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The acyl-CoA thioesterase I (Acot1) is regulated by the peroxisome proliferator-activated receptor alpha and hepatocyte nuclear factor 4 alpha via a distal response element in the promotor.

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Short title: Acot1 regulation by PPARα and HNF4α

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Abbreviations: Acot1, acyl-CoA thioesterase I: PPARα, peroxisome proliferator-activated receptor alpha: PPRE, peroxisome proliferator-response element: DR1, direct repeat 1: HNF4α, hepatic nuclear factor 4 alpha.

Abstract

The cytosolic acyl-CoA 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 coenzyme A. Acot1 was previously shown to be strongly upregulated at mRNA and protein level in rodents by fibrates. In this study, we show that Acot1 mRNA levels were increased 90-fold in liver by treatment with Wy-14,643 and that Acot1 mRNA is also increased 15-fold in the liver of hepatocyte nuclear factor 4 alpha (HNF4α) knockout animals. Our study identified a direct repeat 1 (DR1) located in the Acot1 gene promotor in mouse, which binds the peroxisome proliferator-activated receptor alpha (PPARα) and the HNF4α. Chromatin immunoprecipitation assay (ChIP) showed that the identified DR1 bound PPARα/retinoid X receptor alpha (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 promotor in cells by the PPARα agonist Wy-14,643, following co-transfection with PPARα/RXRα. However, transfection with a plasmid containing HNF4α also resulted in an increase in promotor activity. Taken together, these data show that Acot1 is under regulation by an interplay between of HNF4α and PPARα.

Key words: peroxisome proliferator response element, direct repeat 1, acyl-CoA, lipid metabolism.

Introduction

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 alpha (PPARα), a nuclear receptor identified in 1990 by Issemann and Green, which at that time was shown to be activated by peroxisome proliferators (1). The 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 (2)), lipoprotein metabolism (3), glycerol metabolism (4) and amino acid metabolism (5). Targeted disruption of the PPARα in mouse by Lee et al in 1995 substantiated the role of this nuclear receptor in regulation of lipid homeostasis (6). The binding of the PPARα as a heterodimer with the retinoid X receptor (RXR) to a direct repeat 1 (DR1) element (AGGTCAnAGGTCA or variants thereof) in the promotor/intron of target genes has now been widely established (2). Studies on endogenous ligands for the 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 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 the PPARα (10). Thus the intracellular levels of acyl-CoAs and free fatty acids are important in regulation of this nuclear receptor. This DR1 element that binds the PPARα/RXR heterodimer can also bind another nuclear receptor, the hepatic nuclear factor 4 alpha (HNF4α) as a homodimer (11). The HNF4α (NR2A1) is expressed in liver, kidney, intestine and pancreas (12) and is central to maintenance of hepatocyte differentiation and regulation of genes involved in lipid metabolism (13). The ligands for HNF4α are shown to be various acyl-CoA esters, which act as agonists/antagonists, 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 coenzyme A, and acyl-CoAs/free fatty acids act as either

agonists/antagonists for the PPARα and the HNF4α. These enzymes are called acyl-CoA thioesterases (Acots) (for review see (15)), and a new nomenclature was recently introduced (16). One member of this gene family, the cytosolic acyl-CoA 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 (DEHP) (22). At the same time, the cDNA (17) and gene (19) was cloned from mouse and was also shown to be induced by the peroxisome proliferator clofibrate in liver at both mRNA and protein level (18,19). The 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 upregulated under diabetic conditions in rat liver (23). Although it has been shown that Acot1 is upregulated by fibrates and fasting via the PPARα, an actual peroxisome proliferator-response element has not been identified. In this study, we identify the response element involved in activation of Acot1 by PPARα/RXRα. The response element located between –9600 bp and –9612 bp upstream of the translation start site 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 promotor activity. In vivo, the Acot1 mRNA was induced 15-fold in 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 promotor 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* promotor 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 mice (HNF4α -/-L) aged between 45-56 days as described (13). Ten to twelve-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 sacrificed 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, 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 **C T C G A G** CAGACTTGAAGGCAGATGGTTT-3' and 5'-**C T C G A G** TTTTTCTTCCTTGTGTTGTAATCC-3' (1,861 bp) and 5'-**C T C G A G** CACCGGAGTCACCTGATAGAGTC-3' and, 5'-**CTCGAG**GCCAGGGTGCACAGACTTT-3' (157 bp) with the XhoI sites indicated in bold. The first construct was 1,861 bp in length and corresponded to bp -8659 to -10,520 upstream of the ATG start site in the *Acot1* gene. A shorter construct of 157 bp in length was also generated and corresponded to –9506 to –9663 upstream of the ATG start site. The PCR was performed as follows: 94°C for 30 sec, 61°C for 30 sec, 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 Promotor (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 Quikchange™ site directed mutagenesis kit (Stratagene, La Jolla) according to the

Manufacturer's instructions, using 16 cycles. The primers used were GGGCTGAGTTGGAACTGGG*TTT*AA*AAA*CATGGCAAGCTCTC (and the reverse complement primer) with the mutated bases underlined in italics (constructs named pGL3-Acot1□1861 and pGL3-Acot□157). The PPAR□ and RXR□ plasmids were as described previously (24) and the HNF4□ in pCMV (25) was a kind gift from Dr John Chiang.

Chromatin Immunoprecipitation Assay (ChIP)

Freshly isolated livers were ground to a fine powder under liquid nitrogen and crosslinked in 1% formaldehyde in 1 X phosphate buffered saline (PBS) at 37ºC for 20 min. Crosslinking was terminated with 0.125 M glycine and the cell pellet was washed twice with 1 X 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 were sheared by sonication and the nuclei lysate was cleared by centrifugation at 50,000 x 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 PPAR□ (Geneka) or HNF4□ (K2915 ABcam). The antibody/protein/DNA complex was isolated using magnetic beads conjugated with protein A (New England Biolabs). Following several washes the protein/DNA complex was eluted (50 mM $NaHCO_3$, 1% SDS) from the magnetic beads and crosslinking was reversed by incubation at 65ºC overnight. The samples were incubated with proteinase K for 1 hour at 45ºC, and following protein digestion the DNA was purified using phenol/chloroform/isoamyl alcohol extraction, and 2-5 □L of sample was used for PCR. The PCR was performed for 31 cycles as follows: 94ºC for 3 mins, followed by 94ºC for 30 sec, 56ºC for 30 sec and 72ºC for 1 min, followed by 72ºC for 7 min. Primers used were Acot1 DR1 forward 5'-CACCGGAGTCACCTGATAGAGTC-3' and reverse 5'-GCCAGGGTGCACAGACTTT-3' amplifying an area of 157 bp (~9.6kb upstream of the ATG start site) containing the DR1. The ChIP negative primers amplified an area of 141 bp located ~1.8kb 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, penicillin/streptomycin (100U/ml of each) in an atmosphere of 5% CO_2. HeLa cells were cultured in EMEM (Sigma Corp) with 10% fetal bovine serum, 1mM sodium pyruvate, 1% non-essential amino acids and penicillin/streptomycin (100U/ml of each) in an atmosphere of 5% CO_2. The cells were cultured in 24 well plates and grown to approx. 70% confluence before transfection. The transfections were carried out using Tfx-20 reagent (Promega Corp.), in a ratio of 1:2 (v/v) plasmid to reagent. Cells were transfected with 0.25 μg *Acot1* promotor plasmids pGL3-Acot1-1861 and pGL3-Acot1Δ1861 or pGL3-Acot1-157 and pGL3-Acot1Δ157, 0.25 μg of the PPARα, RXRα or HNF4α expression vectors 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 post-transfection, the cells were treated with 50 μM Wy-14,643 or dimethyl sulfoxide (DMSO) as vehicle where appropriate, as indicated in the figure legends. Cell lysates were assayed for luciferase activity using Luciferase Reporter Gene Assay (Promega Corp.) and for β-galactosidase activity using β-Galactosidase Enzyme Assay System (Promega Corp.). Experiments were carried out in triplicate wells and two to four individual experiments were carried out. 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 capitals. Mutated DR1 oligos were also used with the mutations underlined in italics: 5'-gctgagttggaactGGG*TTT*AA*AAA*CAtggcaagctct-3'. 10 pmol 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., Madison, WI). Gel mobility shift assay incubation mixes (25 µl) contained 10 mM Tris (pH 7.8), 20 mM KCl, 2 µg bovine serum albumin (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 a HNF4α antibody (Ab. HNF4α (C19)X, Santa Cruz Biotechnology, Inc.) as indicated in figure legends. 50,000 cpm of labeled probe was added to each reaction and incubated on ice for 45 minutes. The complexes were resolved on a 5% polyacrylamide gel in 1X Tris Borate EDTA (TBE) 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 Δ/Δ L) aged between 45-56 days as described (13), using Trizol Reagent (Invitrogen Corp.). Ten to twelve-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 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 the Primer Express Software (Applied Biosystems). 1 µg of total RNA from each liver sample was reverse transcribed into cDNA using iScript (Biorad Inc.). The 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 Ct value per treatment was used to calculate relative expression levels of mouse *Acot1* mRNA with the □□CT method.

