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 PPAR α *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 no 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 PPAR α . As expected, other members of the nuclear receptor superfamily also bind to this element and repress the activation mediated by PPAR α , 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 induced by LCFAs at the transcriptional level in hepatocytes of foetal rats [6], or during the foetal–neonatal transition [7,8], the activity, and mRNA levels of CPT II, are already high in the livers of term fetuses. These levels remain 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 (ERR α) [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; ERR α , oestrogen-related receptor α ; FXR, farnesoid X-activated receptor; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; hRXR α , human RXR α ; L-CPT I, liver CPT I; LCFA, 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 ovalbumin 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 PPAR α 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 PPAR α -null animals [4,5]. The constitutive expression of several fatty acid-utilization genes, including CPT II, was also examined in hearts of PPAR $\alpha^{-/-}$ 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 PPAR α . 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 limited role in PPAR α binding, followed by a conserved A/T flanking region. In addition we show that the orphan receptors COUP-TF I and ERR α also bind to this element, modulating the PPAR α *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'-CTAAGATCAGGGATTGGG-GCGTGG-3') and DH74 (5'-GGCTCCATCCTTGCAGAC-TGTGG-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 *Pst*I/*Xba*I, 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) *Pst*I/*Xba*I sites to generate the construct p477CPTII-CAT. Using this construct as a template, a PCR fragment was amplified using oligonucleotides CATF (5'-CTTCCGGCTCGTATGTTGTG-3'; complementary to positions +164 to +183 of pCAT-Basic) and DH221 (5'-ATATGAGATAAAGATAACATGTTATGCACAGTGC-AT-3'; located between positions -29 to +7 of the CPTII gene) and cloned into the *Sma*I site of the pGL3 Basic reporter plasmid (encoding the firefly luciferase reporter gene; Promega). The resulting construct was called pCPTIIA.

Deletion constructs of the 5'-regulatory region were formed as follows. pCPTIIB was formed by PCR amplification with pCPTIIA as a template, using primers DH173 (5'-AAATGCTgc-AGCCAAGAATGGATG-3'; nucleotides in lower case were changed to provide a *Pst*I site), corresponding to positions -286 to -263, and DH221, phosphorylating and cloning the product into the pGL3 Basic *Sma*I site. For plasmids pCPTIIC

and pCPTIID, which were constructed in an identical manner, the forward primers DH175 (5'-CCGCTCtGcAGGGGCTTTC-3'; corresponding to co-ordinates -245 to -226) and DH 87 (5'-GCAGTAGCTcTGCAGGGTTCAGGG-3'; corresponding to co-ordinates -138 to -112; where nucleotides in lower case were changed to provide a *Pst*I site), respectively, and reverse primer DH221 were used. Plasmid pCPTIIE was constructed using primers DH291 (5'-gcacggctagCTAACAACAG-3'; corresponding to co-ordinates -77 to -68) and CPT2Rev (5'-gaattcctcgaGATATGAGATAAAGATAACATG-3'; position +8 to -14; nucleotides in lower case were added to provide *Nhe*I/*Xho*I sites at the 5' and 3' ends, respectively), digestion with *Nhe*I and *Xho*I and cloning into *Nhe*I and *Xho*I sites of pGL3 Basic.

