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Transactivation of the Peroxisome Proliferator-Activated Receptor Is Differentially Modulated by Hepatocyte Nuclear Factor-4

CHRISTOPHER J. WINROW,* SANDRA L. MARCUS,* KENJI S. MIYATA,† BAOWEI ZHANG,† JOHN P. CAPONE,†[1] AND RICHARD A. RACHUBINSKI*[1]

**Department of Anatomy and Cell Biology, University of Alberta, Edmonton, Alberta T6G 2H7, Canada and †Department of Biochemistry, McMaster University, Hamilton, Ontario L8N 3Z5, Canada*

Peroxisome proliferator-activated receptors (PPARs) stimulate the expression of several genes involved in lipid metabolism by binding to specific *cis*-acting peroxisome proliferator-responsive elements (PPREs) via cooperativity with retinoid X receptors. We demonstrate here that hepatocyte nuclear factor-4 (HNF-4), another member of the nuclear hormone receptor superfamily, bound with differing affinities to the PPREs from the genes encoding rat acyl-CoA oxidase and hydratase-dehydrogenase, the first two enzymes of the peroxisomal β-oxidation pathway. In cotransfection assays, HNF-4 repressed rat PPAR-dependent activation of a reporter gene linked to the acyl-CoA oxidase PPRE, either in the absence or presence of the peroxisome proliferator, Wy-14,643. Rat PPAR-dependent activation of a reporter gene linked to the hydratase-dehydrogenase PPRE was less efficiently repressed by HNF-4 in the absence of Wy-14,643 than was activation from the acyl-CoA oxidase PPRE. However, in the presence of Wy-14,643, HNF-4 functioned cooperatively with PPAR to significantly enhance induction from the hydratase-dehydrogenase PPRE. These results suggest that the genes encoding the first two enzymes of the peroxisomal β-oxidation pathway are subject to differential regulation by the interplay of multiple members of the steroid/nuclear hormone receptor superfamily, mitigated in part by the structures of the PPREs and by the presence of activators of PPARs.

PEROXISOME proliferators constitute a large group of xenobiotic chemicals that include the fibrate family of hypolipidemic drugs, herbicides, and phthalate ester plasticizers. Exposure to peroxisome proliferators increases both the number and metabolic capacity of peroxisomes (Lock et al., 1989). The mechanism of action of peroxisome proliferators is of considerable interest because these agents have been shown to induce hepatomegaly, chromosomal aberrations, and ultimately hepatocarcinogenesis in rodents (Reddy et al., 1980; Reddy, 1990; Rao and Reddy, 1991). Peroxisome proliferators do not mutate DNA directly and are therefore classified as nongenotoxic carcinogens.

The pleiotropic cellular responses to peroxisome proliferators are mediated in part by the transcriptional induction of a number of genes for which the products are involved in lipid metabolism. These include the rat acyl-CoA oxidase (AOx) and hydratase-dehydrogenase (HD) genes, which encode the first two enzymes of the peroxisomal fatty acid β-oxidation system (Reddy et al., 1986); the CYP4A6 gene, which encodes a member of the cytochrome P450 fatty acid ω-hydroxylase family (Mueroff et al., 1992); and the gene encoding the liver fatty acid binding protein (Issemann et al., 1992; Kaikaus et al., 1993; Besnard et al., 1993). Transactivation of these peroxisome proliferator-responsive genes is mediated by peroxisome proliferator-activated receptors (PPARs) (Issemann and Green, 1990). PPARs are members of the steroid/thyroid hormone receptor superfamily that bind to specific *cis*-acting peroxi-

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[1]Address correspondence to John P. Capone or Richard A. Rachubinski.

some proliferator-responsive elements (PPREs), resulting in an overall stimulation of transcription of responsive genes (Osumi et al., 1991; Zhang et al., 1992; Tugwood et al., 1992; Marcus et al., 1993). Human (Schmidt et al., 1992), rat (Göttlicher et al., 1992), mouse (Issemann and Green, 1990; Chen et al., 1993; Zhu et al., 1993), and *Xenopus* (Dreyer et al., 1992) have been shown to harbor multiple PPAR-related genes, perhaps reflecting a requirement for different PPARs in regulating specific target genes or in mediating responsiveness to a variety of stimuli. PPARs can be activated by a wide spectrum of structurally diverse peroxisome proliferators, as well as by both synthetic and natural fatty acids (Dreyer et al., 1992; Keller et al., 1993). However, none of these agents binds directly to PPARs, and therefore the true ligands for these receptors remain to be identified.