Results

The *Acot1* is regulated by fibrate treatment via PPAR□ in-vivo.

It has previously been shown that Acot1 is upregulated at mRNA and protein level in rodents by treatment with clofibrate (17-19) and DEHP (21). However, this upregulation at mRNA level was shown only by Northern blot analysis and 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 towards Acot1. The Acot1 was upregulated 90-fold in mouse liver following Wy-14,643 treatment, whereas this upregulation was not evident in the PPAR□-null animals, showing that the regulation of Acot1 by fibrates is PPAR□-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 PPAR□ target gene has previously been shown for the same RNA samples (24). Notably, the Acot1 basal mRNA level was doubled in the PPAR□-null animals.

Identification of a DR1 in the *Acot1* gene promotor

Acot1 is very highly induced in mouse liver by fibrate treatment, however the actual peroxisome proliferator-response element in the *Acot1* gene has not been identified. We had previously sequenced 6 kb of the promotor region using conventional sequencing from a P1 clone known to contain the *Acot1* gene (19). However, this area contained only one putative PPRE approx. 2 kb upstream of the translation start site, which was tested using reporter gene systems and EMSA and was not functional (data not shown). The transcription start site is considered to be about 9 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* promotor sequence and identify an imperfect direct repeat 1 (DR1) between –9600 bp and –9612 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 PPAR□/RXR□. It has also been shown that

the 7 nucleotides in the 5′ flanking region of identified PPREs have an important influence on binding of the PPARα (26). Notably, 5 out of the 7 bp in the 5′-flank of the DR1 element of the *Acot1* promotor were conserved, strongly suggesting that this could be a candidate for a functional PPRE.

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

To evaluate whether the identified DR1 in the *Acot1* promotor could be activated by PPARα ligands in a cell system, promotor constructs were generated in the pGL3 promotor vector, containing an endogenous SV40 promotor. This vector was used to clone fragments of the Acot1 promotor region containing the DR1. One plasmid contained a 1,861 bp fragment of the *Acot1* promotor region from –8659 to –10,520 upstream of the ATG translation start site, containing the DR1 (pGL3-Acot1-1861) and a further 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 promotor construct in HepG2 cells was not increased by Wy-14,643 treatment in the absence of transfected PPARα/RXRα (Fig. 3A). However, following co-transfection with PPARα/RXRα, the promotor 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 promotor activity ($p<0.001$ – 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 promotor (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 promotor 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.

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

To determine if the DR1 identified at –9,600 to -9612 in the *Acot1* promotor could bind directly to PPARα/RXRα, both ChIP and electromobility shift assay (EMSA) were carried out. 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 the DR1 binds to PPARα in-vivo. As a negative control, primers amplifying an area of Acot1 promotor (~1.8kb 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 a strong binding to the Acot1 DR1, showing that this element can bind directly to this heterodimer partnership (Fig. 4B). Addition of 50-fold molar excess of cold Acot1 probe competed out the binding of the α-^{32}P labeled Acot1 probe and supershift was evident in the presence of the RXRα antibody, confirming that the complex identified by EMSA contains the 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 promotor activity of pGL3-Acot1α1861 in a cell system (Fig. 3B).

The *Acot1* promotor is regulated by HNF4α

The HNF4α also binds to a DR1 sequence and we therefore examined the possible involvement of this receptor in control of Acot1 expression. Transfection of HepG2 cells with empty pGL3 promotor vector in the presence of HNF4α

resulted in a significant downregulation of promotor activity (the vector contains an endogenous SV40 promotor), and therefore this system was not suitable for further experiments with HNF4. Therefore HeLa cells were used for further experiments with HNF4. As a positive control, a plasmid expressing the promotor 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 HNF4 in HeLa cells. *Acot1* promotor activity (pGL3-Acot1-157) was doubled by co-transfection with HNF4 , however mutation of the DR1 in pGL3-Acot1150 abolished the upregulation via HNF4 (Fig. 5A). This data identifies that the Acot1 promotor is upregulated by HNF4.

To assess if HNF4 could compete for binding to the DR1 identified, in the presence of PPAR/RXR, HeLa cells were transfected with PPAR/RXR and treated with Wy-14,643, in the presence of varying amounts of HNF4. The Acot1 promotor construct pGL3-Acot1-157 was activated in the presence of PPAR/RXR + 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 g HNF4 and further reduced by transfection with 0.5 g HNF4 (Fig. 5B). The mutated Acot1 promotor pGL3-Acot1157 also showed a very slight downregulation of promotor activity in the presence of co-transfected HNF4, however this was similar to that seen in the empty pGL3 promotor vector.

The Acot1 promotor binds to HNF4 in-vivo and in-vitro.

ChIP and EMSA were also carried out on the same DR1 element using liver extracts from HNF4 floxed and knockout animals, and in-vitro translated HNF4. ChIP confirmed the in-vivo binding of HNF4 to the same DR1 element in the *Acot1* promotor as the PPAR and the specificity of this was again confirmed using liver extracts from the HNF4 knockout mouse (Fig. 6A), although some weak signal was detected in the knockout liver extracts. This may be due to the fact that the HNF4 is a conditional liver specific knockout and there is some very low amount of HNF4protein present in the nucleus (29).

EMSA was also performed using in-vitro translated HNF4α and binding to the DR1 was competed using 50-fold molar excess cold probe and supershifted using a HNF4α antibody (Fig. 6B). Mutation of the DR1 element abolished binding of the 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 promotor of *Acot1* was regulated by HNF4α in HeLa cells, and both ChIP and EMSA confirmed binding of the HNF4α to the DR1 identified, it seemed likely that Acot1 is regulated by HNF4α in liver. The Acot1 mRNA levels in the livers of HNF4α knockout were therefore investigated. Real-time PCR showed that Acot1 mRNA levels were upregulated 15-fold in the HNF4α knockout mouse model, indicating that Acot1 mRNA expression is affected by loss of HNF4α expression in liver (Fig. 6C).

Discussion

We have shown in this study that the *Acot1* is a target gene of both the PPARα and HNF4α using animal models, ChIP and reporter gene assays. The Acot1 is positively regulated by PPARα, through a direct binding to a DR1 element located in the distal promotor. Interestingly this DR1 also binds to HNF4α and results in an upregulation of Acot1 promotor activity. The binding of both 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 transcription of the Acot1 gene, either at basal level or during perturbed lipid metabolism (fibrate treatment or fasting – (this study and (17,18,23)). The basal level of Acot1 mRNA is doubled in the liver of the PPARα knockout mouse (Fig. 1), indicating that the Acot1 promotor is under slight repression by PPARα/RXRα in the fed state. Hayhurst et al showed that the expression of PPARα mRNA is downregulated in the HNF4α knockout mouse (13), which may therefore result in a consequent

upregulation of Acot1 in the HNF4α knockout (due to lower expression levels of PPARα). However considering that Acot1 mRNA is only doubled in response to 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 the 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 increased transcription as a consequence of the loss of HNF4α (13). The scavenger receptor class B type 1 (SR-BI), medium chain acyl-CoA dehydrogenase (MCAD), carnitine palmitoyl transferase II (CPT-II) and 3-hydroxy-3-methyl-glutaryl coenzyme A synthase (HMG-CoA synthase) were upregulated in the livers of HNF4α 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, 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 identifies that Acot1 is another PPARα target gene that is increased in the livers of HNF4α-null mice. As the 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 could regulate levels of ligands for the PPARα and HNF4α in the form of free fatty acids or acyl-CoAs. Although the specific function for Acot1 has not been fully resolved, the hypothesis is that this enzyme may be involved in 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, towards 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 also act as ligands for the PPARs (10). Notably also HNF4α 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). It is therefore tempting to hypothesize that Acot1 can play a central role in regulating long-chain acyl-CoA/free fatty acid levels, which would in turn control intracellular levels of ligands for both the PPAR and HNF4. Preliminary data suggests that ACOT1 is a nucleo-cytoplasmic protein (Lindquist et al, unpublished result) and is therefore 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 the PPAR, this could provide a self-inducing mechanism i.e. Acot1 is upregulated by fibrates/fasting, thereby supplying ligand for PPAR and maintaining its own upregulation. This upregulation could in turn be elegantly counteracted via interplay between both PPAR and the HNF4 acting directly on the *Acot1* promotor. The true in-vivo function of Acot1 however, will hopefully be evident with the production of an Acot1 null mouse model, work that is currently underway in our laboratory.