The PPRE mutant construct pCPTIIA1M was generated by overlap-extension PCR [33]. The first PCR used forward primer DH294 (5'-ttgtgctagcTGAGGGATGATAGATCT-3'; position -462 to -446; lower-case sequence was added to generate an *Nhe*I restriction site) and reverse primer DH293 (5'-CCGT-GCTGAAaGcttTTGACATATAATGAC-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'-TATATGTCAAaagCtTTCAGCA-CGGATTAC-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 *Sma*I site. The PPRE mutant construct pCPTIIAFM was generated in the same way, using oligonucleotides DH294 and DH221, and the overlapping oligonucleotides DH524 (5'-TG-ACCTTCAGCACGccaACTAACAAC-3') and DH525 (5'-GTtgggCGTGCTGAAGGTCATTGACATA-3'). The heterologous promoter plasmid was constructed using pGL3-Promoter [which contains the simian virus 40 (SV40) promoter upstream of the firefly luciferase reporter gene]. It contains a fragment corresponding to co-ordinates -108 to -75 and was formed by cloning the oligonucleotide DH206 (5'-TTATATGTCAAT-GACCTTCAGCACGGATTACTAA-3') annealed to DH207 (5'-cgcgTTAGTAATCCGTGCTGAAGGTCATTGACATA-TAagct-3'; the lower-case sequences were added to generate *Sac*I and *Mlu*I cohesive ends) into the pGL3-Promoter *Sac*I and *Mlu*I 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 μ g of the reporter CPT II-luciferase gene construct and 500 ng of eukaryotic expression vectors encoding PPAR α , RXR α , COUP-TF I and ERR α (respectively: pSG5-mPPAR α , pJCXR8, pRSHCOUP and pERR α) 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 μ M 9-*cis*-retinoic acid, 30 μ M 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 PPAR α , human RXR α (hRXR α), human COUP-TF I and mouse ERR α] 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 PPAR α and δ were amplified by reverse-transcriptase PCR [36] using total RNA from HepG2 cells treated and untreated with 125 μ M linoleic acid (Sigma) for 24 h, respectively. PPAR γ 2 was amplified by PCR using the Human Fat Cell Quick-CloneTM cDNA (Clontech). Oligonucleotides for PCR amplification were designed in agreement with the human PPARs' described sequences (L02932, L07592 and U79012 for human PPAR α , δ and γ 2, respectively) and were designated ALPHA.F (5'-ctgcagctgactGCGATGGTGGACACGG-3'; position -3 to +17; taking +1 as translation initiation) and ALPHA.R (5'-ctgcagcggcgccgCTCAGTACATGTCCCTGTAG-3'; +1407 to +1387) for human PPAR α , DELTA.F (5'-ctgcagcccggtTCAGCCATGGAGCAGCCAC-3'; -6 to +12) and DELTA.R (5'-ctgcagcggcgccgCTTAGTACATGTCCTTGTAG-3'; +1325 to +1306) for human PPAR δ , and GAMMA.2F (5'-ctgcagcccggtGCTGTTATGGGTGAAAC-3'; -6 to +11) and GAMMA.R (5'-ctgcagcggcgccgCTAGTACAAGTCCTTGTAG-3'; +1517 to +1498) for human PPAR γ 2. The lower-case sequences in these six primers were added to generate restriction sites for cloning. The PPAR α PCR product was digested with *Sal*I and *Not*I, cloned into pBluescriptSK+ (Stratagene) *Sal*I/*Not*I sites and sequenced. Finally, the *Sal*I/*Not*I insert was liberated from pBSSK+ and cloned into *Sal*I/*Not*I sites of pGEX-4T-2 (Amersham Biosciences) to yield plasmid pGEXhPPAR α . PPAR δ and PPAR γ 2 PCR products were digested with *Sma*I and *Not*I, cloned into pBSSK+ *Sma*I/*Not*I sites and sequenced. The *Sma*I/*Not*I insert was finally liberated from pBSSK+ and cloned into pGEX-4T-2 *Sma*I/*Not*I sites to yield plasmids pGEXhPPAR δ and pGEXhPPAR γ 2 respectively. *E. coli* BL-21 cells were transformed with the pGEXhPPAR α , pGEXhPPAR δ or pGEXhPPAR γ 2 expression plasmids. Cells were cultured and induced with isopropyl β -D-thiogalactoside as described by the supplier. Purification of the recombinant proteins was assessed as described previously [37].

Electrophoretic mobility shift assay (EMSA)

Unprogrammed lysate (2 μ l) or mouse PPAR α with or without hRXR α (2 μ l) or hCOUP-TF I synthesized *in vitro* was preincubated on ice for 10 min in 10 mM Tris/HCl, pH 8, 0.05% (v/v) Nonidet P-40, 6% glycerol, 1 mM dithiothreitol and 2 μ g of poly(dI-dC). Mouse ERR α synthesized *in vitro* was preincubated on ice for 10 min in 10 mM Tris/HCl, pH 8, 40 mM KCl, 0.05% (v/v) Nonidet P-40, 6% glycerol, 1 mM dithiothreitol, 2 μ g of poly(dI-dC), 0.1 μ g of BSA and 0.1 μ g of salmon sperm DNA. For competition experiments the indicated molar excess of unlabelled double-stranded probe, relative to the labelled probe, was included during preincubation. For supershift analysis 2 μ g of anti-PPAR α or anti-COUP-TF I antibodies (Santa Cruz Biotechnology) was included during preincubation. For binding quantification, labelled probes were incubated with 400 ng of the *E. coli*-expressed and -purified recombinant PPARs and 2 μ l of hRXR α synthesized *in vitro*. The FLPPRE and FL1MPPRE probes resulted from the annealing of oligonucleotides DH206 and DH207, and DH292 and DH293, respectively. The WTPPRE probe resulted from the annealing of oligonucleotides DH489 (5'-ctagcTGACCTTCAGCACGGATTAC-3') and DH490 (5'-

