Control of human carnitine palmitoyltransferase II gene transcription by peroxisome proliferator-activated receptor through a partially conserved peroxisome proliferator-responsive element

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The expression of several genes involved in fatty acid metabolism is regulated by peroxisome proliferator-activated receptors (PPARs). To gain more insight into the control of carnitine palmitoyltransferase (CPT) gene expression, we examined the transcriptional regulation of the human CPT II gene. We show that the 5′-flanking region of this gene is transcriptionally active and binds PPARα in vivo in a chromatin immunoprecipitation assay. In addition, we characterized the peroxisome proliferator-responsive element (PPRE) in the proximal promoter of the CPT II gene, which appears to be a novel PPRE. The sequence of this PPRE contains one half-site which is a perfect consensus sequence (TGACCT) but not clearly recognizable second half-site (CAGCAC); this part of the sequence contains only one match to the consensus, which seems to be irrelevant for the binding of PPARα. As expected, other members of the nuclear receptor superfamily also bind to this element and repress the activation mediated by PPARα, thus showing that the interplay between several nuclear receptors may regulate the entry of fatty acids into the mitochondria, a crucial step in their metabolism.

Key words: fatty acid, gene expression, β-oxidation.

INTRODUCTION

Mitochondrial carnitine palmitoyltransferase (CPT) allows the incorporation of activated long-chain fatty acids (LCFAs) into the mitochondria, where they will be catabolized through β-oxidation. The first reaction, between the acyl-CoA molecule and carnitine, is catalysed by CPT I and takes place on the outside of the mitochondrial membrane, generating free CoA and acylcarnitine. The second reaction, between acylcarnitine and CoA, takes place in the matrix and is catalysed by CPT II on the inside of the inner membrane. The acyl-CoA generated is the substrate for the β-oxidation pathway. Two isotypes of CPT I have been described and are referred to as liver (L-) CPT I and muscle (M-) CPT I, based on the tissues of origin, in which the expression of these genes was first studied [1]. It is generally accepted that the CPT I isotypes are the main locus of control for LCFA oxidation, because these enzymes are sensitive to inhibition by malonyl-CoA. CPT II appears to have a more limited role, since it is not inhibited by this lipogenic intermediate [1]. However, induction of CPT II gene expression has been observed in starved mice [2] and in animals fed with a high-fat diet or peroxisome proliferators [3–5], suggesting that changes in CPT II could be relevant for fatty acid oxidation. Despite these results, the role of LCFAs in the expression of CPT II remains unclear. Thus whereas L-CPT I expression is sensitive to inhibition by malonyl-CoA, CPT II remains high throughout suckling and weaning, regardless of the fat content of the solid diet [8].

The expression of several genes involved in intra- and extra-cellular lipid metabolism, especially in peroxisomal and mitochondrial β-oxidation, is controlled by ligand-activated receptors collectively referred to as peroxisome proliferator-activated receptors (PPARs) [9]. These receptors are members of the nuclear receptor superfamily and are activated by a wide array of peroxisome proliferators as well as by natural and synthetic fatty acids [10,11], anti-diabetic drugs [12], prostaglandin J2 [13] and leukotriene B4 [14]. Activated PPAR, as a heterodimer with retinoid X receptor (RXR), binds to specific cis-acting elements termed peroxisome proliferator-responsive elements (PPREs) [15–17]. The consensus PPREs consist of a direct repeat of the sequence AGGTCA separated by 1 nucleotide (DR1) and flanked by an A/T-rich sequence. The previously described PPREs [18] contain a highly conserved first half-site and a less-conserved second half-site, flanked by the A/T-rich sequence. These peculiarities have been described as conferring polarity to the binding of the nuclear receptor heterodimer [17,18]. Thus PPAR binds to the poorly conserved half-site of the DR1, because it interacts with the A/T-rich flanking sequence of the element, while RXR binds to the conserved half-site of the DR1 (reviewed in [19]).