It is not yet known if the upregulation of Acot1 in the HNF4 knockout mouse liver is a direct result of the loss of HNF4, or alternatively a secondary consequence of the perturbed lipid metabolism in these animals. While Acot1 is upregulated in the absence of HNF4 in mouse liver, the promotor is also slightly upregulated by HNF4, which is a paradox. However, a similar situation exists for the MCAD, which is upregulated in the HNF4 null mouse liver (13), but the promotor is also activated by HNF4 (31). The expression of both Acot1 and MCAD are highly modulated in accordance with fatty acid oxidation rates, and Acot 1 is also co-expressed in liver and kidney (19) with HNF4, which would allow regulation by HNF4 in these tissues. The competition experiments performed in cells indicates that HNF4 can repress the Acot1 promotor activation by ligand-activated PPAR, although the mechanism behind this is not yet understood. As both these receptors have a positive effect on the Acot1

promotor, one would expect that addition of activated PPARα and HNF4α may result in a synergestic increase in promotor activity. However transcriptional regulation by HNF4α is accomplished by interactions with various coactivators or co-repressors, such as histone deacetylae 1 (HDAC1) (a co-repressor), and a very recent study by Stanulovic et al showed that both HNF4α and HDAC1 are present on an upstream enhancer element of the glutamine synthetase (GS) promotor, which results in suppression of GS expression in liver periportal areas (29). Therefore the competition between HNF4α and PPARα for binding to the Acot1 DR1 is likely a complicated interplay between co-activators, co-repressors and ligand availability.

Recent reports using 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 (PXR) agonist (32). Treatment of rats with sesamin, a major lignin in sesame seeds known to have cholesterol and lipid lowering activities, resulted in an approx. 100-fold upregulation of Acot1 in rat liver, although the authors state that this upregulation may be an over-estimation (33). However, given that Acot1 is upregulated 90-fold in liver in response to Wy-14,643, this may not be an over-estimation and may require further investigation. To our knowledge, this upregulation of Acot1 at mRNA by PPARα activators is the highest identified to date. A further study also identified Acot1 mRNA induction by a synthetic liver X receptor (LXR) 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 mRNA and protein level 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 (ACOT3-6). Interestingly, all members of this gene family are targets of the PPARα, however the Acot1 shows the highest level of induction at mRNA level by PPARα activators. Although these

genes are located in close proximity to each other, they all have distinct promotor regions and likely individual PPREs regulating their transcriptional level. The human orthologue of the ACOT1 has recently been characterized and is localized in a cluster on human chromosome 14q24.3, together with mitochondrial and peroxisomal related genes (19,36). The promotor of the human ACOT1 contains several DR1 elements, however we have examined the regulation of the human ACOT1 in liver from bezafibrate treated patients using Northern blot, but no significant upregulation was evident at mRNA level. There are large species differences in response to fibrate treatment and rodents are very susceptible to peroxisome proliferation, hepatomegaly and upregulation of fatty acid oxidation enzymes in response to fibrates. In contrast, humans are not susceptible to the above effects of fibrate treatment, although these fibrates act as very efficient lipid lowering drugs in man (37).

In conclusion, we have identified that *Acot1* is regulated by both the PPAR and HNF4 nuclear receptors, resulting in an activation of promotor activity. In-vivo, both the HNF4 and the PPAR are involved in the basal expression of this gene. Acot1 is therefore 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 are in fact the substrates/products of its own catalysis.

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Figure legends:

Fig. 1: Expression of Acot1 mRNA is regulated by fibrate treatment in mouse liver. Total RNA was isolated from livers of wild-type (PPAR (+/+)) and PPAR-null (PPAR (-/-)) male mice fed 0.1% Wy-14,643 for one 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 Ct method using eukaryotic 18S rRNA as an endogenous control. n=3 ± S.D. ($p<0.003$, unpaired Student's *t-test*).

Fig. 2: The Acot1 promotor contains a direct repeat 1.
The core consensus sequence for the direct repeat 1 (DR1) known to bind the PPAR/RXR heterodimer is shown (DR1 consensus), together with the 5' flanking sequence. The putative DR1 for mouse *Acot1* is shown.

Fig. 3: The *Acot1* promotor is activated by PPAR/RXR. HepG2 cells were transfected with promotor constructs containing empty pGL3 promotor vector, Acox1 PPRE (2 copies) (AOx-tk-Luc) and pGL3-Acot1-1861. **(A)** Cells were transiently transfected with the indicated promotor vector (0.25 g) ± PPAR (0.25 g) and RXR (0.25 g). Empty pcDNA3.1+ vector was used to keep DNA concentrations equal in all transfections. 24 h post transfection, cells were treated with vehicle (DMSO) or 50 M 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 -galactosidase activity. Data shown as mean ± S.E.M for Acox1 and Acot1 ($p<0.001$ for ligand activation (PPAR/RXR + DMSO vs PPAR/RXR + Wy-14,643 - Student's *t-test*)) **(B)** Cells were transiently transfected with the *Acot1* promotor constructs pGL3-Acot1-1861 or pGL3-Acot1 1861 (0.25 g) ± PPAR (0.25 g) and RXR (0.25 g). 24 h post transfection, cells were treated with 50 M Wy-14,643. Transfections were carried

out in triplicate wells in three different experiments. Luciferase activity was normalized to β-galactosidase activity. Data shown as mean ± S.E.M. ($p<0.001$, unpaired Students' *t-test*). Mutated bases in the DR1 are shown underlined in italics.

Fig. 4: The *Acot1* DR1 binds PPARα/RXRα in-vivo and in-vitro. (A) ChIP was carried out on liver nuclei of PPARα wild type (+/+) and PPARα knockout (-/-) mice treated with Wy-14,643, using a PPARα antibody. Following immunoprecipitation, an amplicon of 157 bp of the Acot1 promotor containing the DR1 (located ~ -9.6 kb) was amplifed by PCR. Negative control: an amplicon of 141 bp in the Acot1 promotor (located at ~ -1.8kb and does not contain any DR1). **(B)** Electromobility shift assay (EMSA) was carried out using 1 µg of in-vitro translated PPARα, RXRα and HNF4α. α-^{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 an arrow while the supershift complexes are indicated by an arrowhead.

Fig. 5: The *Acot1* promotor is regulated by HNF4α. (A) HeLa cells were transiently transfected for 48 hours with 0.25 µg of the empty pGL3 promotor vector, OTC-235-Luc as a positive control (28), the *Acot1* promotor construct pGL3-Acot1-157 or the mutated *Acot1* promotor construct pGL3-Acot1Δ157, together with HNF-4α (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 are shown ± S.E.M for OTC-Luc 235 ($p<0.001$, unpaired Students' *t-test*) and ± S.E.M. for Acot 1 ($p<0.001$, unpaired Students' *t-test*). **(B)** HeLa cells were transiently transfected with 0.25 µg of the empty pGL3 promotor vector, the *Acot1* promotor construct pGL3-Acot1-157 or the mutated *Acot1* promotor construct pGL3-Acot1Δ157, PPARα (0.25 µg) and RXRα (0.25 µg), plus addition of HNF-4α (0.25 µg or 0.5 µg) or pcDNA3.1+ (empty vector) (0.25 µg or 0.5 µg). Cells were treated with 50 µM Wy-14,643 and incubated 48 hours. Transfections

were carried out in triplicate wells in two different experiments. Luciferase activity was normalized to β-galactosidase activity.

Fig. 6: The *Acot1* is regulated by HNF4α. (A) ChIP was carried out on liver nuclei of HNF4α flox (F/F) and HNF4α knockout mice (HNF4α ΔL), using a HNF4α antibody (K2915, Abcam). Following immunoprecipitation, an amplicon of 157 bp of the Acot1 promotor containing the DR1 (located ~ -9.6kb upstream of the ATG start site) was amplifed by PCR. Negative control: an amplicon of 141 bp in the Acot1 promotor (located at ~ -1.8kb and does not contain any DR1). **(B)** Electromobility shift assay (EMSA) was carried out using 1 μg of in-vitro translated HNF4α. α-^{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 an arrow while the supershift complex is indicated by an arrowhead. (**C)** Total RNA was isolated from livers of control male HNF4α flox (F/F) or HNF4α knockout mice (HNF4α ΔL). 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 as mean ± S.D. ($p<0.01$, unpaired Students' *t-test*).

