The acyl-CoA thioesterase I is regulated by PPARα and HNF4α via a distal response element in the promoter

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Abstract The cytosolic acyl-coenzyme A thioesterase I (Acot1) is an enzyme that hydrolyzes long-chain acyl-CoAs of C_{12}-C_{20}-CoA in chain length to the free fatty acid and CoA. Acot1 was shown previously to be strongly upregulated at the mRNA and protein level in rodents by fibrates. In this study, we show that Acot1 mRNA levels were increased by 90-fold in liver by treatment with Wy-14,643 and that Acot1 mRNA was also increased by 15-fold in the liver of hepatocyte nuclear factor 4α (HNF4α) knockout animals. Our study identified a direct repeat 1 (DR1) located in the Acot1 gene promoter in mouse, which binds the peroxisome proliferator-activated receptor α (PPARα) and HNF4α. Chromatin immunoprecipitation (ChIP) assay showed that the identified DR1 bound PPARα/retinoid X receptor α (RXRα) and HNF4α, whereas the binding in ChIP was abrogated in the PPARα and HNF4α knockout mouse models. Reporter gene assays showed activation of the Acot1 promoter in cells by the PPARα agonist Wy-14,643 after cotransfection with PPARα/RXRα. However, transfection with a plasmid containing HNF4α also resulted in an increase in promoter activity. Together, these data show that Acot1 is under regulation by an interplay between HNF4α and PPARα.—Dongol, B., Y. Shah, I. Kim, F. J. Gonzalez, and M. C. Hunt. **The acyl-CoA thioesterase I is regulated by PPARα and HNF4α via a distal response element in the promoter. *J. Lipid Res.* 2007.** 48: **1781–1791.**

Supplementary key words peroxisome proliferator response element • peroxisome proliferator-activated receptor α • direct repeat 1 • acyl-coenzyme A • lipid metabolism • hepatic nuclear factor 4α

Nuclear receptors are ligand-activated transcription factors that regulate the expression of a myriad of genes by binding to specific DNA elements. One example is the peroxisome proliferator-activated receptor α (PPARα), a nuclear receptor identified in 1990 by Issemann and Green (1), which at that time was shown to be activated by peroxisome proliferators. PPARα is involved in the transcriptional control of numerous genes involved in β-oxidation of fatty acids in mitochondria and peroxisomes, bile acid metabolism and inflammation control (for review, see Ref. 2), lipoprotein metabolism (3), glycerol metabolism (4), and amino acid metabolism (5). Targeted disruption of the PPARα in mouse by Lee et al. (6) in 1995 substantiated the role of this nuclear receptor in the regulation of lipid homeostasis. The binding of the PPARα as a heterodimer with the retinoid X receptor (RXR) to a direct repeat 1 (DR1) element (AGGTCAn-AGGTCA or variants thereof) in the promoter/intron of target genes is now widely established (2). Studies of endogenous ligands for PPARα showed that this nuclear receptor is activated by a variety of free fatty acids, such as linoleic acid, linolenic acid, and arachidonic acid, but is also weakly activated by saturated long-chain fatty acids (7–9). More recently, acyl-CoAs, the activated form of fatty acids, were also shown to activate PPARα (10). Thus, the intracellular levels of acyl-CoAs and free fatty acids are important in the regulation of this nuclear receptor. This DR1 element that binds the PPARα/RXR heterodimer can also bind another nuclear receptor, hepatic nuclear factor 4α (HNF4α), as a homodimer (11). HNF4α (NR2A1) is expressed in liver, kidney, intestine, and pancreas (12) and is central to the maintenance of hepatocyte differentiation and the regulation of genes involved in lipid metabolism (13). The ligands for HNF4α have been shown to be various acyl-CoA esters, which act as activators/inhibitors, depending on the chain length and degree of saturation (14).

Interestingly, there is a family of enzymes that hydrolyze acyl-CoAs to the free fatty acid and CoA, and acyl-CoAs/free fatty acids act as either agonists or antagonists for PPARα and HNF4α. These enzymes are called acyl-CoA thioesterases (Acots) (for review, see Ref. 15), and a new

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Abbreviations: Acot1, acyl-coenzyme A thioesterase I; ChIP, chromatin immunoprecipitation; DR1, direct repeat 1; EMSA, electromobility shift assay; HNF4α, hepatic nuclear factor 4α; MCAD, medium-chain acyl-coenzyme A dehydrogenase; OTC, ornithine transcarbamylase; PPARα, peroxisome proliferator-activated receptor α; PPRE, peroxisome proliferator response element; RXR, retinoid X receptor.

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nomenclature was recently introduced (16). One member of this gene family, the cytosolic acyl-coenzyme A thioesterase I (Acot1, but previously known as CTE-I), was identified as a peroxisome proliferator-induced enzyme in rodent liver (17–20).

The Acot1 cDNA was initially cloned from rat (21) and was shown to be induced in liver by the peroxisome proliferator di(2-ethylhexyl) phthalate (22). At the same time, the cDNA (17) and gene (19) were cloned from mouse and were also shown to be induced by the peroxisome proliferator clofibrate in liver at both the mRNA and protein levels (18, 19). Acot1 is mainly expressed in liver, kidney, heart, and lung and is upregulated by fasting in kidney (18). Acot1 is also strongly upregulated by fasting in liver and heart in a partly PPARα-independent manner (18) and is upregulated under diabetic conditions in rat liver (23). Although it has been shown that Acot1 is upregulated by fibrates and fasting via PPARα, an actual peroxisome proliferator-response element (PPRE) has not been identified. In this study, we identify the response element involved in the activation of Acot1 by PPARα/RXRα. The response element located between −9,600 and −9,612 bp upstream of the translation start site of ATG in Acot1 bound to the PPARα/RXRα in vivo and in vitro and was activated in a cell system in the presence of Wy14,643, a PPARα ligand. Interestingly, the same DR1 element also bound HNF4α, resulting in a slight increase of promoter activity. In vivo, the Acot1 mRNA was induced by 15-fold in the HNF4α knockout mouse, suggesting that Acot1 may be regulated by HNF4α. Our data show that Acot1 is regulated by a delicate interplay between PPARα/RXRα and HNF4α.

MATERIALS AND METHODS

Identification of a DR1 in the promoter of the *Acot1* gene

The gene structure of the mouse *Acot1* had previously been identified from a P1 clone purchased from Genome Systems, Inc. (19). Approximately 6 kb of the genomic sequence of *Acot1* upstream of the ATG start site was sequenced from this phage clone. This 6 kb sequence was used to blast against the mouse genomic sequence (www.ncbi.nlm.nih.gov) to obtain the full sequence of the *Acot1* promoter region. Computer-assisted and visual analysis was used to identify putative response elements.

Animals and treatment

Liver was excised from male HNF4α floxed (F/F) and HNF4α knockout (HNF4α ΔL) mice aged between 45 and 56 days as described (13). Ten to 12 week old wild-type or PPARα-null male mice on a pure Sv/129 genetic background were treated for 1 week with 0.1% (w/w) Wy-14,643 (Calbiochem-Novabiochem International) as described previously (24). Animals were euthanized by CO_2 asphyxiation followed by cervical dislocation, and liver was excised and stored at −70°C for preparation of total RNA. All studies were carried out with ethical permission from the Animal Ethics Board in Stockholm, Sweden.