Recent studies on the gene encoding medium-chain acyl-CoA dehydrogenase have shown that several members of the nuclear receptor superfamily interact specifically with the nuclear receptor-responsive element NRRE-1, including chicken ovalbumin promoter [20], PPAR [21], retinoic acid receptor/RXR [22], apolipoprotein regulatory protein-1 and hepatocyte nuclear factor-4 [23] and oestrogen-related receptor α (ERRα) [24]. During development and in the hypertrophied and failing heart, the rate of fatty acid utilization and the expression of genes

Abbreviations used: ChIP, chromatin immunoprecipitation; COUP-TF, chicken ovalbumin promoter transcription factor; CPT, carnitine palmitoyltransferase; DR1, direct repeat of the sequence AGGTCA separated by 1 nucleotide; EMSA, electrophoretic mobility shift assay; ERRα, oestrogen-related receptor α; FXR, farnesoid X-activated receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; hRXRα, human RXRα; L-CPT I, liver CPT I; LCF, long-chain fatty acid; M-CPT I, muscle CPT I; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-responsive element; RXR, retinoid X receptor; SV40, simian virus 40.

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encoding fatty acid oxidation, such as medium-chain acyl-CoA dehydrogenase, are similar and correlate with changes in PPAR and chicken ovulbin promoter transcription factor (COUP-TF) expression. This suggests that the expression of PPAR and COUP-TF nuclear receptors controls the transcription of genes for fatty acid oxidation enzymes [25]. The interplay between nuclear receptors is also observed in the regulation of several genes such as the acyl-CoA synthetase [26], M-CPT I [27] and mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase [28–30].

Mice lacking PPARα show altered constitutive or induced expression of genes encoding several mitochondrial and peroxisomal fatty acid-catabolizing enzymes [31]. The expression of CPT II is increased in wild-type mice fed Wy-14,643 or ciprofibrate and this induction is lost in the PPARα-null animals [4,5]. The constitutive expression of several fatty acid-utilization genes, including CPT II, was also examined in hearts of PPARα+/− mice [31]; the expression of CPT II is decreased in these animals. Starvation also induces the immunoreactive CPT II protein by 2.3-fold in the kidney of wild-type mice, and this induction is lost in the knock-out animal [2]. All these data are consistent with our findings of a PPRE in the 5′-flanking region.

Here we show by a chromatin immunoprecipitation (ChIP) assay that the CPT II gene promoter is occupied in vivo by PPARα. We localized the PPRE in the human CPT II gene promoter. The sequence of this PPRE contains one half-site which is a perfect consensus sequence (TGACCT), but no clearly recognizable second half-site (CAGCAC), which appears to play a role in PPARα binding, followed by a conserved A/T flanking region. In addition we show that the orphan receptors COUP-TF I and ERRα also bind to this element, modulating the PPARα trans-activation of the CPT II promoter. These results confirm the relevance of nuclear receptors in the transcriptional control of mitochondrial fatty acid oxidation.

**EXPERIMENTAL**

**Plasmids**

The human CPT II gene 5′-regulatory region was amplified using the oligonucleotides DH73 (5′-CTAAGATCAGGATTGGG- GCAGGG-3′) and DH74 (5′-GGCTCCATCTTGGCGAGACGTGGG-3′), corresponding to positions −514 to −490 and +84 to +107, respectively, of the human CPT II gene [32]. Human genomic DNA was used as a template. The PCR product was digested with PstI/XhoI, and a product containing the human CPT II 5′-regulatory region from position −477 to +7 was cloned into pCAT-Basic (reporter plasmid from Promega encoding for chloramphenicol acetyltransferase) PstI/XhoI sites to generate the construct p477CPTIIICAT. Using this construct as a template, a PCR fragment was amplified using oligonucleotides CATF (5′-CTTCCGGGCTCTGTGATTGG-3′) and CATR (5′-ATATGAGATCCACATGTGATGTGG-3′) corresponding to co-ordinates −108 to +75 and was inserted into pGL3-Basic (reporter pGL3 Basic site). The resulting plasmid was called pCPTIIA.

Deletion constructions of the 5′-regulatory region were performed as follows. pCPTIIIB was formed by PCR amplification with pCPTIIA as a template, using primers DH173 (5′-AAATGCTgcAGCACAAGATGAGTG-3′; nucleotides in lower case were changed to provide a PstI site), corresponding to positions −286 to −263, and DH221, phosphorylating and cloning the product into the pGL3 Basic Smal site. For plasmids pCPTIIC and pCPTIID, which were constructed in an identical manner, the forward primers DH175 (5′-CCGCTCtGcAGGCGTTC-3′; corresponding to co-ordinates −245 to −226) and DH87 (5′-CAAGTCTATGAGGGCTCAGG-3′; corresponding to co-ordinates −138 to −112; where nucleotides in lower case were changed to provide a PstI site), respectively, and reverse primer DH221 were used. Plasmids pCPTIIIE was constructed using primers DH291 (5′-gaacgtgaCTAACAAGAC-3′; corresponding to co-ordinates −77 to −68) and CPT2Rev(5′-gaacctcgtGAATATGAAGTATACGAT-3′; position +8 to −14; nucleotides in lower case were added to provide NheI and XhoI and cloning into NheI and XhoI sites of pGL3 Basic.