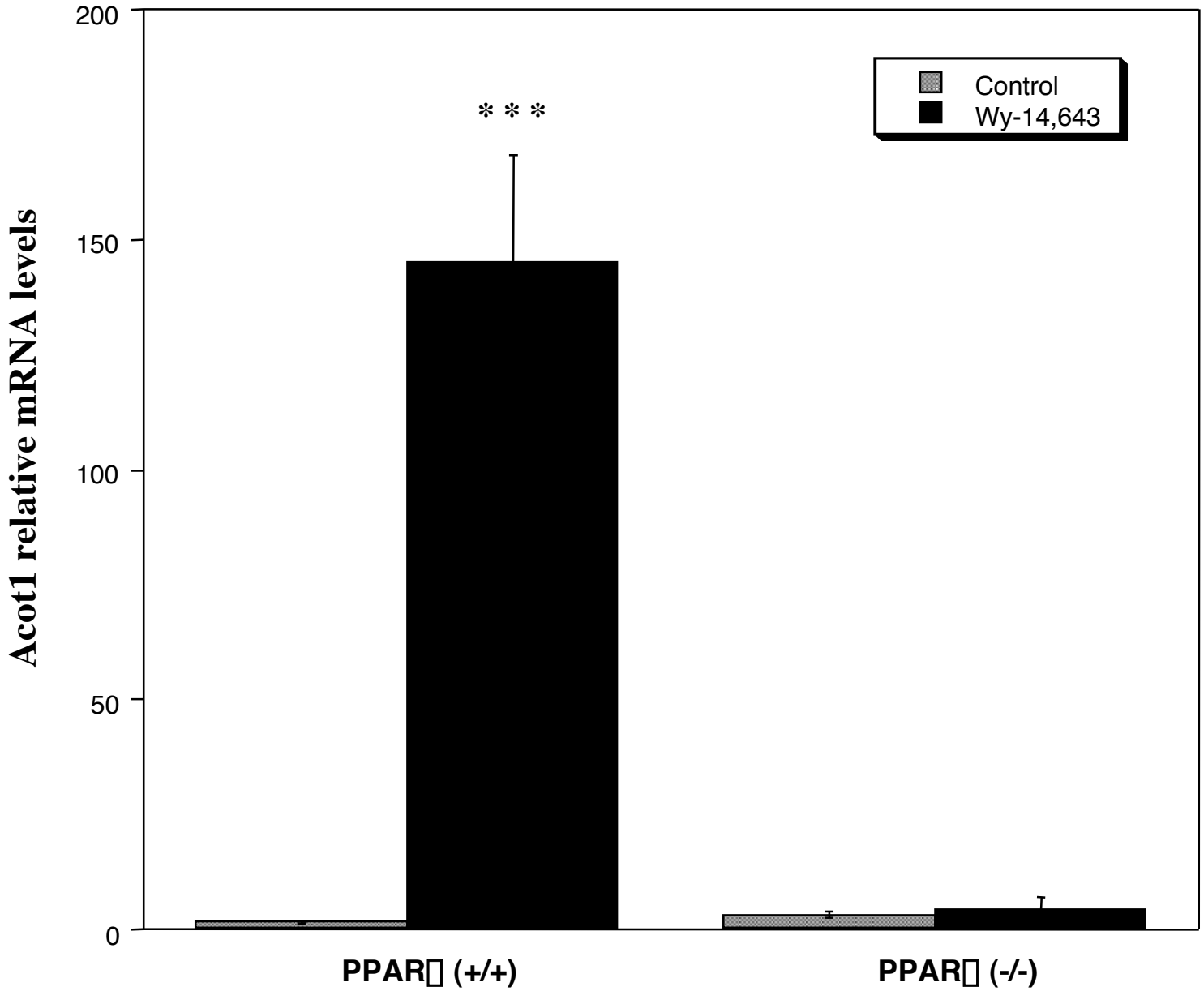
References

1. Issemann I. and Green S. 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature*. **347:** 645-650.
2. Mandard S., Müller M. and Kersten S. 2004. Peroxisome proliferator-activated receptor α target genes. *Cell. Mol. Life Sci.* **61:** 393-416.
3. Améen C., Edvardsson U., Ljungberg A., Asp L., Åkerblad P., Tuneld A., Olofsson S.-O., Lindén D. and Oscarsson J. 2005. Activation of peroxisome proliferator-activated receptor α increases the expression and activity of microsomal triglyceride transfer protein in the liver. *J. Biol. Chem.* **280:** 1224-1229.
4. Patsouris D., Mandard S., Voshol P. J., Escher P., Soon Tan N., Havekes L. M., Koenig W., Marz W., S. T., Wahli W., Müller M. and Kersten S. 2004. PPARα governs glycerol metabolism. *J. Clin. Invest.* **114:** 94-103.
5. Kersten S., Mandard S., Escher P., Gonzalez F., Tafuri S., Desvergnes B. and Wahli W. 2001. The peroxisome proliferator-activated receptor α regulates amino acid metabolism. *FASEB J.* **15:** 1971-1978.
6. Lee S. S., Pineau T., Drago J., Lee E. J., Owens J. W., Kroetz D. L., Fernandez-Salguero P. M., Westphal H. and Gonzalez F. J. 1995. Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol. Cell. Biol.* **15:** 3012-3022.
7. Forman B. M., Chen J. and Evans R. M. 1997. Hypolipidemic drugs, polyunstaurated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 4312-4317.
8. Kliewer S. A., Sundseth S. S., Jones S. A., Brown P. J., Wisely G. B., Koble C. S., Devchand P., Wahli W., Willson T. M., Lenhard J. M. and Lehmann J. M. 1997. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. *Proc. Natl. Acad. Sci. U.S.A.* **94:** 4318-4323.
9. Göttlicher M., Widmark E., Li Q. and Gustafsson J. Å. 1992. Fatty acids activate a chimera of the clofibric acid-activated receptor and the glucocorticoid receptor. *Proc. Natl. Acad. Sci. U.S.A.* **89:** 4653-4657.
10. Hostetler H. A., Petrescu A. D., Kier A. B. and Schroeder F. 2005. Peroxisome proliferator-activated receptor α (PPARα) interacts with high affinity and is conformationally responsive to endogenous ligands. *J. Biol. Chem.* **280:** 18667-18682.
11. Nishiyama C., Hi R., Osada S. and Osumi T. 1998. Functional interactions between nuclear receptors recognizing a common sequence element, the direct repeat motif spaced by one nucleotide (DR-1). *J. Biochem. (Tokyo).* **123:** 1174-1179.
12. Sladek F. M., Zhong W. M., Lai E. and Darnell J., J. E. 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **12B:** 2353-2365.