Reporter gene assay constructs

Two constructs in the 5′ flanking region of mouse *Acot1*, containing the putative DR1, were constructed by PCR from a genomic P1 clone containing the *Acot1* gene (19) using the following primers: 5′-**CTCGAG**CAGACTTGAAGGCAGATGGTTT-3′ and 5′-**CTCGAG**TTTTTCTTCCTTGTGTTGTAATCC-3′ (1,861 bp) and 5′-**CTCGAG**CACCGGAGTCACCTGATAGAGTC-3′ and 5′-**CTCGAG**GCCAGGGTGCACAGACTTT-3′ (157 bp), with the *Xho*I sites indicated in boldface. The first construct was 1,861 bp in length and corresponded to bp −8,659 to −10,520 upstream of the ATG start site in the *Acot1* gene. A shorter construct of 157 bp was also generated and corresponded to −9,506 to −9,663 upstream of the ATG start site. PCR was performed as follows: 94°C for 30 s, 61°C for 30 s, 72°C for 3 min (or 1 min for 157 bp), and 72°C for 10 min. The PCR products were cloned into the luciferase reporter vector pGL3 promoter (constructs named pGL3-Acot1-1861 and pGL3-Acot1-157). Sequences were verified using the ABI Prism Dye Terminator Ready-Reaction Kit (Perkin-Elmer). Mutations were introduced into the DR1 element using the Quikchange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions using 16 cycles. The primers used were 5′-GGGCTGAGTTGGAACTGGG-*TTT*AA*AAA*CATGGCAAGCTCTC-3′ (and the reverse complement primer), with the mutated bases underlined in italics (constructs named pGL3-Acot1Δ1861 and pGL3-AcotΔ157). The PPARα and RXRα plasmids were as described previously (24), and HNF4α in pCMV (25) was a kind gift from Dr. John Chiang.

Chromatin immunoprecipitation assay

Freshly isolated livers were ground to a fine powder under liquid nitrogen and cross-linked in 1% formaldehyde in 1× PBS at 37°C for 20 min. Cross-linking was terminated with 0.125 M glycine, and the cell pellet was washed twice with 1× PBS. Nuclei were isolated and lysed in an SDS lysis buffer (50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors). Chromatin was sheared by sonication, and the nuclear lysate was cleared by centrifugation at 50,000 *g* for 30 min. The soluble chromatin was diluted 10-fold (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, and 167 mM NaCl) and immunoprecipitated with primary antibody for PPARα (Geneka) or HNF4α (K2915; Abcam). The antibody/protein/DNA complex was isolated using magnetic beads conjugated with protein A (New England Biolabs). After several washes, the protein/DNA complex was eluted (50 mM $NaHCO_3$ and 1% SDS) from the magnetic beads, and cross-linking was reversed by incubation at 65°C overnight. The samples were incubated with proteinase K for 1 h at 45°C, and after protein digestion, the DNA was purified using phenol-chloroform-isoamyl alcohol extraction; 2–5 μl of sample was used for PCR. PCR was performed for 31 cycles as follows: 94°C for 3 min, followed by 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by 72°C for 7 min. Primers used were Acot1 DR1 forward (5′-CACCGGAGTCACCTGATAGAGTC-3′) and reverse (5′-GCCAGGGTGCACAGACTTT-3′) amplifying an area of 157 bp (∼9.6 kb upstream of the ATG start site) containing the DR1. The chromatin immunoprecipitation (ChIP) negative primers amplified an area of 141 bp located ∼1.8 kb upstream of the ATG start methionine, which does not contain any DR1. PCR products were resolved on a 2% agarose gel.

Cell culture and transfections

HepG2 cells were routinely cultured in EMEM (Sigma Corp.), L-glutamine, 10% fetal bovine serum, and penicillin/streptomycin (100 U/ml each) in an atmosphere of 5% CO_2. HeLa cells were cultured in EMEM (Sigma Corp.) with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% nonessential amino acids, and penicillin/streptomycin (100 U/ml each) in an atmosphere of 5% CO_2. The cells were cultured on 24-well plates and grown to

~70% confluence before transfection. The transfections were carried out using Tfx-20 reagent (Promega Corp., Madison, WI) in a ratio of 1:2 (v/v) plasmid to reagent. Cells were transfected with 0.25 μg of *Acot1* promoter plasmids pGL3-Acot1-1861 and pGL3-Acot1Δ1861 or pGL3-Acot1-157 and pGL3-Acot1Δ157, 0.25 μg of the PPARα, RXRα, or HNF4α expression vector, and 0.3 μg of the pSV-β-galactosidase control vector. DNA concentrations were kept constant using pcDNA3.1(+) (empty vector) where appropriate. Twenty-four hours after transfection, the cells were treated with 50 μM Wy-14,643 or DMSO as vehicle where appropriate, as indicated in the figure legends. Cell lysates were assayed for luciferase activity using the Luciferase Reporter Gene Assay (Promega Corp.) and for β-galactosidase activity using the β-Galactosidase Enzyme Assay System (Promega Corp.). Experiments were carried out in triplicate wells, and two to four individual experiments were performed. Luciferase activity was normalized to β-galactosidase activity.

Electromobility shift assay

Oligonucleotides (Cybergene AB, Huddinge, Sweden) corresponding to the DR1 for the mouse *Acot1* were as follows: 5′-gctgagttggaactGGGGCAAAGTTCAtggcaagctct-3′ (plus the reverse complement primer), with the core sequence of the DR1 site indicated in uppercase letters. Mutated DR1 oligonucleotides were also used, with the mutations underlined in italics: 5′-gctgagttggaactGGG*TTT*AA*AAA*CAtggcaagctct-3′. Ten picomoles of each primer was annealed to give double-stranded probes and labeled with [γ-^{32}P]ATP (Amersham Biosciences) using T7 polynucleotide kinase (Promega Corp.). In vitro-translated PPARα, RXRα, and HNF4α were synthesized using the TNT Coupled Reticulocyte Lysate System (Promega Corp.). Gel mobility shift assay incubation mixes (25 μl) contained 10 mM Tris (pH 7.8), 20 mM KCl, 2 μg of BSA, 10% glycerol, 500 ng of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech), and 1 μl of in vitro-translated PPARα, RXRα, or HNF4α. Competition experiments were performed with 50-fold molar excess of unlabeled specific probes, and supershift was carried out using an RXRα antibody (a kind gift from Dr. Pierre Chambon) or an HNF4α antibody [Ab. HNF4α (C19)X; Santa Cruz Biotechnology, Inc.] as indicated in the figure legends. Labeled probe (50,000 cpm) was added to each reaction and incubated on ice for 45 min. The complexes were resolved on a 5% polyacrylamide gel in 1× Tris-borate-EDTA, and the gel was dried and exposed to X-ray film.