The PPRE mutant construct pCPTIIA1M was generated by overlap-extension PCR [33]. The first PCR used forward primer DH294 (5′-ttggctagTGAGAAATGGATG-3′; position −462 to −446; lower-case sequence was added to generate a NheI restriction site) and reverse primer DH293 (5′-CCGCTTGATAGTTGATATAATGAC-3′; position −82 to −112; nucleotides in underlined lower case correspond to those changed from the wild-type sequence). The second PCR used forward primer DH292 (5′-TCTATGCAAGGCGTGGATTGC-3′), which overlapped with DH293, and reverse primer DH221. After the subsequent overlap-extension reaction, the PCR product was phosphorylated and cloned into the pGL3 Basic Smal site. The PPRE mutant construct pCPTIIAFM was generated in the same way, using oligonucleotides DH294 and DH221, and the overlapping oligonucleotides DH524 (5′-TGACCTTCAGGCGAGCTAACAAG-3′) and DH525 (5′-GGACGTGCTGCTGCTTGAAGTATTACTAATAAAATT-3′), which were annealed to DH207 (5′-cgcgtCAGGCTGCTGCTATTACTAATAAAATT-3′; the lower-case sequences were added to generate SacI and MluI cohesive ends) into the pGL3-Promoter SacI and MluI sites. The resulting plasmid was called pCPTII-PPRE. Automatic sequencing using the fluorescent terminator kit (PerkinElmer) was performed to check all constructs.

**Cell culture and transfections**

HepG2 and HeLa cells were cultured in minimal essential medium supplemented with non-essential amino acids and 10% fetal bovine serum. CaCo-2 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with non-essential amino acids and 10% fetal bovine serum. Cells were co-transfected by the calcium phosphate method [34,35] with 2 μg of the reporter CPT II-luciferase gene construct and 500 ng of eukaryotic expression vectors encoding PPARα, RXRα, COUP-TF I and ERRα (respectively: pSG5-mPPARα, pJCXR8, pRSHCOUP and pERRα) or an equal amount of salmon sperm DNA. In all experiments 40 ng of pRL (encoding the cytomegalovirus promoter and Renilla luciferase) was included as an internal control in transfections. Experiments with ligands included either vehicle (DMSO) or ligand (1 μM 9-cis-retinoic acid, 30 μM Ly-171883 or Wy-14,643). Cell extracts were prepared by passive lysis and luciferase assays were performed following the instructions of the Dual Luciferase Reporter Assay System (Promega).

Luciferase activity was measured using the TD-20/20 Luminometer (Turner Designs).
**In vitro transcription and translation**
cDNAs for the receptors [mouse PPARα, human RXRα (hRXRα), human COUP-TF I and mouse ERRα] and pSG5 as an unprogrammed lysate were transcribed and translated using a commercially available kit according to the instructions of the manufacturer (Promega).