13. Hayhurst G. P., Lee Y.-H., Lambert G., Ward J. M. and Gonzalez F. J. 2001. Hepatocyte Nuclear Factor 4 (Nuclear Receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. *Mol. Cell. Biol.* **21:** 1393-1403.
14. Hertz R., Magenheim J., Berman I. and Bar-Tana J. 1998. Fatty acyl-CoA thioesters are ligands of hepatic nuclear factor-4. *Nature*. **392:** 512-516.
15. Hunt M. C. and Alexson S. E. H. 2002. The role acyl-CoA thioesterases play in mediating intracellular lipid metabolism. *Prog. Lipid Res.* **41:** 99-130.
16. Hunt M. C., Yamada J., Maltais L. J., Wright M. W., Podesta E. J. and Alexson S. E. H. 2005. A revised nomenclature for mammalian acyl-CoA thiosterases/hydrolases. *J. Lipid Res.* **46:** 2029-2032.
17. Lindquist P. J. G., Svensson L. T. and Alexson S. E. H. 1998. Molecular cloning of the peroxisome proliferator-induced 46-kDa cytosolic acyl-CoA thioesterase from mouse and rat liver. *Eur. J. Biochem.* **251:** 631-640.
18. Hunt M. C., Lindquist P. J. G., Peters J. M., Gonzalez F. J., Diczfalusy U. and Alexson S. E. H. 2000. Involvement of the peroxisome proliferator-activated receptor alpha (PPAR) in regulation of long chain acyl-CoA thioesterases. *J. Lipid Res.* **41:** 814-823.
19. Hunt M. C., Nousiainen S. E. B., Huttunen M. K., Orii K., Svensson L. T. and Alexson S. E. H. 1999. Peroxisome proliferator-induced long chain acyl-CoA thioesterases comprise a highly conserved novel multi-gene family involved in lipid metabolism. *J. Biol. Chem.* **274:** 34317-34326.
20. Yamada J., Matsumoto I., Furihata T., Sakuma M. and Suga T. 1994. Purification and properties of long-chain acyl-CoA hydrolases from the liver cytosol of rats treated with peroxisome proliferator. *Arch. Biochem. Biophys.* **308:** 118-125.
21. Yamada J., Suga K., Furihata T., Kitahara M., Watanabe T., Hosokawa M., Satoh T. and Suga T. 1998. cDNA cloning and genomic organization of peroxisome proliferator-inducible long-chain acyl-CoA hydrolase from rat liver cytosol. *Biochem. Biophys. Res. Commun.* **248:** 608-612.
22. Svensson L. T., Engberg S. T., Aoyama T., Usuda N., Alexson S. E. H. and Hashimoto T. 1998. Molecular cloning and characterization of a mitochondrial peroxisome proliferator-induced acyl-CoA thioesterase from rat liver. *Biochem. J.* **329:** 601-608.
23. Yamada J., Kuramochi Y., Takoda Y., Takagi M. and Suga T. 2003. Hepatic induction of mitochondrial and cytosolic acyl-coenzyme A hydrolases/thioesterases under conditions of diabetes and fasting. *Metabolism.* **52:** 1527-1529.
24. Hunt M. C., Yang Y. Z., Eggertsen G., Carneheim C. M., Gåfvels M., Einarsson C. and Alexson S. E. H. 2000. The peroxisome proliferator-activated receptor (PPAR) regulates bile acid biosynthesis. *J. Biol. Chem.* **37:** 28947-28953.
25. Crestani M., Sadeghpour A., Stroup D., Galli G. and Chiang J. Y. 1998. Transcriptional activation of the cholesterol 7alpha-hydroxylase gene (CYP7A) by nuclear hormone receptors. *J. Lipid Res.* **39:** 2192-2200.
26. Juge-Aubry C., Pernin A., Favez T., Burger A. G., Wahli W., Meier C. A. and Desvergnes B. 1997. DNA binding properties of peroxisome proliferator-

activated receptor subtypes on various natural peroxisome proliferator response elements. *J. Biol. Chem.* **272:** 25252-25259.

27. Guardiola-Diaz H. M., Rehnmark S., Usuda N., Albrektsen T., Feltkamp D., Gustafsson J. Å. and Alexson S. E. H. 1999. Rat peroxisome proliferator-activated receptors and brown adipose tissue function during cold acclimatization. *J. Biol. Chem.* **274:** 23368-23377.
28. Inoue Y., Hayhurst G. P., Inoue J., Mori M. and Gonzalez F. 2002. Defective ureagenesis in mice carrying a liver-specific disruption of hepatocyte nuclear factor 4 (HNF4). *J. Biol. Chem.* **277:** 25257-25265.
29. Stanulovic V. S., Kyrmizi I., Kruithof-de Julio M., Hoogenkamp M., Vermeulen J. L. M., Ruijter J. M., Talianidis I., Hakvoort T. B. M. and Lamers W. H. 2007. Hepatic HNF4 deficiency induces periportal expression of glutamine synthetase and other pericentral enzymes. *Hepatology*. **45:** 433-444.
30. Huhtinen K., O'Byrne J., Lindquist P. J. G., Contreras J. A. and Alexson S. E. H. 2002. The peroxisome proliferator-induced cytosolic type I acyl-CoA thioesterase (CTE-I) is a serine-histidine-aspartic acid alpha/beta hydrolase. *J. Biol. Chem.* **277:** 3424-3432.
31. Carter M. E., Gulick T., Raisher B. D., Caira T., Ladias J. A. A., Moore D. D. and Kelly D. P. 1993. Hepatocyte nuclear factor-4 activates medium chain acyl-CoA dehydrogenase gene transcription by interacting with a complex regulatory element. *J. Biol. Chem.* **268:** 13805-13810.
32. Hartley D. P., Dai X., He Y. D., Carlini E. J., Wang B., Huskey S. W., Ulrich R. G., Rushmore T. H., Evers R. and Evans D. C. 2004. Activators of the rat pregnane X receptor differentially modulate hepatic and intestinal gene expression. *Mol. Pharmacol.* **65:** 1159-1171.
33. Tsuruoka N., Kidokoro A., Matsumoto I., Abe K. and Kiso Y. 2005. Modulating effect of sesamin, a functional lignan in sesame seeds, on the transcriptional levels of lipid-and alcohol-metabolizing enzymes in rat liver: A DNA microarray study. *Biosci. Biotechnol. Biochem.* **69:** 179-188.
34. Stulnig T. M., Steffensen K. R., Gao H., Reimers M., Dahlman-Wright K., Schuster G. U. and Gustafsson J. Å. 2002. Novel roles of Liver X receptors exposed by gene expression profiling in liver and adipose tissue. *Mol. Pharmacol.* **62:** 1299-1305.
35. Westin M. A. K., Alexson S. E. H. and Hunt M. C. 2004. Molecular cloning and characterization of two mouse peroxisome proliferator-activated receptor alpha (PPAR)-regulated peroxisomal acyl-CoA thioesterases. *J. Biol. Chem.* **279:** 21841-21848.
36. Hunt M. C., Rautanen A., Westin M. A. K., Svensson L. T. and Alexson S. E. H. 2006. Analysis of mouse and human acyl-CoA thioesterase (ACOT) gene clusters shows that convergent functional evolution results in a reduced number of peroxisomal ACOTs. *FASEB J.* **20:** 1855-1864.
37. Barbier O., Pineda Torra I., Duguay Y., Blanquart C., Fruchart J.-C., Glineur C. and Staels B. 2002. Pleiotropic actions of peroxisome proliferator-activated receptors in lipid metabolism and atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.* **22:** 717-726.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150
100
50
0
* * *
Control
Wy-14,643
PPARα (+/+)
PPARα (-/-)