Real-time PCR

Total RNA was prepared from the livers of four male HNF4α floxed (F/F) and four HNF4α knockout mice (HNF4α ΔL) aged between 45 and 56 days as described (13) using Trizol Reagent (Invitrogen Corp.). Ten to 12 week old wild-type or PPARα-null male mice on a pure Sv/129 genetic background were treated for 1 week with 0.1% (w/w) Wy-14,643 (Calbiochem-Novabiochem International) as described previously (24). Total RNA was isolated from liver samples using the QuickPrep® Total RNA Extraction Kit (Amersham Biosciences) and DNase treated using RNeasy Protect (Qiagen). Mouse Acot1-specific PCR primers and probe (forward primer, 5′-CTGGCGCATGCAGGATC-3′; reverse primer, 5′-GGCACTTTTCTTGGATAGCTCC-3′; 5′-FAM-labeled TaqMan TAMRA probe, 5′-TGGGTTCAATCCAGCTGCGAGAAATAAAG-3′) were designed in the 3′ untranslated region of the *Acot1* gene using Primer Express Software (Applied Biosystems). One microgram of total RNA from each liver sample was reverse-transcribed into cDNA using iScript (Bio-Rad, Inc.). PCR amplifications were performed in triplicate using TaqMan Universal PCR Master Mix (Applied Biosystems). The relative amount of *Acot1* mRNA was quantified in liver tissue using single-plex real-time PCR analysis in an ABI PRISM® 7000 Sequence Detection System using eukaryotic 18S rRNA as an endogenous control (Applied Biosystems). The average threshold cycle value per treatment was used to calculate relative expression levels of mouse *Acot1* mRNA with the $^{\Delta\Delta Ct}$ method.

RESULTS

Acot1 is regulated by fibrate treatment via PPARα in vivo

It has been shown that Acot1 is upregulated at mRNA and protein levels in rodents by treatment with clofibrate (17–19) and di(2-ethylhexyl) phthalate (21). However, this upregulation at the mRNA level was shown only by Northern blot analysis; to quantitatively examine the level of induction of Acot1 mRNA in mouse liver by Wy-14,643, real-time PCR was carried out using specific primers toward Acot1. Acot1 was upregulated by 90-fold in mouse liver after Wy-14,643 treatment, whereas this upregulation was not evident in the PPARα-null animals, showing that the regulation of Acot1 by fibrates is PPARα-dependent (**Fig. 1**). To confirm that treatment of the animals with Wy-14,643 was successful, the upregulation of acyl-CoA oxidase, a well-known PPARα target gene, has been shown for the same RNA samples (24). Notably, the Acot1 basal mRNA level was doubled in the PPARα-null animals.

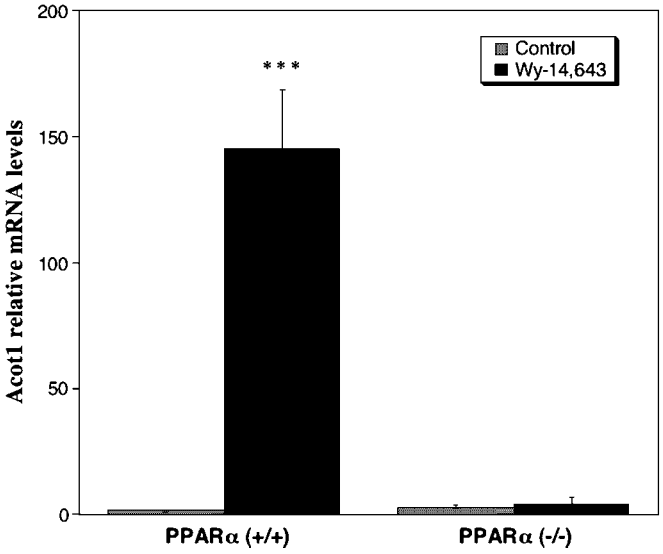

Fig. 1. Expression of acyl-coenzyme A thioesterase I (Acot1) mRNA is regulated by fibrate treatment in mouse liver. Total RNA was isolated from livers of wild-type peroxisome proliferator-activated receptor α (PPARα) (+/+) and PPARα-null (−/−) male mice fed 0.1% Wy-14,643 for 1 week. The relative mRNA levels of Acot1 were measured using TaqMan quantitative real-time PCR. mRNA levels for individual animals were measured in triplicate. The wild-type control value for one animal was set to 1. Calculations were performed according to the ΔΔCt method using eukaryotic 18S rRNA as an endogenous control. n = 3; error bars represent ±SD. *** $P < 0.003$, by unpaired Student's *t*-test.

Identification of a DR1 in the *Acot1* gene promoter

Acot1 is very highly induced in mouse liver by fibrate treatment; however, the actual PPRE in the *Acot1* gene has not been identified. We had previously sequenced 6 kb of the promoter region using conventional sequencing from a P1 clone known to contain the *Acot1* gene (19). However, this area contained only one putative PPRE ~2 kb upstream of the translation start site, which was tested using reporter gene systems and electromobility shift assay (EMSA) and was not functional (data not shown). The transcription start site is considered to be approximately nine nucleotides upstream of the ATG translation start site in Acot1 (19). The availability of the complete mouse genomic sequence enabled us to obtain the *Acot1* promoter sequence and identify an imperfect DR1 between −9,600 and −9,612 bp upstream of the ATG start site (**Fig. 2**). This DR1 was a good candidate to be a PPRE, as DR1 elements have been shown to bind to the heterodimer partnership PPARα/RXRα. It has also been shown that the seven nucleotides in the 5′ flanking region of identified PPREs have an important influence on binding of the PPARα (26). Notably, five of the seven base pairs in the 5′ flank of the DR1 element of the *Acot1* promoter were conserved, strongly suggesting that this could be a candidate for a functional PPRE.

The DR1 in the *Acot1* promoter is activated by Wy-14,643

To evaluate whether the identified DR1 in the *Acot1* promoter could be activated by PPARα ligands in a cell system, promoter constructs were generated in the pGL3 promoter vector containing an endogenous SV40 promoter. This vector was used to clone fragments of the Acot1 promoter region containing the DR1. One plasmid contained 1,861 bp fragment of the *Acot1* promoter region from −8,659 to −10,520 upstream of the ATG translation start site, containing the DR1 (pGL3-Acot1-1861), and another plasmid contained a mutated DR1 (pGL3-Acot1Δ1861). Cotransfection of these plasmids was carried out together with plasmids containing the PPARα and RXRα, as described in Materials and Methods. The activity of the pGL3-Acot1-1861 promoter construct in HepG2 cells was not increased by Wy-14,643 treatment in the absence of transfected PPARα/RXRα (**Fig. 3A**). However, after cotransfection with PPARα/RXRα, the promoter activity was increased significantly in the presence of PPARα/RXRα alone ($P < 0.001$). Addition of Wy-14,643, a PPARα ligand, resulted in a further increase in promoter activity ($P < 0.001$ for − pcDNA3.1 + Wy-14,643 vs. PPARα/RXRα + Wy-14,643). A construct containing two copies of the acyl-CoA oxidase PPRE (AOx-tk-Luc) was used as a positive control (27), which showed an induction by PPARα/RXRα + Wy-14,643 ($P < 0.001$) similar to that observed in the Acot1 native promoter (Fig. 3A). We also introduced mutations into the Acot1 DR1 (pGL3-Acot1Δ1861) to abolish binding of the PPARα/RXRα heterodimer. Transfection of HepG2 cells with the wild-type pGL3-Acot1-1861 vector resulted in a significant increase in promoter activity in the presence of PPARα/RXRα + Wy-14,643; however, mutation of the DR1 site abolished this activation (Fig. 3B), confirming that the identified DR1 is responsible for activation by PPARα/RXRα.

