I footprint analysis of protein-binding domains in the CPT-Iα proximal promoter

A 400 bp fragment of the CPT-Iα promoter from nt −383 to +19 was ^{32}P-labelled on the top strand (Top). The CPT-Iα-labelled probe was incubated with proteins isolated from rat liver nuclei (RLNE) and digested with DNase I. Protected regions of the CPT-Iα promoter are indicated on the right with boxes and labelled. Sp1 and NF-Y protein binding sites are indicated, as are the protected regions I, II, III and IV. The G-ladder is shown for the labelled top strand probe. The same CPT-Iα fragment was labelled on the bottom strand and analysed as indicated above.

supershifted. These results indicated that both isoforms were present in rat liver nuclear extract and were able to bind this element. The antibody to Sp4 was not able to form a supershifted complex (results not shown). Similar experiments were conducted with the oligomer representing the element Sp1(#2) (Figure 5B), and essentially identical results were obtained. Since our transfections were conducted in HepG2 cells, we also tested the binding of nuclear proteins isolated from HepG2 nuclei to the Sp1 sites in the CPT-Iα promoter. The antibodies to Sp1 and Sp3 supershifted the binding activity in HepG2 cell nuclear extract (results not shown). These results indicate that the same proteins were binding to the *CPT-Iα* Sp1 sites.

The transcription factor NF-Y frequently binds to CCAAT boxes. Therefore we tested the ability of an NF-Y antibody to alter the binding of nuclear proteins to the oligomer representing the CCAAT box in the *CPT-Iα* gene (Figure 5C). The NF-Y antibody supershifted the major protein–DNA complex indicating NF-Y is the major protein binding to this element. The supershift experiments were also performed with HepG2 nuclear extract and similar results, with respect to NF-Y binding, were obtained (results not shown). Based on computer searches for consensus DNA transcription-factor binding elements, footprinted regions I–IV did not appear to contain any homologous sequences. The identity of the proteins that bind these regions has not been determined.