tcgaGTAATCCGTGCTGAAGGTCAg-3'; nucleotides in lower case in these primers and those shown below were added to provide *Nhe*I/*Xho*I sites at the 5' and 3' ends, respectively). The 1MPPRE probe resulted from the annealing of oligonucleotides DH491 (5'-ctagcagCtTTCAGCACGGATTAC-3'; the nucleotides in underlined lower case correspond to those changed from the wild-type sequence) and DH492 (5'-tcgaGTAATCCGTGCTGAAGGcttg-3'). The 2MPPRE probe resulted from the annealing of oligonucleotides DH493 (5'-ctagcTGACCTTCAGtACG-GATTAC-3') and DH494 (5'-tcgaGTAATCCGTaCTGAAGG-TCAg-3'), and FMPPRE resulted from the annealing of oligonucleotides DH495 (5'-ctagcTGACCTTCAGCACGccaAC-3') and DH496 (5'-tcgaGTtgggCGTGCTGAAGGTCAg-3'). The ERRsite probe was produced by the annealing of oligonucleotides DH588 (5'-ctagCAATGACCTTCAGC-3') and DH589 (5'-tcga-GCTGAAGGTCAATTG-3'). ³²P-labelled probe (2 ng) was then added and the incubation was continued for 15 min at room temperature. The final volume for all reactions was 20 μ l. Samples were electrophoresed at 4 °C on a 4.5% polyacrylamide gel in 0.5 $\times$ TBE buffer (45 mM Tris/45 mM boric acid/1 mM EDTA, pH 8.0). The retarded complexes were quantified using Storm PhosphorImager[®] (Amersham Biosciences).

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% (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 μ g of anti-PPAR α antibody (Santa Cruz Biotechnology) or 20 μ g 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 μ l of water and 10 μ l aliquots were used as template for PCR reaction. Primers used for promoter amplification were DH176 (5'-CTCCTGCA-GAAATCCTTTCTAATATCC-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-GCACATCTGAAGTAC-3'; +1321 to +1340 of the human CPT II cDNA) and DH583 (5'-ACGGCAGTAGAGCCATCC-TTG-3'; position +1618 to +1598 of the human CPT II cDNA).

RESULTS

The human CPT II gene contains a PPAR α -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 PPAR α expression vector (pSG5-mPPAR α) and a hRXR α expression vector (pJCXR8) in HeLa cells (Figure 1A). Addition of PPAR α ligands (Ly-171883 or Wy-14,643) and RXR α ligands (9-*cis*-retinoic acid) further increased the transcriptional activity of the promoter.

PPAR α 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 PPAR α (Figure 2),

Figure 1 Transcriptional activation of the human CPT II gene promoter by PPARα/RXRα and ligands

Localization of the human CPT II gene PPRE by progressive deletion analysis. **(A)** pCPTIIA plasmid, containing the 5' regulatory region of the human CPT II gene from position -477 to +7 cloned in front of the luciferase reporter gene was transfected alone or co-transfected with expression vectors for PPARα and RXRα into HeLa cells in the absence or presence of Ly-171883 (30 μM)/9-*cis*-retinoic acid (1 μM) or Wy-14,643 (30 μM)/9-*cis*-retinoic acid (1 μM). The Figure shows means ± S.D. of *Renilla* normalized luciferase activity from three independent transfections with two plates each. Statistical analysis of the data (Student's *t* test) confirmed that the increase in transcriptional activity caused by addition of ligands was significant ($P < 0.05$ when compared with luciferase activity in the presence of PPARα/RXRα and no treatment). No significant difference was found between treatments with Ly-171883 and Wy-14,643 ($P > 0.05$). **(B)** Deleted constructs of the human CPT promoter. Numbers on the left denote the 5'-deletion end point of the construct. **(C)** Luciferase reporter constructs containing the indicated position of the 5'-flanking region of the CPT II gene were co-transfected with or without pSG5-mPPARα into HepG2 cells. Means ± S.D. of *Renilla* normalized luciferase activity are shown from three independent transfections with two plates each, expressed as fold induction relative to the activity of each construct in the absence of PPARα.

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

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.