	5' flank	Core DR1 sequence
DR-1 consensus	CAAAACT GG T	AGGTCA A AGGTCA T
Acot1	TGGAACT	GGGGCA A AGTTCA

Fig. 2. The Acot1 promoter contains a direct repeat 1 (DR1). The core consensus sequence for the DR1 known to bind the PPARα/retinoid X receptor α (RXRα) heterodimer is shown (DR1 consensus), together with the 5′ flanking sequence. The putative DR1 for mouse *Acot1* is shown.

The Acot1 DR1 binds PPARα/RXRα in vivo and in vitro

To determine whether the DR1 identified at −9,600 to −9,612 in the *Acot1* promoter could bind directly to PPARα/RXRα, both ChIP and EMSA were carried out. The ChIP assay was carried out using wild-type and knockout animals for PPARα (6, 13), whereas EMSA was carried out using in vitro-translated PPARα/RXRα, as described previously (24).

ChIP resulted in the binding of PPARα to the Acot1 DR1 identified in wild-type mouse liver extract, and the binding was increased by treatment with Wy14,643 (**Fig. 4A**, upper panel). In the PPARα-null mouse liver extract, no binding to the Acot1 DR1 was evident, showing that DR1 binds to PPARα in vivo. As a negative control, primers amplifying an area of the Acot1 promoter (~1.8 kb upstream of the ATG start site) not containing the DR1 showed no binding by PPARα in either wild-type or knockout liver nuclei (Fig. 4A, lower panel). Using EMSA, neither PPARα nor RXRα bound to the Acot1 DR1 element individually; however, in the presence of both PPARα and RXRα, there was strong binding to the Acot1 DR1, showing that this element can bind directly to this heterodimer partnership (Fig. 4B). Addition of a 50-fold molar excess of cold Acot1 probe competed out the binding of the γ-^{32}P-labeled Acot1 probe, and supershift was evident in the presence of the RXRα antibody, confirming that the complex identified by EMSA contains RXRα. Mutations introduced into the DR1 abolished binding by PPARα/RXRα to the DR1 or any supershift by the RXRα antibody (Fig. 4B). The lack of binding to the mutated DR1 in EMSA also substantiates the lack of induction of promoter activity of pGL3-Acot1Δ1861 in a cell system (Fig. 3B).

The *Acot1* promoter is regulated by HNF4α

HNF4α also binds to a DR1 sequence; therefore, we examined the possible involvement of this receptor in the control of Acot1 expression. Transfection of HepG2 cells with empty pGL3 promoter vector in the presence of HNF4α resulted in a significant downregulation of promoter activity (the vector contains an endogenous SV40