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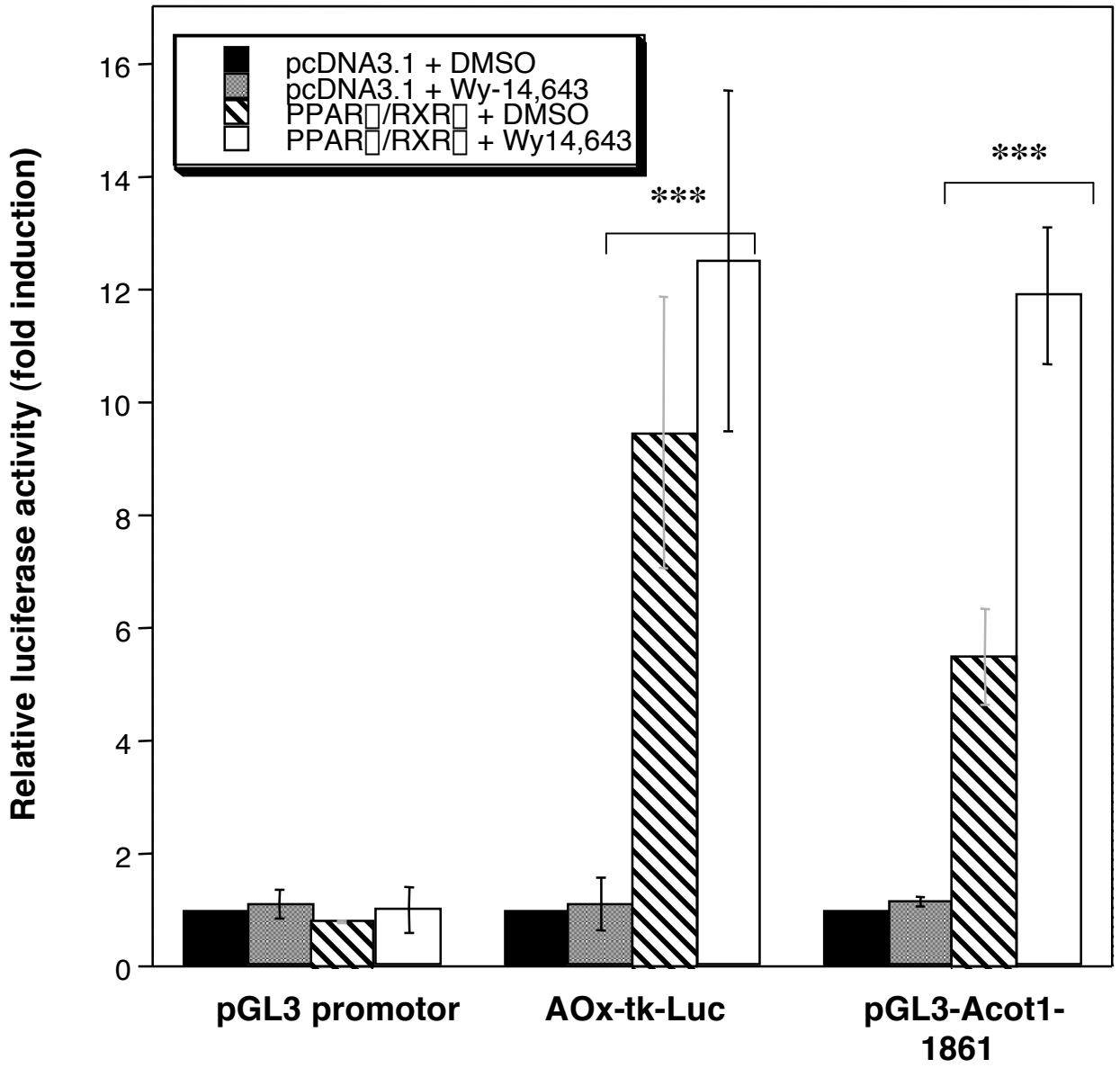
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4
6
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AOx-tk-Luc
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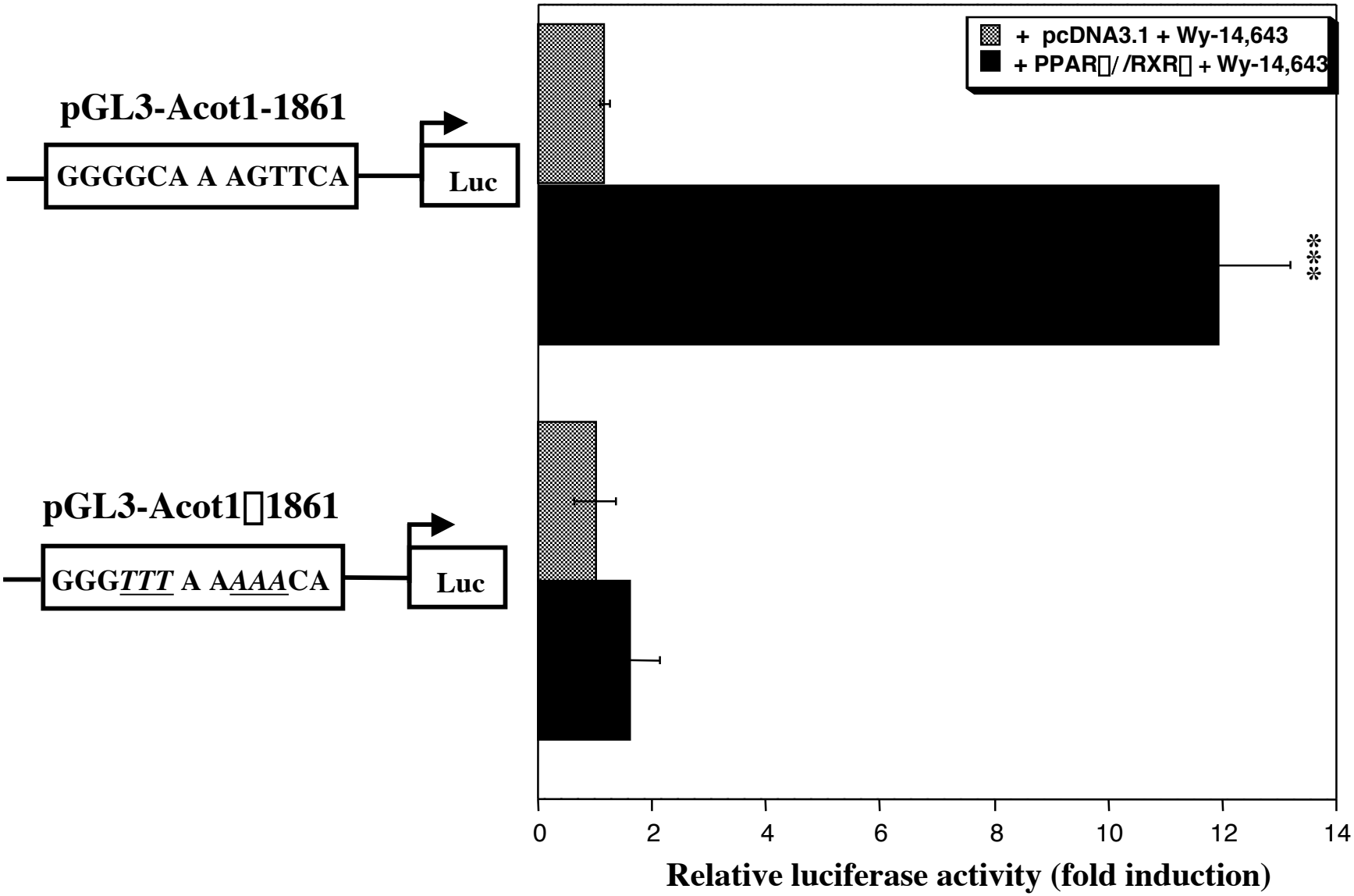
B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Relative luciferase activity (fold induction)