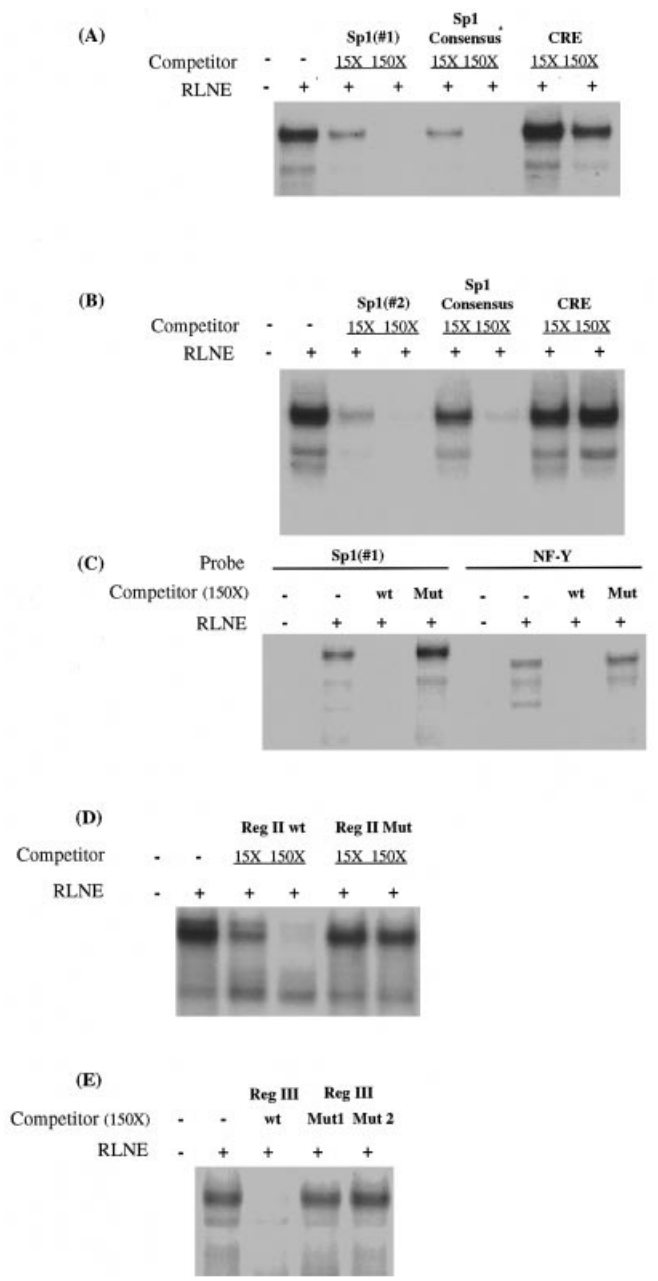

Figure 4 Characterization of protein binding sites in the CPT-Iα promoter

The binding of proteins to protected regions in the promoter was characterized with gel-mobility assays. (**A**) An oligomer representing the *CPT-Iα* Sp1(#1) site was labelled with [^{32}P]dCTP and incubated with proteins isolated from rat liver nuclei (RLNE). The resulting complexes were resolved on a non-denaturing acrylamide gel. Excess unlabelled competitor oligomers for the Sp1(#1), Sp1 consensus sequence and CRE consensus sequence were added to the binding reactions as indicated above each lane. The bands shown represent the protein–DNA complexes. Sequences for the Sp1 and CRE oligomers are given in the Experimental section. (**B**) An oligomer representing the *CPT-Iα* Sp1(#2) site was labelled and used in gel-mobility assays as indicated above each lane. (**C**) Labelled oligomers corresponding to the *CPT-Iα* Sp1(#1) and the *CPT-Iα* CCAAT box sequences were shifted with rat liver nuclei (RLNE) in a gel-mobility assay. An excess of unlabelled wild-type or mutant oligomer for each site was added as indicated above each lane. (**D**) A labelled probe of region II footprinted sequence was used in a gel-mobility assay as described above. An excess of competitor representing either wild-type (Reg II wt) or mutant oligomer (Reg II Mut) was added as is indicated above each lane. (**E**) An oligomer for footprinted sequence region III (Reg III) was labelled and used in a gel-mobility assay as described for region II, using an excess of competitor wild-type (wt) or mutant oligomers (Mut 1 or Mut 2). The sequences of the wild-type oligomers and mutant competitors are given in the Experimental section.