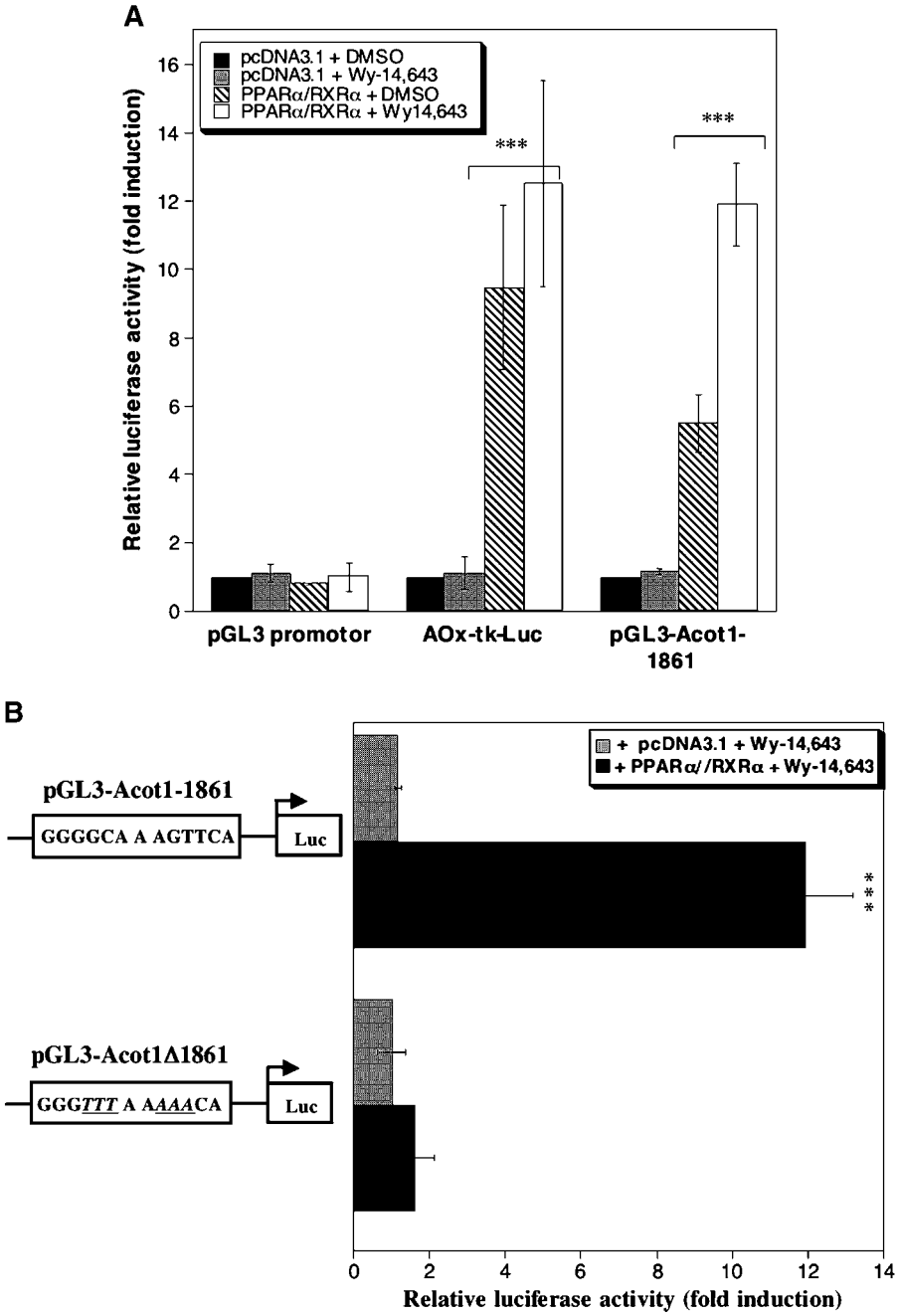

Fig. 3. The *Acot1* promoter is activated by PPARα/RXRα. HepG2 cells were transfected with promoter constructs containing empty pGL3 promoter vector, acyl-CoA oxidase (Acox1) peroxisome proliferator response element (PPRE; two copies) (AOx-tk-Luc), and pGL3-Acot1-1861. A: Cells were transiently transfected with the indicated promoter vector (0.25 μg) with or without PPARα (0.25 μg) and RXRα (0.25 μg). Empty pcDNA3.1+ vector was used to keep DNA concentrations equal in all transfections. At 24 h after transfection, cells were treated with vehicle (DMSO) or 50 μM Wy-14,643 for a further 24 h. Transfections were carried out in triplicate wells in three or four different experiments. Luciferase activity was normalized to β-galactosidase activity. Data shown are means ± SEM for Acox1 and Acot1. *** $P < 0.001$ for ligand activation (PPARα/RXRα + DMSO vs. PPARα/RXRα + Wy-14,643 by Student's *t*-test). B: Cells were transiently transfected with the *Acot1* promoter constructs pGL3-Acot1-1861 and pGL3-Acot1Δ1861 (0.25 μg) with or without PPARα (0.25 μg) and RXRα (0.25 μg). At 24 h after transfection, cells were treated with 50 μM Wy-14,643. Transfections were carried out in triplicate wells in three different experiments. Luciferase activity was normalized to β-galactosidase activity. Mutated bases in DR1 are underlined in italics. Data shown are mean ± SEM. *** $P < 0.001$ by unpaired Student's *t*-test.

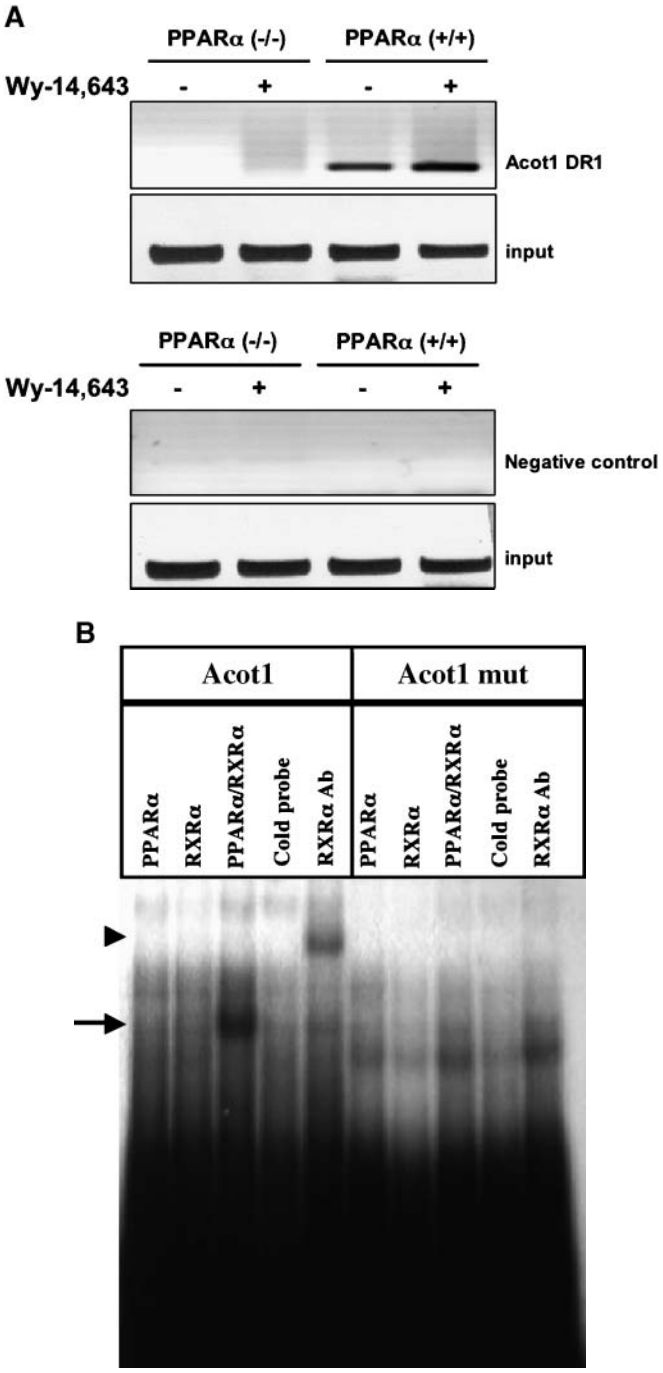

Fig. 4. The *Acot1* DR1 binds PPARα/RXRα in vivo and in vitro. A: Chromatin immunoprecipitation (ChIP) was carried out on liver nuclei of PPARα wild-type (+/+) and PPARα knockout (−/−) mice treated with Wy-14,643 using a PPARα antibody. After immunoprecipitation, an amplicon of 157 bp of the Acot1 promoter containing DR1 (located at ∼−9.6 kb) was amplified by PCR. Negative control was an amplicon of 141 bp in the Acot1 promoter (located at ∼−1.8 kb) that does not contain any DR1. B: Electromobility shift assay (EMSA) was carried out using 1 μg of in vitro-translated PPARα, RXRα, and hepatic nuclear factor 4α (HNF4α). γ-^{32}P-labeled probes for the Acot1 DR1 or mutated DR1 (Acot1 mut) were used. Cold probe was a 50-fold molar excess of unlabeled probe. Supershift experiments were carried out using an RXRα antibody (Ab). The PPARα/RXRα heterodimer is indicated by the arrow, and the supershift complexes are indicated by the arrowhead.

promoter); therefore, this system was not suitable for further experiments with HNF4α. As a result, HeLa cells were used for further experiments with HNF4α. As a positive control, a plasmid expressing the promoter of the hepatic ornithine transcarbamylase (OTC) gene in pGL3 Basic (OTC-235-Luc) was used (28), which was upregulated 2.5-fold by transfection with HNF4α in HeLa cells. *Acot1* promoter activity (pGL3-Acot1-157) was doubled by cotransfection with HNF4α; however, mutation of DR1 in pGL3-Acot1Δ150 abolished the upregulation via HNF4α (**Fig. 5A**). These data indicate that the Acot1 promoter is upregulated by HNF4α.

To assess whether HNF4α could compete for binding to the DR1 identified in the presence of PPARα/RXRα, HeLa cells were transfected with PPARα/RXRα and treated with Wy-14,643 in the presence of varying amounts of HNF4α. The Acot1 promoter construct pGL3-Acot1-157 was activated in the presence of PPARα/RXRα + Wy-14,643 (similar to that seen with pGL3-Acot1-1861 in HepG2 cells), whereas this activity was reduced significantly in the presence of 0.25 μg of HNF4α and was reduced further by transfection with 0.5 μg of HNF4α (Fig. 5B). The mutated Acot1 promoter pGL3-Acot1Δ157 also showed a very slight downregulation of promoter activity in the presence of cotransfected HNF4α; however, this was similar to the effect seen in the empty pGL3 promoter vector.