**Expression and purification of human PPARs in Escherichia coli**
Human PPARα and δ were amplified by reverse-transcriptase PCR [36] using total RNA from HepG2 cells treated and untreated with 125 μM linoleic acid (Sigma) for 24 h, respectively. PPARγ was amplified by PCR using the Human Fat Cell Quick-Clone® cDNA (Clontech). Oligonucleotides for PCR amplification were designed in agreement with the human PPARs’ described sequences (L02932, L07592 and U79012 for human PPARα, δ and γ, respectively) and were designated ALPHA.F (5'-ctgagttgcgcGCGATGGAGCAGG-3'; position -3 to +17; taking +1 as translation initiation) and ALPHA.R (5'-ctgagttgcgcCTACGTAATCTGCTGTA-3'; +1407 to +1387) for human PPARα, DELTA.F (5'-ctgagttgcgcTCAGCAGGAGCACGAGC-3'; -6 to +12) and DELTA.R (5'-ctgagttgcgcTTAGTACATGTCCTTGTTAG-3'; +1325 to +1306) for human PPARδ, and GAMMA.2F (5'-ctgagttgcgcCTACGTAATCTGCGAGCCAC-3'; -6 to +11) and GAMMA.R (5'-ctgagttgcgcCTAGTACAGGTCTCTGTAG-3'; +1517 to +1498) for human PPARγ.2 The lower-case sequences in these six primers were added to generate restriction sites for cloning. The PPARα PCR product was digested with Sall and NotI, cloned into pBlueScriptSK+ (Stratagene) Sall/NotI sites and sequenced. Finally, the Sall/NotI insert was liberated from pBSSK+ and cloned into Sall/NotI sites of pGEX-4T-2 (Amersham Biosciences) to yield plasmid pGEXhPPARα. PPARδ and PPARγ2 PCR products were digested with Sall and NotI, cloned into pBSSK+ Sall/NotI sites and sequenced. The Sall/NotI insert was finally liberated from pBSSK+ and cloned into pGEX-4T-2 Sall/NotI sites to yield plasmids pGEXhPPARδ and pGEXhPPARγ2 respectively.

**ChIP assays**
CaCo-2 cells were fixed with formaldehyde at 37 °C for 10 min. Cells were harvested and incubated in nuclear lysis buffer [5 mM Pipes/KOH, pH 8.0, 85 mM KCl and 0.5 μg/ml (v/v) Nonidet P-40] for 10 min in ice. Nuclei were pelleted by centrifugation, and soluble chromatin was further isolated as described in [38]. The chromatin solution was sonicated and precleared with salmon sperm DNA/Protein G-Sepharose. The precleared chromatin solution was incubated for 16 h with 20 µg of anti-PPARα antibody (Santa Cruz Biotechnology) or 20 µg of preimmune serum. Immune complexes were collected with salmon sperm DNA/Protein G-Sepharose, washed and eluted in 1% SDS/0.1 M NaHCO₃. Cross-links were reversed and chromatin-associated proteins were digested with proteinase K. The DNA was recovered by phenol/chloroform extraction and ethanol precipitation. Pellets were resuspended in 50 µl of water and 10 µl aliquots were used as template for PCR reaction. Primers used for promoter amplification were DH176 (5'-CTCCTGCA-GAAATCTTTTCTATATCC-3', located at position -186 to -159 of the human CPT II 5'-regulatory region) and DH221. Primers used for exon 4 amplification were DH582 (5'-TCCAG-GCATACTGAAATCG-3'; +1321 to +1340 of the human CPT II cDNA) and DH583 (5'-ACGGCATGAGCACATCC-TTG-3'; position +1618 to +1598 of the human CPT II cDNA).

**RESULTS**

**The human CPT II gene contains a PPARα-response element**
The expression of luciferase from a construct containing the 5'-regulatory sequence of the human CPT II gene (−477 to +7) is increased by co-transfection of a mouse PPARα expression vector (pSG5-mPPARα) and a hRXRα expression vector (pJXCR8) in HeLa cells (Figure 1A). Addition of PPARα ligands (Ly-17883 or Wy-14,643) and RXRα ligands (9-cis-retinoic acid) further increased the transcriptional activity of the promoter.

**PPARα binds the human CPT II gene promoter in vivo**
We were able to immunoprecipitate the human CPT II gene promoter using an antibody against human PPARα (Figure 2),
amplifying a region from −186 to +7 of the human CPT II gene 5′-regulatory region by PCR. The PCR product was detected in input and in anti-hPPARα-immunoprecipitated chromatin, but was not detected when preimmune serum was incubated with chromatin. No PCR product was detected in the immunoprecipitated samples when oligonucleotides for exon 4 amplification were used. These results indicate that the immunoprecipitation of the human CPT II gene 5′-regulatory region with anti-hPPARα is specific.