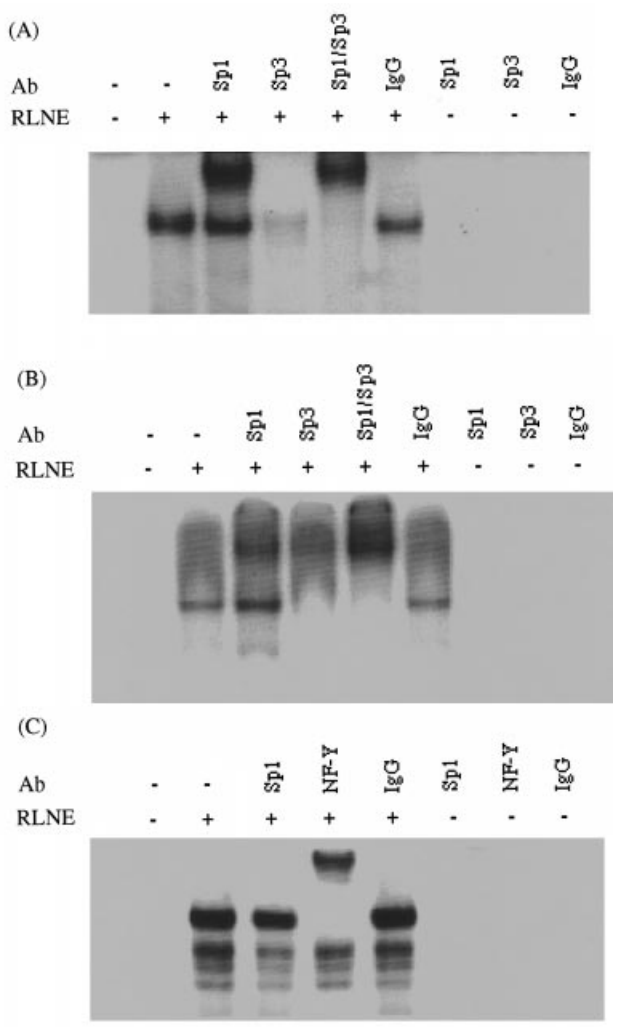

Figure 5 Identification of Sp1, Sp3 and NF-Y binding to the CPT-Iα promoter

Gel-mobility supershift assays were used to identify proteins that bound the *CPT-Iα* gene. Double-stranded oligonucleotides containing the Sp1 and CCAAT box sequences of the CPT-Iα promoter were labelled with [α^{32}P]dCTP. The probes were incubated with nuclear proteins isolated from rat liver (RLNE). The sequences of the probes are given in the Experimental section. (**A**) Antibodies (Ab) to either Sp1 or Sp3 were added to the binding mixture containing the 3′-most Sp1 site probe Sp1(#1) as indicated above each lane. IgG was added as a control. The bands represent the shifted complexes. The free probe is not shown. (**B**) Binding reactions were conducted with the Sp1(#2) site as described above. (**C**) Binding reactions were conducted with the CCAAT-box probe as described above and antibodies (Ab) to either NF-Y or Sp1 were added as indicated.

The contribution of each specific protein binding site to basal expression of the *CPT-Iα* gene was determined by site-directed mutagenesis of each site in the context of −1653/+19 CPT-Iα–Luc vector. Each Sp1 site, the NF-Y site, and footprinted regions I, II and III were disrupted individually. The mutations introduced had been shown previously to disrupt the binding of nuclear factors (Figure 4). In addition, two double mutants were created, in which both Sp1 sites or the Sp1(#1) and the NF-Y site were mutated. These CPT-Iα–Luc vectors were transiently transfected into HepG2 cells and luciferase assays were performed. Luciferase activity of the mutated protein binding sites was normalized to the wild-type −1653/+19 CPT-Iα–Luc vector (Figure 6).

We observed a significant decrease in basal expression when either the Sp1(#2) site or the NF-Y site was mutated. The relative expression of CPT-Iα–Luc vectors with mutations in the Sp1(#2) site and NF-Y site was 24±6% and 31±8% respectively. The disruption of the Sp1(#1) site caused a decrease in expression to 65±4%. The mutation of regions I, II, and III resulted in vectors that expressed at relative levels of 82±10%, 63±12% and 89±4% respectively. A further decrease in basal activity of the CPT-Iα promoter occurred when both Sp1 sites or Sp1(#1) and NF-Y sites were mutated simultaneously (5±1% and 16±2% respectively). These results indicated that Sp1 and NF-Y have a critical role in regulating the basal expression of the *CPT-Iα* gene. We wished to determine whether Sp1 or Sp3 was responsible for stimulating transcription of *CPT-Iα*. Sp3 has been reported to have both inhibitory and stimulatory roles [22]. Other studies have found that Sp1 and Sp3 have a synergistic effect on the trans-activation of various genes [23–25]. The −1653/+19 CPT-Iα–Luc vector used in the present study was co-transfected with mammalian expression vectors for Sp1 and Sp3 [21], but overexpression of either protein did not alter transcription of the *CPT-Iα* gene (results not shown). It is likely that both proteins stimulate transcription of the *CPT-Iα* gene and are present in great abundance in HepG2 cells.