The Acot1 promoter binds to HNF4α in vivo and in vitro

ChIP and EMSA were also carried out on the same DR1 element using liver extracts from HNF4α floxed and knockout animals and in vitro-translated HNF4α. ChIP confirmed the in vivo binding of HNF4α to the same DR1 element in the *Acot1* promoter as the PPARα, and the specificity of this was again confirmed using liver extracts from the HNF4α knockout mouse (**Fig. 6A**), although some weak signal was detected in the knockout liver extracts. This may be attributable to the fact that HNF4α is a conditional liver-specific knockout and there is some very low amount of HNF4α protein present in the nucleus (29). EMSA was also performed using in vitro-translated HNF4α, and binding to the DR1 was competed out using a 50-fold molar excess of cold probe and supershifted using an HNF4α antibody (Fig. 6B). Mutation of the DR1 element abolished the binding of HNF4α or any supershift by the HNF4α antibody. These results confirm the identification of a DR1 element in *Acot1* that can bind both PPARα/RXRα and HNF4α in vivo and in vitro.

As the promoter of *Acot1* was regulated by HNF4α in HeLa cells and both ChIP and EMSA confirmed the binding of HNF4α to the DR1 identified, it seemed likely that Acot1 was regulated by HNF4α in liver. Therefore, Acot1 mRNA levels in the livers of HNF4α knockout animals were investigated. Real-time PCR showed that Acot1 mRNA levels were upregulated by 15-fold in the HNF4α knockout mouse model, indicating that Acot1 mRNA expression is affected by the loss of HNF4α expression in liver (Fig. 6C).

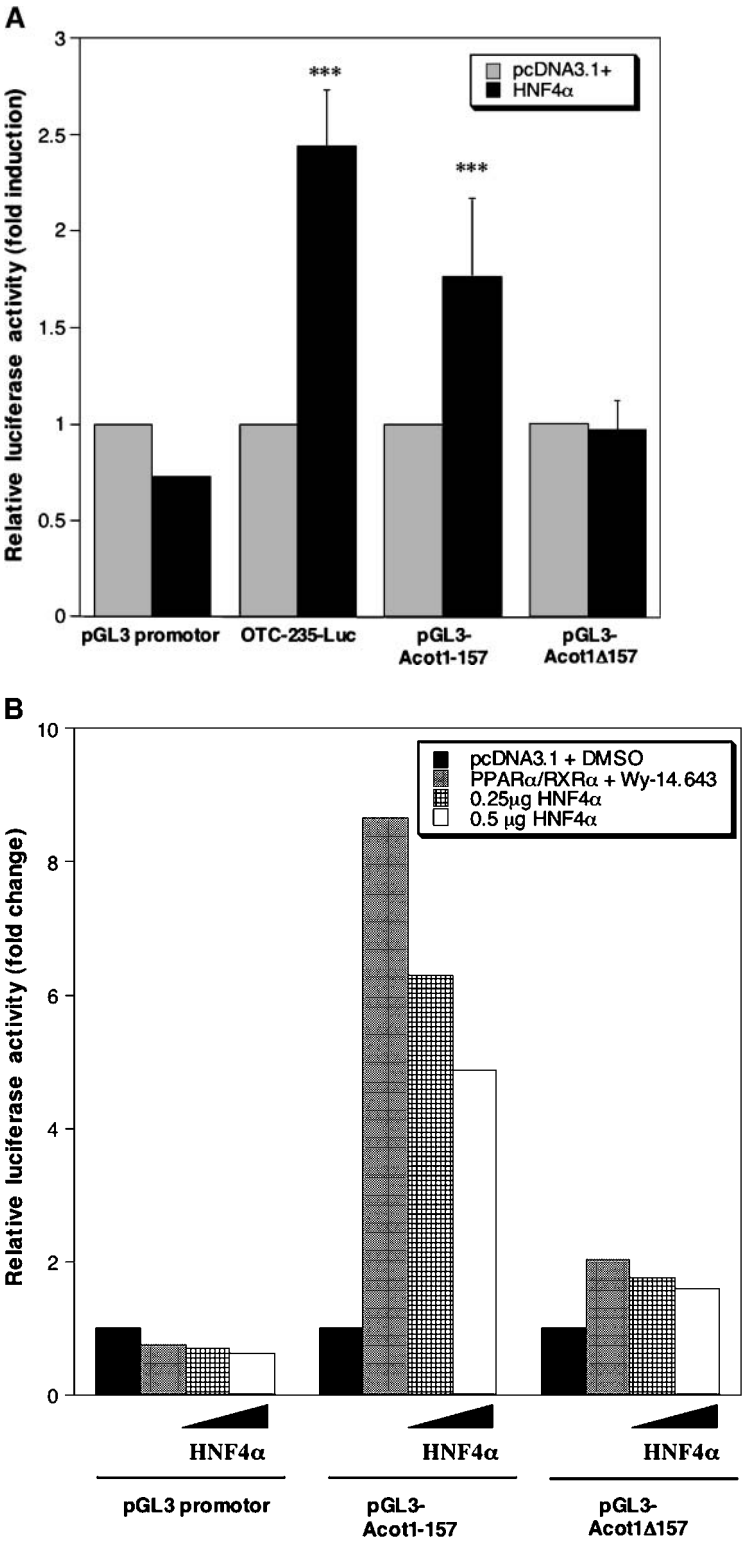

Fig. 5. The *Acot1* promoter is regulated by HNF4α. A: HeLa cells were transiently transfected for 48 h with 0.25 μg of the empty pGL3 promoter vector, ornithine transcarbamylase (OTC)-235-Luc, as a positive control (28), the *Acot1* promoter construct pGL3-Acot1-157, or the mutated *Acot1* promoter construct pGL3-Acot1Δ157, together with HNF4α (0.25 μg) or pcDNA3.1+ (empty vector) (0.25 μg). Transfections were carried out in triplicate wells in two or three different experiments. Luciferase activity was normalized to β-galactosidase activity. Data shown are ±SEM for OTC-235-Luc and Acot1. *** $P < 0.001$ by unpaired Student's *t*-test. B: HeLa cells were transiently transfected with 0.25 μg of the empty pGL3 promoter vector, the *Acot1* promoter construct pGL3-Acot1-157, or the mutated *Acot1* promoter construct pGL3-Acot1Δ157, PPARα (0.25 μg) and RXRα (0.25 μg), plus HNF4α (0.25 or 0.5 μg) or pcDNA3.1+ (empty vector) (0.25 or 0.5 μg). Cells were treated with 50 μM Wy-14,643 and incubated for 48 h. Transfections were carried out in triplicate wells in two different experiments. Luciferase activity was normalized to β-galactosidase activity.