**Localization of the PPRE**

To locate the PPRE, progressively larger 5′ deletions of the 5′-flanking region of CPT II were used (Figure 1B). These constructs were tested in co-transfection experiments with pSG5-mPPARα using human hepatoma HepG2 cells. Nucleotides up to position −138 were deleted without loss of the response to PPARα, which was eliminated by further deletion up to nucleotide −80 (Figure 1C). Sequence comparison of CPTII promoter from position −138 to −80 with a consensus PPRE (Figure 3A) shows, besides the inverted repeat IR-1, previously identified as a functional farnesoid X-activated receptor (FXR)/RXR-binding site [39], the presence of a putative PPRE in which the second half-site was poorly conserved. Figure 3(B) shows that a probe including this putative element (FLPPRE) did not form a retardation complex with either mouse PPARα or hRXRα (Figure 3B, lane 3) or hRXRα (Figure 3B, lane 9) alone, whereas the inclusion of both mouse PPARα and hRXRα in the EMSA resulted in a prominent complex (Figure 3B, lane 4). The specificity of binding was studied by competition assays. Figure 3(B) also shows that the addition of increasing amounts of an unlabelled CPTII probe was able to eliminate the labelled complex (Figure 3B, lanes 5 and 6). However, a probe in which the conserved half-site was mutated (FL1MPPRE) was unable to produce PPARα/RXRα complex (results not shown) or to eliminate the complex formed with the wild-type probe (Figure 3B, lanes 7 and 8).

When the characterized sequence was inserted into a pGL3-Promoter vector (a plasmid containing the luciferase gene under the control of an SV40 promoter) this sequence conferred PPARα responsiveness to the otherwise unresponsive SV40 promoter (Figure 4A), indicating that this sequence is sufficient to mediate the PPARα response. These data indicate that the PPRE is located between −108 and −75, a region which contains an imperfect direct repeat separated by one nucleotide, followed by an AT-rich sequence (TGACCTTCAGCACGGATTAC) corresponding to co-ordinates −97 to −78, which seems to mediate the response to PPARα. The introduction of four point mutations into the conserved half-site eliminated the binding of PPARα (Figure 3B), and when introduced in the context of pCPTIIA it

**Figure 2** PPARα occupies the CPT II gene promoter in vivo

ChIP assays were carried out by immunoprecipitating CaCo-2 cell chromatin with an antibody against human PPARα (PPARα Ab) or preimmune serum (PI). Immunoprecipitated chromatin was amplified with oligonucleotides DH176 and DH221, which amplified from −186 to +7 (promoter), or oligonucleotides DH582 and DH583, which amplified the human CPT II exon 4. Input represents 1% of total chromatin. A negative control (H2O) for PCR is also shown. The data are representative of three independent experiments.
obliterates both basal activity and the induction by PPAR\(_\alpha\) (Figure 4B). These data indicate that the localized element is necessary for both maintaining basal activity of the promoter and mediating the PPAR\(_\alpha\) response. The introduction of four point mutations into the A/T-rich flanking region, which also eliminated the binding of PPAR\(_\alpha\) (see below), abolished the response to PPAR\(_\alpha\), but did not affect the basal activity of the promoter.

**COUP-TF I and ERR\(_\alpha\) bind to the same DNA region in the CPT II gene as PPAR\(_\alpha\) and repress the induction of the CPT II gene by PPAR\(_\alpha\)**

We performed gel mobility-shift assays to analyse whether other members of the nuclear receptor superfamily bind to the PPRE of the CPT II gene (Figure 5A). For this experiment we used a probe (WTTPRE) that only contained the DR1 and the flanking region of the element (positions -97 to -78). COUP-TF I and ERR\(_\alpha\) transcribed and translated in vitro produced a prominent complex with a DNA probe containing the DNA sequence to which PPAR\(_\alpha\)/RXR\(_\alpha\) bind (Figure 5A, lanes 3 and 4, respectively). In transactivation experiments both COUP-TF I and ERR\(_\alpha\) were able to repress the transcriptional activity of the promoter and to repress the activation produced by PPAR\(_\alpha\)/RXR\(_\alpha\) (Figure 5B). To study the binding of these factors by supershift assays with nuclear extracts, we used CaCo-2 cell nuclear extracts as a source of these proteins. In the presence of CaCo-2 cell extracts, the WTTPRE probe generated a specific complex (a in Figure 5C) that was eliminated in the presence of a 200-fold molar excess of WTTPRE probe (Figure 5C, lane 7), but was not affected by the addition of 200-fold molar excess of an unrelated probe (Figure 5C, lane 8). Addition of anti-PPAR\(_\alpha\) (Figure 5C, lane 3) or anti-COUP-TF I (Figure 5C, lane 4) antibodies reduced the abundance of the complex, and a supershifted band was generated in the presence of anti-PPAR\(_\alpha\) antibody. Incubation with both antibodies further reduced the specificity of the effects was assayed by incubating with an unrelated antibody (Figure 5C, lane 6). A minor complex (b in Figure 5C) was also detected that could correspond to ERR\(_\alpha\). When we used a probe containing just the ERR\(_\alpha\) site (the first direct repeat of the element followed by three flanking nucleotides, identical with the ERR consensus), which does not bind PPAR or COUP-TF I, complex b (Figure 5C, lane 9) was clearly detected. This band has a similar mobility to the in vitro-synthesized mouse ERR\(_\alpha\) complex (Figure 5C, lane 10).