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 (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 5 The human CPT II gene PPRE interacts with other nuclear receptors

(A) EMSA of the CPT II gene PPRE with PPAR α /RXR α , COUP-TF I and ERR α . 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, ERR α or mouse PPAR α /hRXR α (lanes 3–5 respectively) and analysed by EMSA. (B) COUP-TF I and ERR α repress the induction of the CPT II gene by PPAR α . Luciferase reporter construct containing from -514 to +7 of CPT II gene was co-transfected with expression vectors for mouse PPAR α and hRXR α in the absence or presence of increasing amounts of COUP-TF I or ERR α (lanes 3–6, transfected with 62.5, 125, 250 and 500 ng of plasmid encoding COUP-TF I; lanes 8–11, transfected with 62.5, 125, 250 and 500 ng of plasmid encoding ERR α) into HepG2 cells. The effect of COUP-TF I or ERR α alone was also tested, by transfecting 500 ng of plasmid encoding COUP-TF I or ERR α (lanes 7 and 12). Means $\pm$ S.D. of *Renilla* normalized luciferase activity, from three independent transfections with two plates each, are

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 $\pm$ 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 PPARs and 2 μ l of *in vitro*-synthesized RXR α , or 2 μ l of *in vitro*-synthesized COUP-TF I. Retarded complexes were quantified using a PhosphorImager[®]. Panel 1, the binding of the three PPAR isotypes to the wild-type probe (WTPPRE) taking the binding of PPAR γ 2 as 100%. The binding of PPAR α , δ and γ 2 isotypes, COUP-TF I and ERR α 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 ($P > 0.05$).

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 μ g of nuclear extract in the absence or presence of specific antibodies against PPAR α (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 μ g of CaCo-2 nuclear extract, lane 10 contains 2 μ l of *in vitro*-synthesized mouse ERR α and lane 11 shows free probe.

the element and caused about a 40 % reduction in the binding of PPAR δ and γ 2, but no significant differences were found between the binding of PPAR α 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 ERR α 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 PPAR α /RXR α (Figure 1A). The addition of PPAR α and RXR α 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 PPAR α in cell culture (Figure 1C). Using EMSA (Figure 3), PPAR α -unresponsive SV40 promoter chimaeric constructs (Figure 4A) and site-directed mutagenesis (Figure 4B), we localized an element sufficient and necessary to mediate the PPAR α 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 PPAR α -knockout mice are controversial. Aoyama et al. [4] and Watanabe et al. [40] reported that the immunoreactive CPT II protein present in the liver of mice lacking PPAR α was not diminished when compared with the wild-type mice, while Hashimoto et al. [2] reported a 50 % decrease of CPT II protein in the liver of PPAR α -knockout mice and a 30 % decrease in kidney. In hearts of PPAR α ^{-/-} mice the constitutive expression of CPT II gene is reduced by about 77 % compared with the wild-type animals [40]. The fact that HepG2 cells express low levels of PPAR α [41] and that the mutation of the element in the conserved half-site abolishes the basal activity of this gene in HepG2 cells (Figure 4B) raises the possibility that factors other than PPAR α are involved in maintaining the basal promoter activity of the gene throughout the binding to the same *cis* element. According to that, introduction of mutations on the flanking region affected the PPAR binding but did not affect the basal activity of the promoter, indicating that the basal activity is maintained by other factor(s) different from PPAR in HepG2 cells.

The knockout data suggest that the importance of PPAR α in the regulation of the CPT II gene expression depends on the tissue in which CPT II is expressed, pointing to an interplay of factors regulating the expression of the gene. Besides the heterodimer PPAR/RXR, the sequence described in this report can be recognized by other members of the nuclear receptor superfamily. COUP-TF I and ERR α (Figure 5), nuclear orphan receptors involved in the control of fatty acid-metabolism-related genes, are able to bind to the CPT II promoter *in vitro* and repress the activation induced by PPAR α , providing a way of modulating the PPAR α induction. We wanted to demonstrate that nuclear receptors present in nuclear extract from cells expressing PPAR α , RXR α , COUP-TF I and ERR α were able to bind the characterized element. We used nuclear extracts from CaCo-2 cells because low levels of endogenous PPAR α have been described in HepG2 cells [41], and we detected good levels of endogenous PPAR α in CaCo-2 cells by reverse-transcriptase PCR (results

not shown). Using nuclear extracts from CaCo-2 cells we demonstrate that PPAR α and COUP-TF I bind to the same element (Figure 5C). When a probe (ERRsite) that does not bind PPAR or COUP-TF I but contains the ERR α -binding site was used, a complex appears that could correspond to ERR α . These results raise the possibility of competition between the PPAR α /RXR α heterodimer and either COUP-TF I or ERR α binding to the same region and controlling the CPT II gene expression.