Sp1 has an important role in directing transcription from TATA-less genes. Sp1 assists in the recruitment of TATA factors and other transcriptional initiation factors to the promoter [26,27]. In the promoters of rat growth hormone and human placental lactogen B genes, Sp1 contributes to both basal transcriptional activity and to thyroid hormone responsiveness [28,29]. The ratio of Sp1 protein to activator protein (AP)-2 is involved in the developmental regulation of K3 keratin gene transcription [30]. An interaction between Sp1 and the CCAAT-enhancer binding protein (C/EBP) β has been reported to regulate basal gene expression in a rat liver *CYP450* gene [31]. NF-Y is present in the promoters of many TATA-less genes and may have a similar role to Sp1 [26,32]. NF-Y enhances the thyroid hormone responsiveness of the *S14* gene through functional interaction with the thyroid hormone receptor [33]. Additionally, NF-Y and Sp1 co-operate to regulate transcription of the rat fatty acid synthase gene through a physical interaction [34]. The various functions attributed to NF-Y and Sp1 suggest that these factors may participate in the developmental and hormonal aspects of *CPT-Iα* transcriptional regulation as well as basal gene expression.

At birth, the expression of *CPT-Iα* is increased five-fold in the liver [7]. C/EBP α induces the expression of several genes encoding metabolic enzymes, such as phosphoenolpyruvate carboxykinase and glycogen synthase in the liver at birth [35,36]. We co-transfected our CPT-Iα–Luciferase vectors with a dominant negative C/EBP vector called A-C/EBP [37]. This vector did not decrease the expression of CPT-Iα–Luc suggesting that C/EBP proteins do not regulate *CPT-Iα* expression (results not shown). Other factors must be involved in the induction of this gene at birth. Glucocorticoids are additionally responsible for inducing phosphoenolpyruvate carboxykinase and tyrosine aminotransferase at birth [38]. However, dexamethasone had only a small effect on *CPT-Iα* transcription (results not shown).

The proximal promoter of the *CPT-Iα* gene is similar to the promoters of other acyl transferases and enzymes involved in fatty acid oxidation. The medium-chain acyl-CoA dehydrogenase promoter is TATA-less with Sp1 playing a prominent role in basal expression [39]. The liver carnitine octanoyltransferase (peroxisomal CPT) gene does not have a TATA element, but contains CCAAT motifs and GC boxes for Sp1 binding [40]. The promoter of *CPT-Iβ* has been cloned from several species.