DISCUSSION

We have shown in this study that *Acot1* is a target gene of both PPARα and HNF4α using animal models, ChIP, and reporter gene assays. Acot1 is positively regulated by PPARα through direct binding to a DR1 element located in the distal promoter. Interestingly, this DR1 also binds to HNF4α and results in an upregulation of Acot1 promoter activity. The binding of both of these receptors to the Acot1 DR1 element is intriguing, and ChIP shows that this element can be occupied by either nuclear receptor in vivo. Most likely, competition between these two receptors can regulate the transcription of the *Acot1* gene, either at the basal level or during perturbed lipid metabolism (fibrate treatment or fasting) (17, 18, 23, this study). The basal level of Acot1 mRNA is doubled in the liver of the

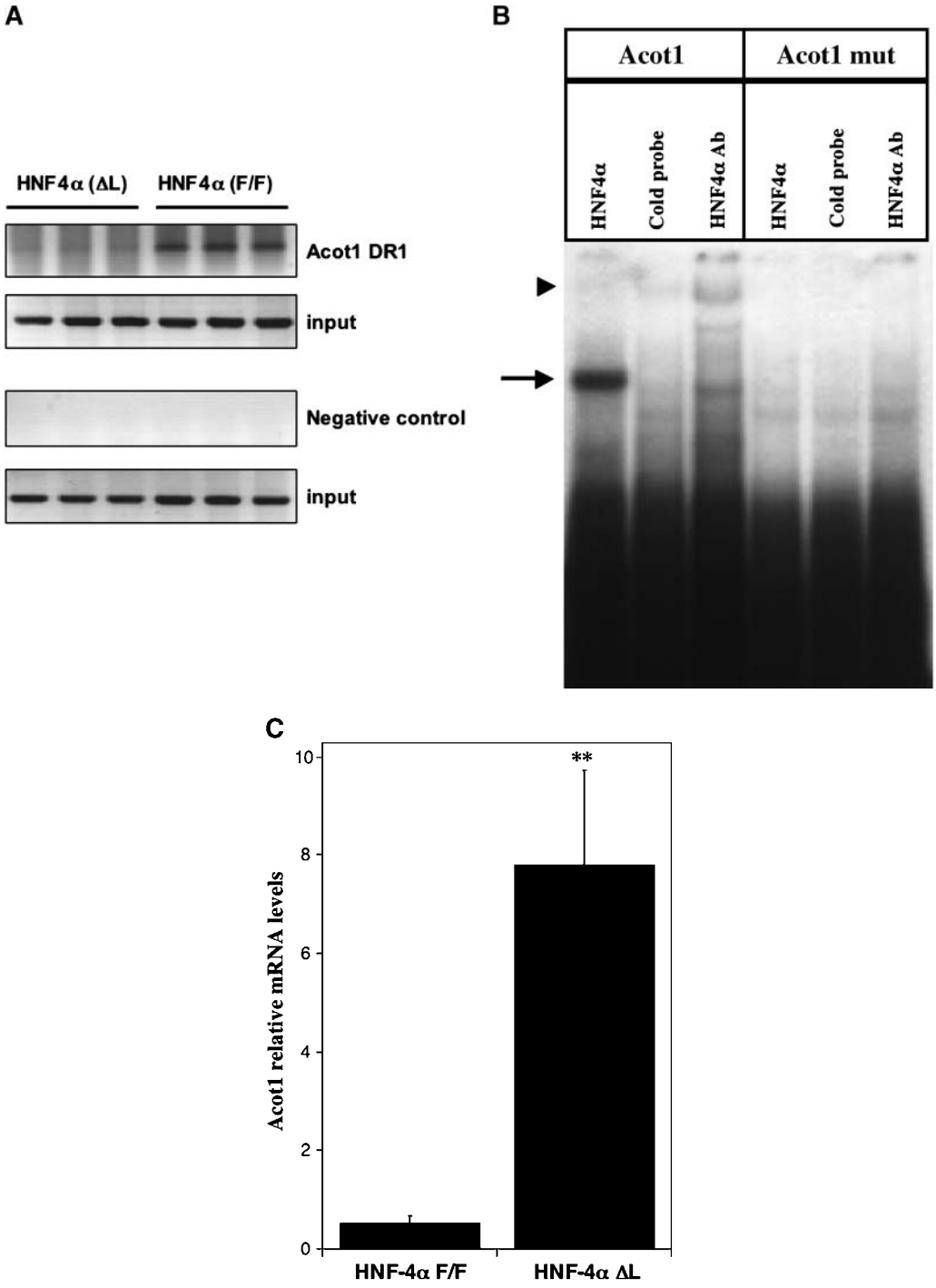

Fig. 6. *Acot1* is regulated by HNF4α. A: ChIP was carried out on liver nuclei of HNF4α flox (F/F) and HNF4α knockout (HNF4α ΔL) mice using an HNF4α antibody (K2915; Abcam). After immunoprecipitation, an amplicon of 157 bp of the Acot1 promoter containing the DR1 (located at ~−9.6 kb upstream of the ATG start site) was amplified by PCR. Negative control was an amplicon of 141 bp in the Acot1 promoter (located at ~−1.8 kb) that does not contain any DR1. B: EMSA was carried out using 1 μg of in vitro-translated HNF4α. γ-^{32}P-labeled probes for the Acot1 DR1 or mutated DR1 (Acot1 mut) were used. Cold probe was a 50-fold molar excess of unlabeled probe. Supershift experiments were carried out using an HNF4α antibody (Ab). The HNF4α homodimer is indicated by the arrow, and the supershift complex is indicated by the arrowhead. C: Total RNA was isolated from livers of control male HNF4α flox (F/F) or HNF4α knockout (HNF4α ΔL) mice. The relative mRNA levels of Acot1 were measured using quantitative real-time PCR, and mRNA for individual animals was measured in triplicate. The control value for one floxed animal was set to 1. Calculation were performed according to the ΔΔCt method using eukaryotic 18S rRNA as an endogenous control. n = 4; data shown are means ± SD. ** $P < 0.01$ by unpaired Student's *t*-test.

PPARα knockout mouse (Fig. 1), indicating that the Acot1 promoter is under slight repression by PPARα/RXRα in the fed state. Hayhurst et al. (13) showed that the expression of PPARα mRNA is downregulated in the HNF4α knockout mouse, which may result in a consequent upregulation of Acot1 in the HNF4α knockout (caused by lower expression levels of PPARα). However, considering that Acot1 mRNA is only doubled in response to a complete loss of PPARα, the reduced level of PPARα in the HNF4α knockout mouse could not account for the corresponding induction of Acot1 mRNA in HNF4α-null mice (15-fold) and therefore is more likely a consequence of the loss of HNF4α.