**Characterization of human CPT II gene PPRE**

To characterize the sequence of the human CPT II gene PPRE, we performed gel-shift assays (Figure 6) with wild-type and mutated probes, in the presence of PPAR\(_\alpha\), \(\gamma\)2 and RXR\(_\alpha\). Probe 1MPPRE contained four point mutations in the first half-site of the responsive element, and this mutation dramatically reduced the binding of all PPAR isoforms to the element. Probe 2MPPRE contained a point mutation in the second half-site of
The human CPT II gene PPRE interacts with other nuclear receptors

(A) EMSA of the CPT II gene PPRE with PPARα/RXRα, COUP-TF I and ERRα. The labelled WTPPRE probe (–97 to –78) was incubated with water (lane 1), unprogrammed (pSG5) reticulocyte lysate (lane 2), and in vitro–translated COUP-TF I, ERRα or mouse PPARα/hRXRα (lanes 3–5 respectively) and analysed by EMSA. (B) COUP-TF I and ERRα repress the induction of the CPT II gene by PPARα. Luciferase reporter construct containing from –514 to +7 of the CPT II gene was co-transfected with expression vectors for mouse PPARα and hRXRα in the absence or presence of increasing amounts of COUP-TF I or ERRα (lanes 3–6, transfected with 62.5, 125, 250 and 500 ng of plasmid encoding COUP-TF I; lanes 7–11, transfected with 62.5, 125, 250 and 500 ng of plasmid encoding ERRα) or in the presence of PPARα (lanes 3–6) and hRXRα (lanes 7–11) in HepG2 cells. The effect of COUP-TF I or ERRα alone was also tested, by transfecting 500 ng of plasmid encoding COUP-TF I or ERRα (lanes 7 and 12). Means ± S.D. of Renilla normalized luciferase activity, from three independent transfections with two plates each, are expressed as relative luciferase activity, with the activity in the absence of expression vectors set at 1. (C) Lanes 1–8 show EMSA with CaCo-2 nuclear extracts and the WTPPRE probe. Lane 1, free probe; lanes 2–8, 10 μg of nuclear extract in the absence or presence of specific antibodies against PPARα (lanes 3 and 5), COUP-TF I (lanes 4 and 5) or an unrelated antibody (anti-Gal4; lane 6). Lane 7 also contains a 200-fold molar excess of unlabelled WTPPRE probe and lane 8 also contains a 200-fold molar excess of an unlabelled unrelated probe. Lanes 9–11 show EMSA with CaCo-2 nuclear extracts and the ERRsite probe. Lane 9 contains 10 μg of CaCo-2 nuclear extract, lane 10 contains 2 μl of in vitro-synthesized mouse ERRα and lane 11 shows free probe.

Figure 6 Characterization of the human CPT II gene PPRE

(A) The nucleotide sequence between –98 and –78 (WTPPRE), consensus sequence (CONS) and mutated probes in which only the mutated bases are specified. Arrows indicate the motifs and their orientation. (B) Means ± S.D. from three independent EMSAs, performed with equal amounts of the labelled probes shown in (A), in the absence or presence of 400 ng of E. coli-expressed and purified PPARα and 2 μl of in vitro-synthesized hRXRα or 2 μl of in vitro-synthesized COUP-TF I. Retarded complexes were quantified using a PhosphorImager®. Panel 1, the binding of the three PPAR isoforms to the wild-type probe (WTPPRE) taking the binding of PPARγ2 as 100%. The binding of PPARα, δ and γ isoforms, COUP-TF I and ERRα to the mutated probes is represented in panels 2–6 respectively, taking the binding of each receptor to the wild-type probe as 100%. *No statistical significance when compared with binding to the wild-type probe (P > 0.05).
the element and caused about a 40% reduction in the binding of PPARδ and γ2, but no significant differences were found between the binding of PPARα to the wild-type probe and to the second half-site mutated probe. Finally, introduction of four point mutations in the A/T-rich flanking region caused at least a 65% decrease in the binding of all PPARs. Binding of COUP-TF I to the wild-type and mutated probes was also quantified. Mutation of the conserved half-site almost abolished the binding of COUP-TF I to the element. Mutation of the second half-site reduced the binding by 60%, but mutation of the A/T-rich flanking site of the element had no significant effect on the COUP-TF-I binding to the element. Binding of ERRα was dramatically affected by mutation of the conserved half-site, but the mutations in the second half-site and flanking region did not affect the binding.