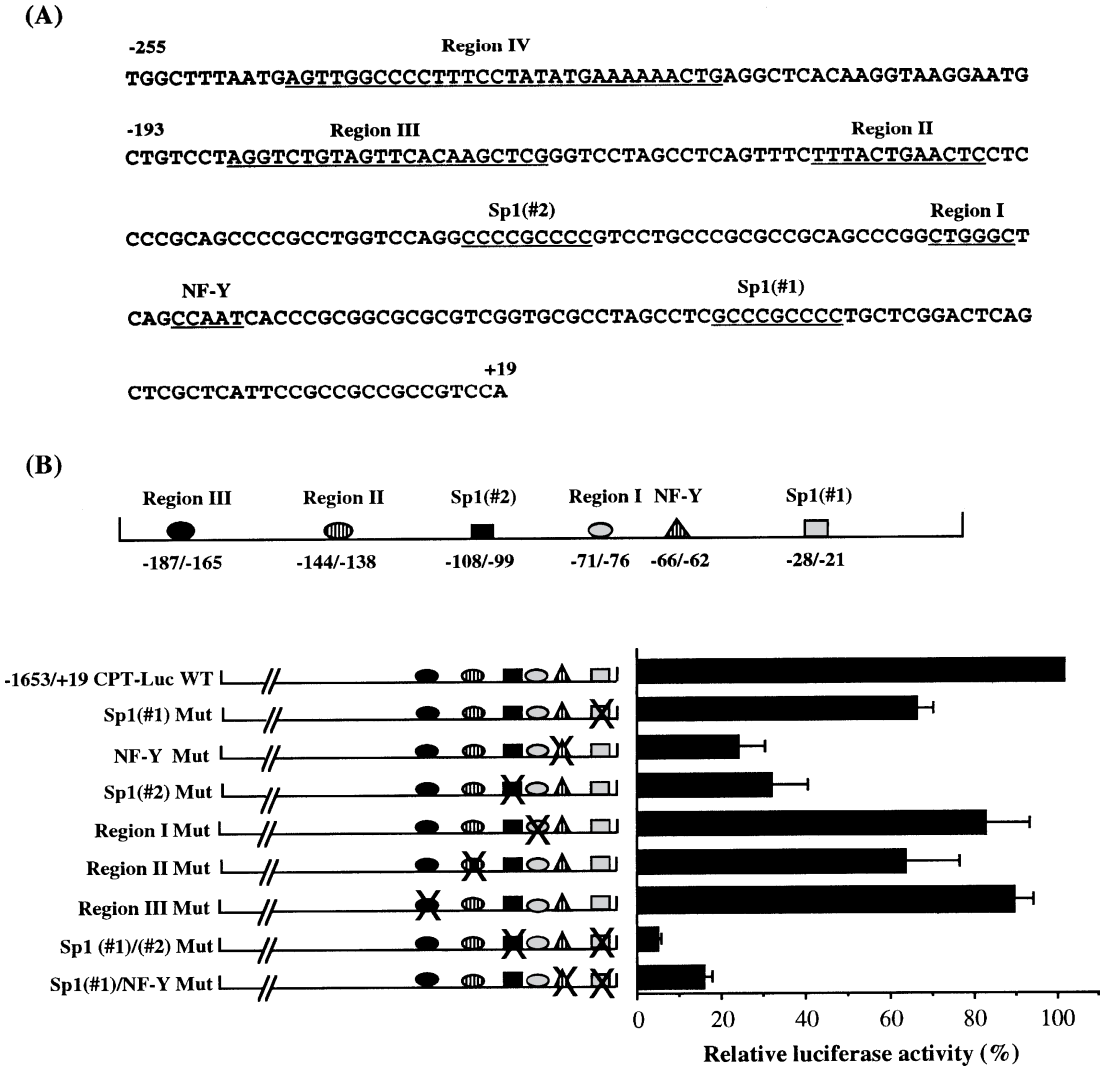

Figure 6 Contribution of protein binding sites in the CPT-Iα promoter to basal expression

Protein binding sites in the CPT-Iα promoter were mutated in context of the −1653/+19 CPT-Iα–Luc vector. (**A**) The sequence of the CPT-Iα promoter from nt −255 to +19 is provided with Sp1 sites, the CCAAT box and the footprinted regions I, II, III and IV (underlined). (**B**) A model of the protein binding sites is shown and X indicates the mutated sites. The vectors were transiently transfected into HepG2 cells essentially as described in the legend to Figure 2, except that the pRL–CMV *Renilla* vector provided a transfection control. The luciferase activity of each mutated construct was compared with the wild-type −1653/+19 CPT-Iα–Luc vector. Luciferase activity was normalized by correcting for protein concentration and *Renilla* luciferase activity. Each transfection was conducted in duplicate and repeated four to six times and the results shown are the means ± S.E.

Human *CPT-Iβ* utilizes two start sites of transcription yielding alternative exons, while the rat *CPT-Iβ* gene has a single first exon. [11,20,41]. In both cases, the promoters are TATA-less. *CPT-Iβ* is expressed only in skeletal muscle, cardiac myocytes and adipose tissue whereas *CPT-Iα* is expressed in a variety of other tissues [1,2]. Our studies did not identify any sequences that might be involved in the tissue-specific regulation of the *CPT-Iα* gene. Our CPT-Iα–Luc vectors were well expressed in the L6 muscle cell line (results not shown). In summary, we have characterized the proximal CPT-Iα promoter and identified elements that regulate basal gene expression. Sp1, Sp3 and NF-Y bound to the proximal promoter and played a major role in driving basal expression of this gene.

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