The HNF4α-null mouse model shows altered lipid homeostasis, with a number of genes showing decreased transcription and others showing increased transcription as a consequence of the loss of HNF4α (13). The scavenger receptor class B type 1, medium-chain acyl-coenzyme A dehydrogenase (MCAD), carnitine palmitoyl transferase II, and HMG-CoA synthase were upregulated in the livers of HNF4α knockout mice, and interestingly, the three latter genes are all PPARα target genes. As steady-state levels of PPARα mRNA are decreased in the livers of the HNF4α knockout animal but some PPARα target genes are still upregulated, it was suggested that increased concentrations of PPARα ligands in the form of free fatty acids may account for these effects (13). Our study indicates that Acot1 is another PPARα target gene that is increased in the livers of HNF4α-null mice. As ACOT1 can hydrolyze acyl-CoAs to free fatty acid and CoA, it is tempting to speculate that the upregulation of Acot1 in the HNF4α knockout mouse could regulate levels of ligands for PPARα and HNF4α in the form of free fatty acids or acyl-CoAs. Although the specific function of ACOT1 has not been fully resolved, the hypothesis is that this enzyme may be involved in the regulation of ligand supply for nuclear receptors such as the PPARs and in shuttling fatty acids away from esterification and triglyceride formation in the cytosol toward oxidation (15). Esterification and triglyceride formation require the CoA ester of fatty acids, whereas free fatty acids and CoA esters may act as ligands for PPARs. The more potent ligands for PPARs are identified as free fatty acids (e.g., linoleic acid, linolenic acid, and arachidonic acid), whereas straight-chain saturated long-chain fatty acids are weaker ligands in vitro.

It was also recently shown that acyl-CoA esters of fatty acids can act as ligands for the PPARs (10). Notably, HNF4α also can be activated or inhibited by acyl-CoAs, depending on chain length and degree of saturation (14), although it is also active in the absence of endogenous ligand. ACOT1 hydrolyzes acyl-CoAs to the free fatty acid and CoA, and recombinant mouse ACOT1 hydrolyzes saturated straight-chain acyl-CoAs such as myristoyl-CoA (C_{14}-CoA), palmitoyl-CoA (C_{16}-CoA), and stearoyl-CoA (C_{18}-CoA) but is also active on unsaturated acyl-CoAs such as palmitoleoyl-CoA ($C_{16:1}$-CoA), oleoyl-CoA ($C_{18:1}$-CoA), and linoleoyl-CoA ($C_{18:2}$-CoA), although to a lesser extent (30). Therefore, it is tempting to hypothesize that Acot1 can play a central role in regulating long-chain acyl-CoA/free fatty acid levels, which in turn would control intracellular levels of ligands for both PPARα and HNF4α. Preliminary data suggest that ACOT1 is a nucleocytoplasmic protein (P. J. G. Lindquist et al., unpublished results) and therefore is localized in the nucleus at the point where it could act in ligand supply for nuclear receptors. With *Acot1* itself being a positive target gene of PPARα, this could provide a self-inducing mechanism (i.e., *Acot1* is upregulated by fibrates/fasting, thereby supplying ligand for PPARα and maintaining its own upregulation). This upregulation could in turn be elegantly counteracted via the interplay between PPARα and HNF4α acting directly on the *Acot1* promoter. Hopefully, the true in vivo function of *Acot1* will be evident with the production of an *Acot1*-null mouse model, work that is currently under way in our laboratory.

It is not yet known whether the upregulation of *Acot1* in the HNF4α knockout mouse liver is a direct result of the loss of HNF4α or, alternatively, a secondary consequence of the perturbed lipid metabolism in these animals. Although *Acot1* is upregulated in the absence of HNF4α in mouse liver, the promoter is also slightly upregulated by HNF4α, which is a paradox. However, a similar situation exists for the MCAD, which is upregulated in the HNF4α-null mouse liver (13), although the promoter is also activated by HNF4α (31). The expression of both Acot1 and MCAD is highly modulated in accordance with fatty acid oxidation rates, and Acot1 is also coexpressed in liver and kidney (19) with HNF4α, which would allow regulation by HNF4α in these tissues. Competition experiments performed in cells indicate that HNF4α can repress Acot1 promoter activation by ligand-activated PPARα, although the mechanism behind this is not yet understood. As both of these receptors have a positive effect on the Acot1 promoter, one would expect that the addition of activated PPARα and HNF4α may result in a synergistic increase in promoter activity. However, transcriptional regulation by HNF4α is accomplished by interactions with various coactivators or corepressors, such as histone deacetylase 1 (a corepressor), and a very recent study by Stanulovic et al. (29) showed that both HNF4α and histone deacetylase 1 are present on an upstream enhancer element of the glutamine synthetase promoter, which results in the suppression of glutamine synthetase expression in liver periportal areas. Therefore, the competition between HNF4α and PPARα for binding to the Acot1 DR1 is likely a complicated interplay between coactivators, corepressors, and ligand availability.

Recent reports from DNA microarray studies have identified *Acot1* induction by several different treatments. In female rat liver and small intestine, *Acot1* was induced by dexamethasone, a rodent pregnane X receptor agonist (32). Treatment of rats with sesamin, a major lignin in sesame seeds known to have cholesterol- and lipid-lowering activities, resulted in an ~100-fold upregulation of *Acot1* in rat liver, although the authors state that this upregulation may be an overestimation (33). However, given that Acot1 is upregulated by 90-fold in liver in response to Wy-14,643, this may not be an overestimation

and may require further investigation. To our knowledge, this upregulation of *Acot1* at the mRNA level by PPARα activators is the highest identified to date. A further study also identified *Acot1* mRNA induction by a synthetic liver X receptor agonist in liver and brown adipose tissue in mouse (34). Thus, *Acot1* is a highly regulated gene in several different tissues and can be rapidly regulated at the mRNA and protein levels under different physiological conditions.

Acot1 is a member of a gene family of acyl-CoA thioesterases, with six members of this gene family localized in a cluster on mouse chromosome 12 D3 (19, 35). These gene products result in proteins localized in cytosol (ACOT1), mitochondria (ACOT2), and peroxisomes (ACOT3–ACOT6). Interestingly, all members of this gene family are targets of PPARα, although *Acot1* shows the highest level of induction at the mRNA level by PPARα activators. Although these genes are located in close proximity to each other, they all have distinct promoter regions and likely individual PPREs regulating their transcriptional level. The human ortholog of ACOT1 was characterized recently and is localized in a cluster on human chromosome 14q24.3, together with mitochondria- and peroxisome-related genes (19, 36). The promoter of the human ACOT1 contains several DR1 elements; however, we have examined the regulation of human ACOT1 in liver from bezafibrate-treated patients using Northern blot analysis, and no significant upregulation was evident at the mRNA level. There are large species differences in response to fibrate treatment, and rodents are very susceptible to peroxisome proliferation, hepatomegaly, and the upregulation of fatty acid oxidation enzymes in response to fibrates. In contrast, humans are not susceptible to these effects of fibrate treatment, although these fibrates act as very efficient lipid-lowering drugs in humans (37).

In conclusion, we have determined that *Acot1* is regulated by both the PPARα and HNF4α nuclear receptors, resulting in an activation of promoter activity. In vivo, both HNF4α and PPARα are involved in the basal expression of this gene. Therefore, *Acot1* is regulated via a complex interplay between these nuclear receptors and would be regulated in response to changes in ligand concentrations of both acyl-CoAs and free fatty acids, which in fact are the substrates/products of its own catalysis.

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