**DISCUSSION**

The human CPT II gene 5′-regulatory region responds to PPARα/RXRα (Figure 1A). The addition of PPARα and RXRα ligands slightly but significantly increased the transcriptional activity of the promoter. Luciferase reporter constructs containing deletions of the promoter downstream from position −138 did not respond to PPARα in cell culture (Figure 1C). Using EMSA (Figure 3), PPARα-unresponsive SV40 promoter chimaeric constructs (Figure 4A) and site-directed mutagenesis (Figure 4B), we localized an element sufficient and necessary to mediate the PPARα response. This element is also necessary for maintaining the basal transcriptional activity of the human CPT II gene promoter in HepG2 cells. The results concerning the basal expression of CPT II in the PPARα-knockout mouse are controversial. Aoyama et al. [4] and Watanabe et al. [40] reported that the immunoreactive CPT II protein present in the liver of wild-type mice, while Hashimoto et al. [2] reported a 50% reduction in the binding of CPT II gene promoter in HepG2 cells. The results concerning the transcriptional activity of the gene throughout the binding to the same element. Binding of ERRα was dramatically affected by mutation of the conserved half-site, but the mutations in the second half-site and flanking region did not affect the binding.

Although PPARα has been proposed as a mediator of the transcriptional effects of LCFAst and fibrates (reviewed in [19]), in foetal hepatocytes LCFAst had no effect on the level of the CPT II transcript, which was already high in these cells, in contrast with the strong induction it exerts on L-CPT I mRNA. However, the peroxisome proliferator, clofibrate, increased the levels of both CPT II and L-CPT I mRNA in foetal hepatocytes [6]. The lack of induction of CPT II in response to fatty acids in foetal hepatocytes or after birth may be associated with the high levels of both CPT II and L-CPT I mRNA in foetal hepatocytes.
expression of this gene observed at these stages. Recent studies [43] propose that fatty acids may regulate transcription in a PPARα-independent way, suggesting independent pathways for peroxisome proliferators and LCFA responses. This could explain that CPT II is a target gene for PPARα but not for LCFArs. However, this explanation seems unlikely as the PPARα-knockout loses the CPT II induction by starvation in kidney [2], indicating that the adaptive response to fasting, and probably to LCFArs, is mediated by PPARα.

Mice lacking the PPARαs show an altered constitutive or induced expression of genes encoding several mitochondrial fatty acid-catalyzing enzymes [31]. Although hepatic fatty acid oxidation is dramatically impaired in fasted PPARα-null mice, the L-CPT I mRNA levels are not so clearly diminished [44,45], indicating that PPARα does not play a crucial role in the L-CPT I response to fasting. If the expression of the L-CPT I in the knockout is not the cause of the diminished β-oxidation during fasting, then a low rate of fatty acid oxidation could be caused by the lack of induction of CPT II. Recent results point out that impaired β-oxidation could also be caused by the lack of induction of the HMG-CoA mitochondrial synthase, which leads to the accumulation of acetyl-CoA or β-oxidation intermediates that are strong inhibitors of acyl-CoA dehydrogenases [46].

This study shows that the human CPT II gene is a target of PPARα. This nuclear receptor mediates its effect through a PPRE that contains a poorly conserved second half-site and a conserved first half-site that seems to act as a core for the binding of other nuclear receptors such as COUP-TF I or ERRα. These results, together with the identification of a functional FXR/RXR-binding element [39] sharing one half-site with the proposed PPRE, indicate that an interplay of factors is regulating the human CPT II gene expression and confirm the relevance of nuclear receptors controlling mitochondrial fatty acid oxidation.

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