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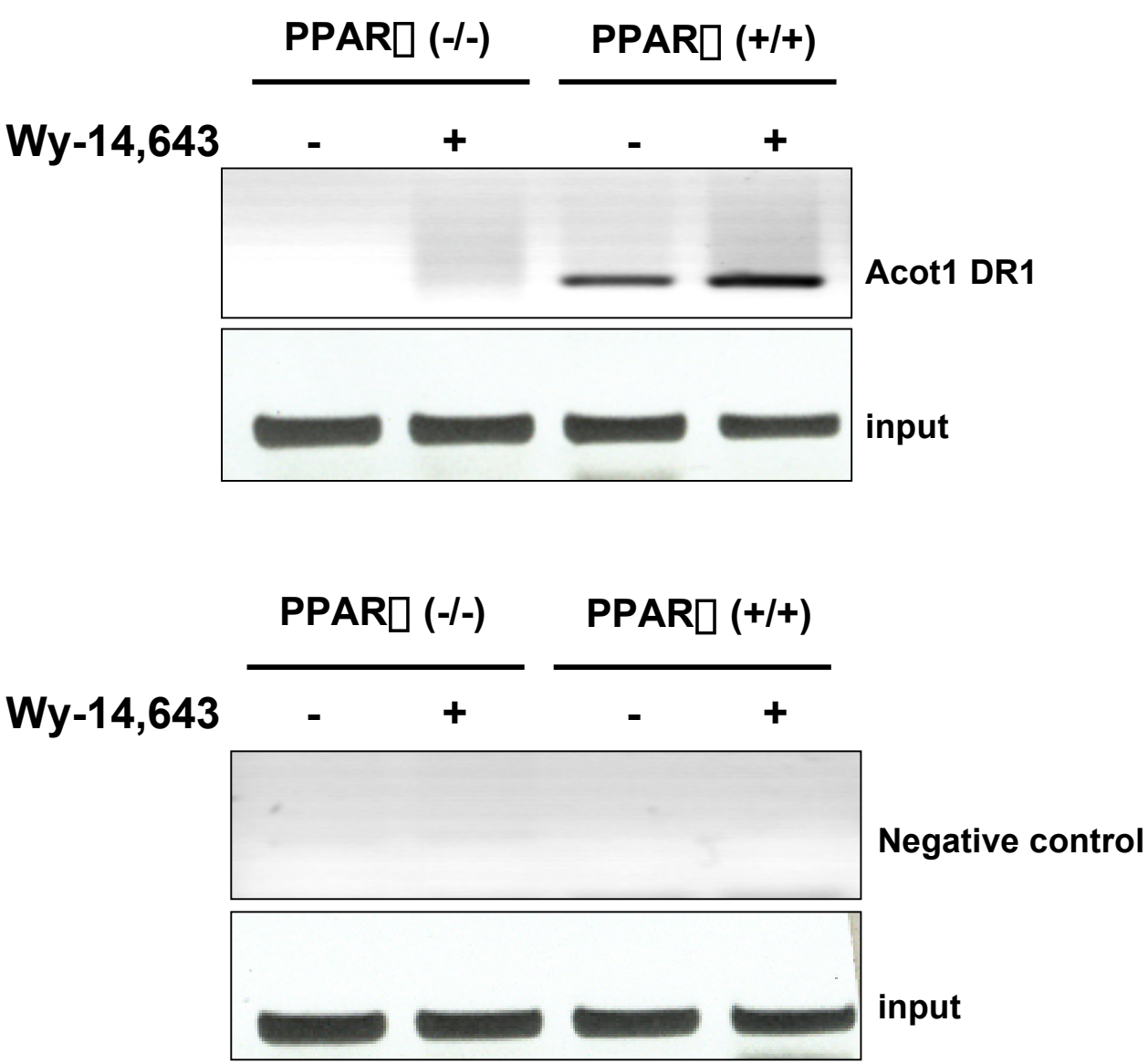
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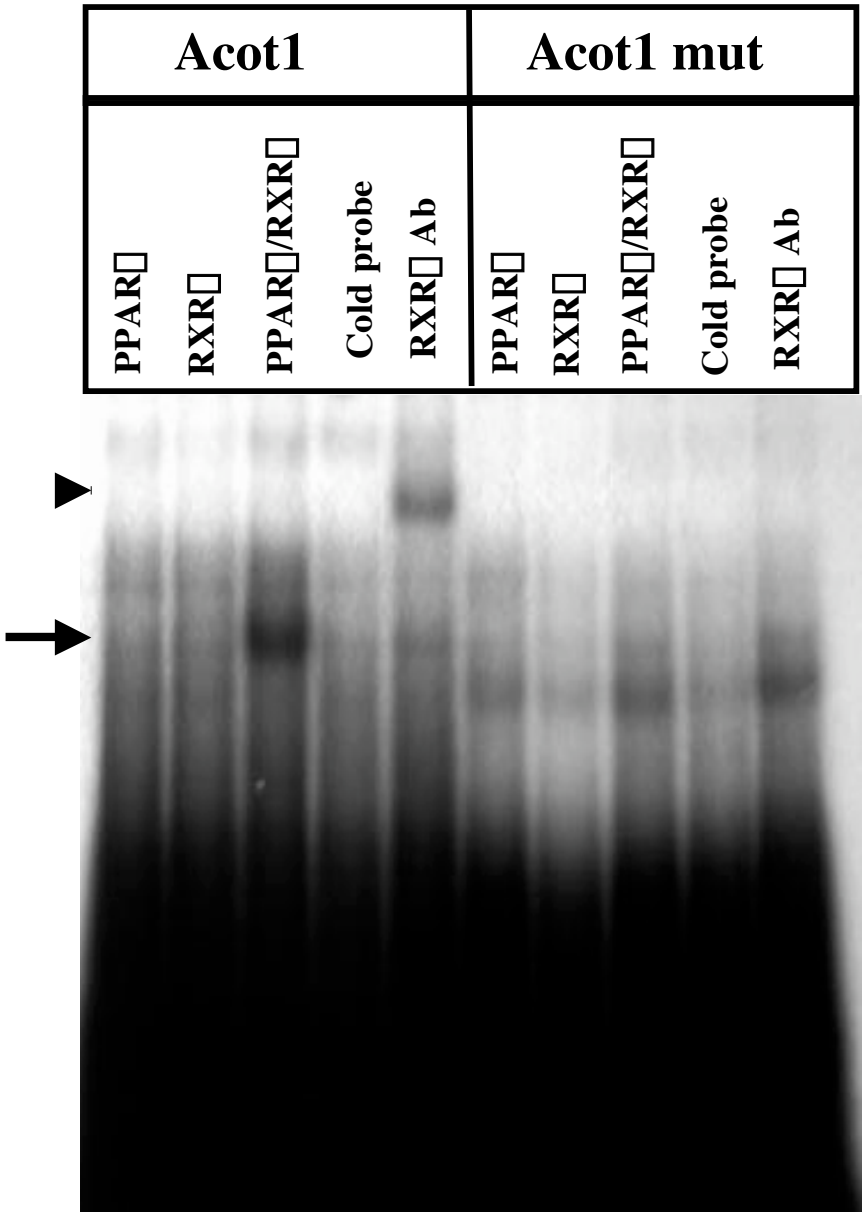
B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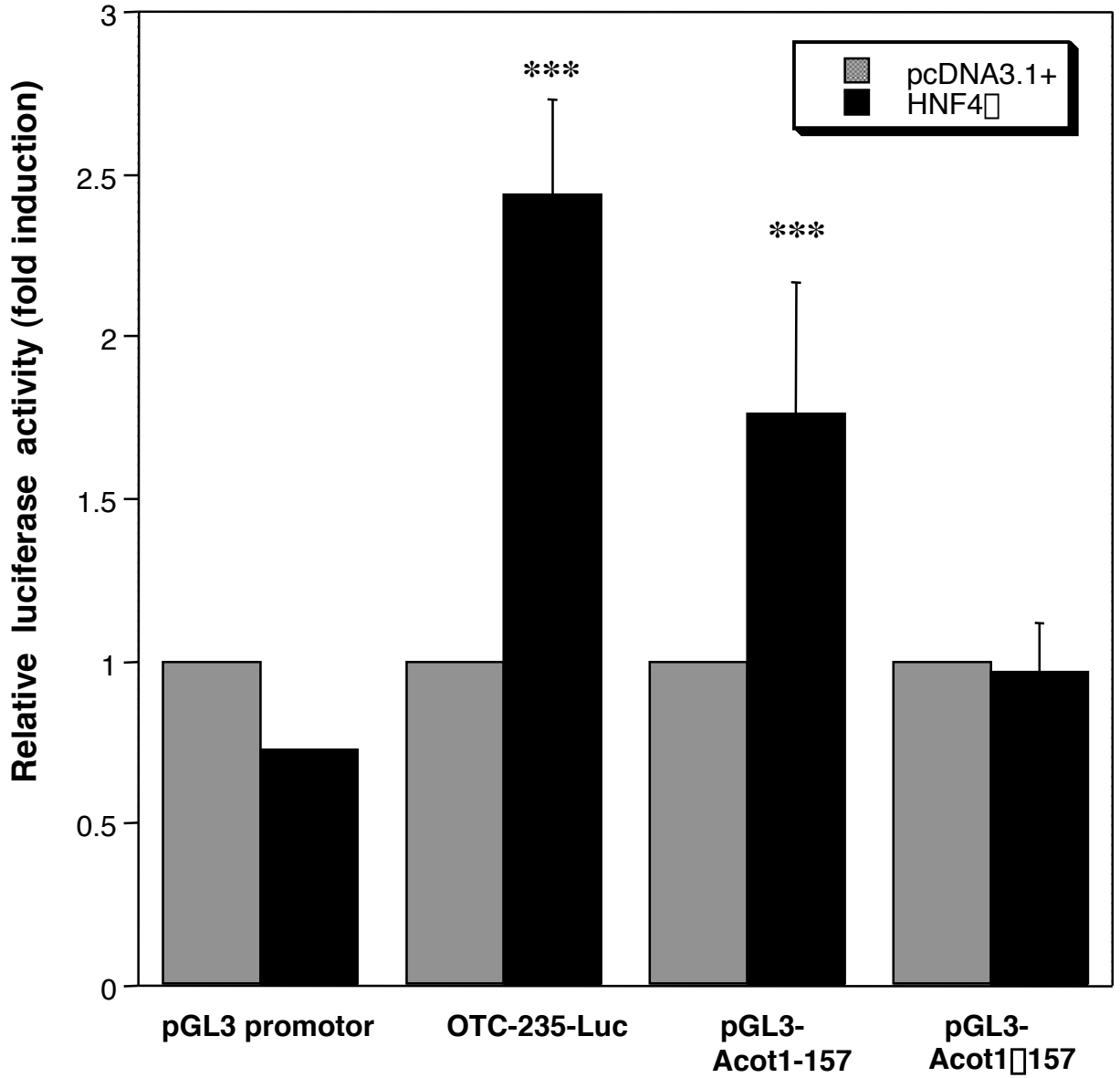
Relative luciferase activity (fold induction)
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2
1.5
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0.5