We found that the human CPT II gene PPRe was located between positions -98 and -78. This sequence appears to be a novel PPRe. It contains one half-site which is a perfect consensus sequence (TGACCT) and has no clearly recognizable second half-site (CAGCAC), containing only one match to the consensus, followed by an A/T-rich flanking region. The second half-site is the least conserved of all the PPRes described thus far, as only one base is conserved. To further characterize the element, we performed EMSA with mutated probes (Figure 6) and purified human PPAR α , δ and γ 2. As in most of the described PPRes [18], the CPT II PPRe binds more efficiently to PPAR γ 2 than α or δ . Introducing mutations in the first half-site dramatically diminished the binding of the three PPAR isotypes, due to the incapability of RXR for binding to the mutated half-site. Mutation of the single conserved nucleotide of the second half-site had no significant effect on the binding of PPAR α , but reduced the PPAR δ and γ 2 binding. This result indicates that the binding of PPAR α to the PPRe does not depend on the conservation of a second half-site, while PPAR δ and γ 2 binding depends more on its conservation. Mutation of the flanking region decreased the binding of all PPARs. These data confirm that, as in other PPRes, the binding of PPARs to the element is more dependent on the flanking region composition than on the second half-site itself. The introduction of mutations in the first half-site or flanking region in the context of the whole promoter (Figure 4) abolished the PPAR α responsiveness of the promoter, correlating function and binding. The binding of COUP-TF I to the element is extremely dependent on the first half-site conservation and completely independent on the composition of the flanking region. ERR α has been described to bind as a monomer to an extended half-site (5'-TCAAGGTCA-3' [24,42]). In agreement with this, mutations in the first half-site dramatically affected the binding, but mutation in the second half-site and flanking region did not affect it. Thus, the first half-site seems to be a common element extremely important for the binding of different nuclear receptors to the element, and this explains its conservation. The novelty of the characterized element is the presence of a poorly conserved second half-site, whose composition is not relevant for the binding of PPAR α .

In order to demonstrate the *in vivo* relevance of this partially conserved element, we performed ChIP assays using an antibody against PPAR α (Figure 2). We performed the assay in CaCo-2 cells, due to the low levels of endogenous PPAR α described in HepG2 and the good levels of endogenous PPAR α detected in CaCo-2. We immunoprecipitated the human CPT II gene promoter using the anti-PPAR α antibody, indicating that the described element binds PPAR α *in vivo*.

Although PPAR α has been proposed as a mediator of the transcriptional effects of LCFAs and fibrates (reviewed in [19]), in foetal hepatocytes LCFAs 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

expression of this gene observed at these stages. Recent studies [43] propose that fatty acids may regulate transcription in a PPAR α -independent way, suggesting independent pathways for peroxisome proliferators and LCFA responses. This could explain that CPT II is a target gene for PPAR α but not for LCFAs. However, this explanation seems unlikely as the PPAR α -knock-out loses the CPT II induction by starvation in kidney [2], indicating that the adaptive response to fasting, and probably to LCFAs, is mediated by PPAR α .

Mice lacking the PPAR α show an altered constitutive or induced expression of genes encoding several mitochondrial fatty acid-catabolizing enzymes [31]. Although hepatic fatty acid oxidation is dramatically impaired in fasted PPAR α -null mice, the L-CPT I mRNA levels are not so clearly diminished [44,45], indicating that PPAR α 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/and 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 PPAR α . 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 ERR α . 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.

We are indebted to Dr Ronald M. Evans, Dr Stephen Green, Dr Ming-Jer Tsai and Dr Vincent Giguère for supplying the expression vectors for RXR α , PPAR α , COUP-TF I and ERR α , respectively. This research was supported by grants PB97-0958 and SAF2001-2923-C02-01 from the Dirección General de Investigación Científica y Técnica and the Fundació la Marató de TV3. N.C. is the recipient of a predoctoral fellowship from the Ministerio de Educación y Ciencia.

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Received 31 May 2002/22 October 2002; accepted 31 October 2002

Published as BJ Immediate Publication 31 October 2002, DOI 10.1042/BJ20020851