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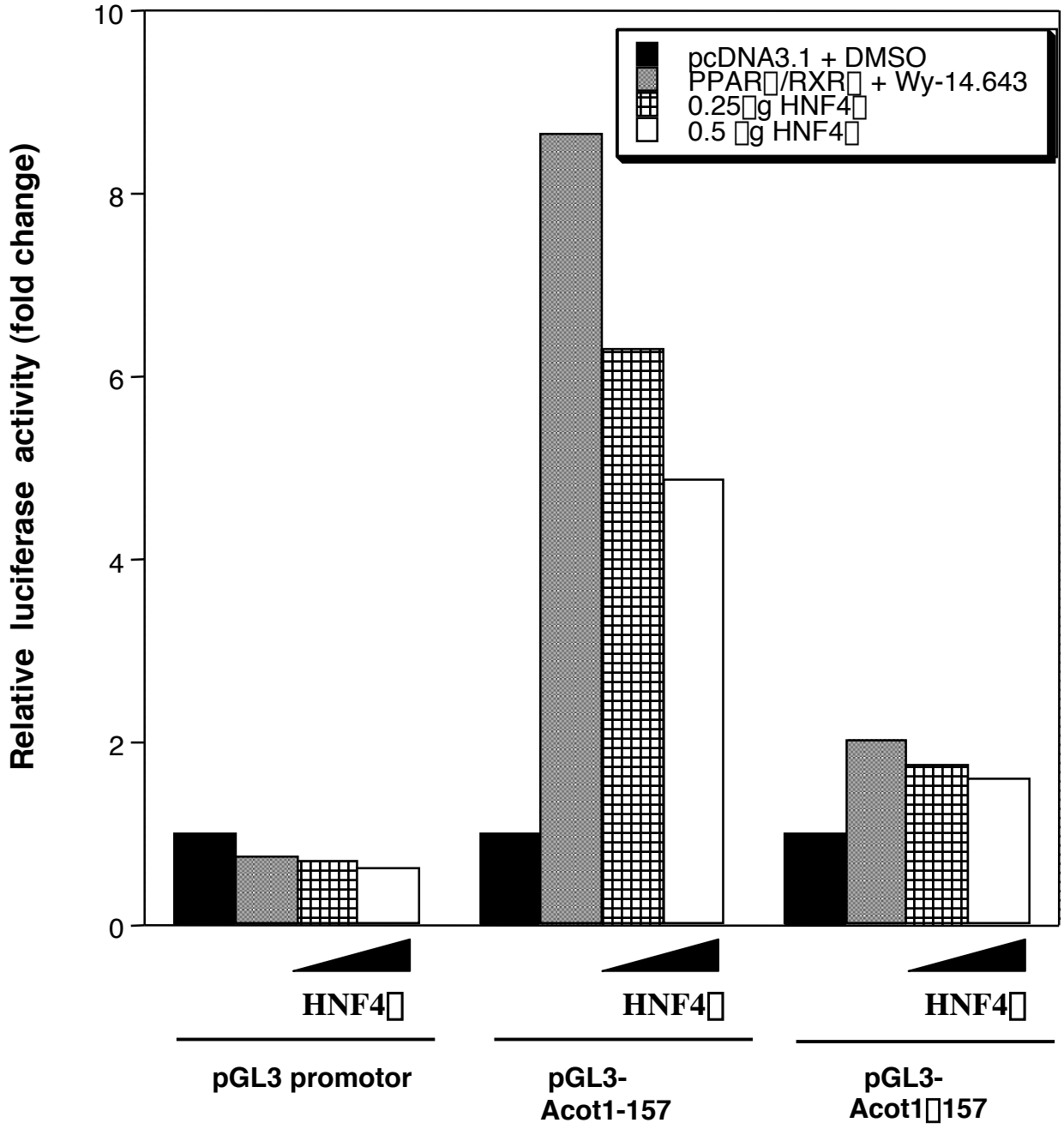
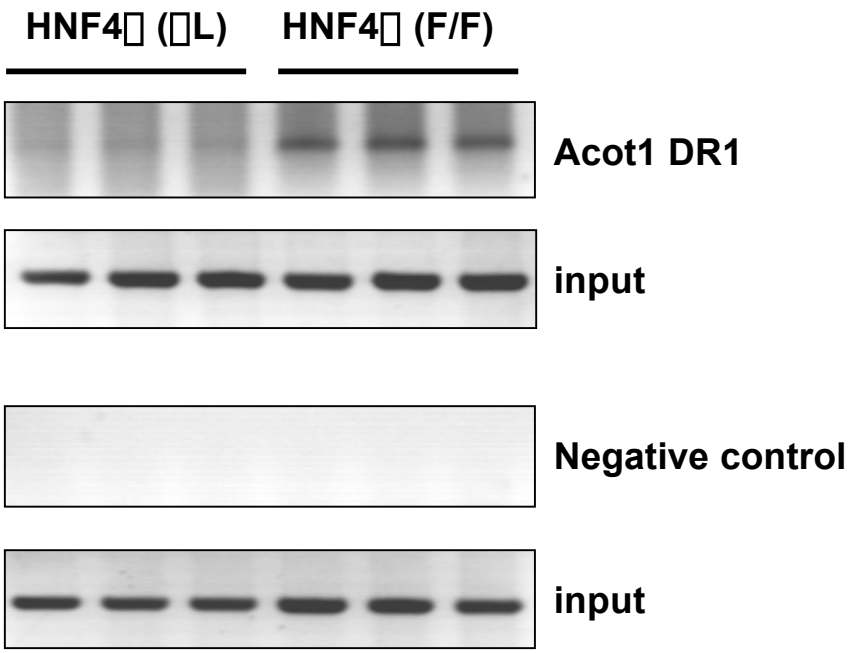
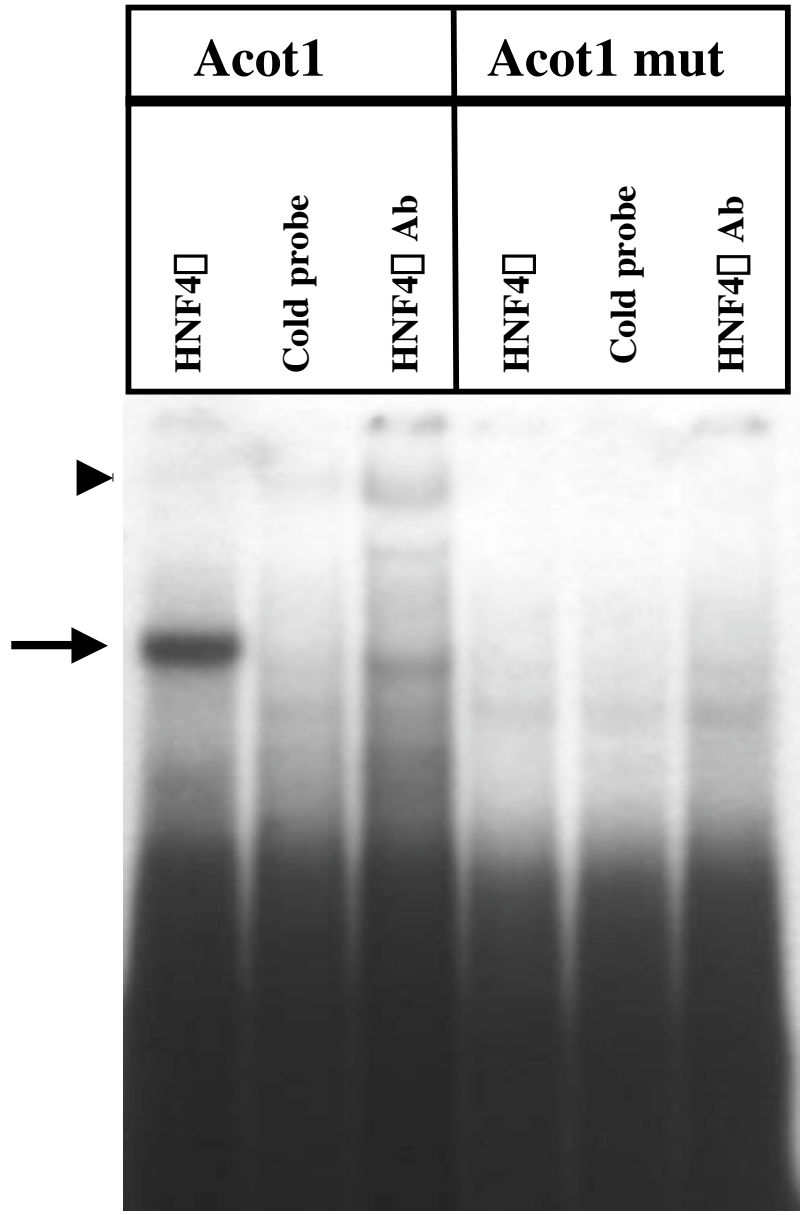
pGL3-Acot1-157
pGL3-Acot1157

B


Relative luciferase activity (fold change)
10
8
6
4
2
0
pcDNA3.1 + DMSO
PPAR/RXR + Wy-14.643
0.25 g HNF4
0.5 g HNF4
HNF4
HNF4
HNF4
pGL3 promotor
pGL3-
Acot1-157
pGL3-
Acot1 157


A
HNF4α (αL)
HNF4α (F/F)
Acot1 DR1
input
Negative control
input


B
Acot1
Acot1 mut
HNF4α
Cold probe
HNF4α Ab
HNF4α
Cold probe
HNF4α Ab

C