The natural PPREs characterized to date consist of direct repeats of the core half-site motif TGACCT, which is also found in the cognate response elements of other nuclear hormone receptors of this class including the thyroid, retinoic acid, vitamin D, and 9-*cis*-retinoic acid (RXR) receptors (Umesono et al., 1991). Target specificity is determined in part by the sequence of the half-sites, the number and relative spacing of their direct repeats, and the ability of some receptors to bind to cognate response elements as homodimers and/or heterodimers (Lucas and Granner, 1992; Mader et al., 1993). However, it is not uncommon for different nuclear hormone receptors to bind to a particular DNA-response element and vice versa. The potential for promiscuous binding, combinatorial interactions, and crosstalk among receptors serves to modulate transcription of hormone-responsive genes in multiple ways, thereby contributing to both the complexity and the diversity in signaling pathways. Indeed, we and others have demonstrated that PPARs bind to PPREs as heterodimers with 9-*cis*-retinoic acid receptors, implying that the peroxisome proliferator and retinoid signaling pathways converge (Kliewer et al., 1992; Gearing et al., 1993; Marcus et al., 1993). Recently, we have shown that COUP-TF, an orphan member of the nuclear hormone receptor superfamily, binds to the HD-PPRE and can antagonize rat (r) PPAR-mediated transactivation from this PPRE in vivo (Miyata et al., 1993). Therefore, PPAR function is dependent upon interactions with, and can be subject to modulation by, other members of the nuclear hormone receptor superfamily.

Hepatocyte nuclear factor-4 (HNF-4) is another orphan member of the nuclear hormone receptor family that binds to TGACCT-related elements (Sladek et al., 1990). HNF-4 is a liver-enriched factor that plays an important role in the transcriptional regulation of several genes for which the products are involved in diverse metabolic pathways. These include the genes encoding apoAI, apoAII, apoB, and apoCIII (Ladias et al., 1992; Mietus-Snyder et al., 1992), α_1-antitrypsin and transthyretin (Costa et al., 1988; 1989), ornithine transcarbamylase (Nishiyori et al., 1994), and some members of the cytochrome P450 hydroxylase superfamily (Chen et al., 1994). HNF-4 mRNA is most abundant in liver and kidney and low in brain, spleen, and lung, a pattern of expression closely matching peroxisome proliferator tissue specificities and PPAR abundance (Issemann and Green, 1990; Dreyer et al., 1992). Because peroxisomes are responsible for the metabolism of long-chain fatty acids, and the PPREs of both the AOx and HD genes share significant homology with the consensus HNF-4 target site, we explored the possibility that HNF-4 might play a role in the regulation of these genes. We demonstrate here that HNF-4 present in extracts of peroxisome proliferator-responsive rat hepatoma cells or synthesized in vitro bound strongly to the AOx-PPRE and with lower affinity to the HD PPRE. HNF-4 on its own had no effect on the transcription of reporter genes linked to the AOx- or HD-PPRE; however, it repressed both the peroxisome proliferator-independent and peroxisome proliferator-dependent transactivation from the AOx-PPRE by rPPAR. Surprisingly, although rPPAR-dependent transactivation of a HD-PPRE-linked reporter gene was also repressed by HNF-4, its activity was potentiated in the presence of a peroxisome proliferator Wy-14,643. Our results suggest that HNF-4 plays an important role in the regulation of genes encoding enzymes of the peroxisomal β-oxidation pathway by differentially modulating transactivation by PPARs.

MATERIALS AND METHODS

Plasmids

The luciferase reporter plasmids pCPS*luc*, pHD(X3)*luc*, and pAOx(X2)*luc* and the effector plasmids expressing the full-length cDNAs for rPPAR and human RXRα have been described (Zhang et al., 1993; Marcus et al., 1993). The plasmid pCH110 (Pharmacia) containing the *lacZ* gene under the control of the SV40 early promoter was included in all transfections to normalize for the efficiency of transfection. The eukaryotic expression vector pSG5 containing a full-length cDNA encoding rat HNF-4 was provided by Dr. Frances Sladek (University of California, Riverside).

Transfection and Measurement of Luciferase Activity

All transfections were carried out with BSC40 monolayer cells by the calcium phosphate method followed by a dimethyl sulfoxide shock (Marcus et al., 1993). Cells were maintained in medium without phenol red and containing 5% charcoal-stripped fetal bovine serum for 24 h prior to and during transfection. Transfections contained 5 μg of either the pHD(X3)*luc* or pAOx(X2)*luc* reporter gene construct and, where indicated, 2 μg of the rPPAR expression vector, 2 μg of RXRα expression vector, and varying amounts of the HNF-4 expression vector. Effector plasmid dosage was kept constant by the addition of appropriate amounts of the corresponding empty vectors. All transfections contained 0.5 μg of pCH110. Total DNA was kept at 20 μg/plate by the addition of sonicated salmon sperm DNA. The peroxisome proliferator Wy-14,643 (a 100× stock in dimethyl sulfoxide) was added to fresh medium to a final concentration of 0.1 mM. Cell extracts were prepared 48 h posttransfection, and luciferase activity was measured as before (Zhang et al., 1992).

In Vitro Transcription/Translation

Transcription of cDNAs encoding rPPAR, RXRα, and HNF-4 followed by translation in rabbit reticulocyte lysate was performed as described (Miyata et al., 1993) using a commercially available kit (Promega). In vitro synthesis of proteins used in gel retardation analyses was carried out with unlabeled methionine.

Gel Retardation Analysis

Nuclear extracts from monolayer cultures of rat hepatoma H4IIEC3 cells were prepared and gel retardation analysis was performed as previously described (Zhang et al., 1993). The following probes were used: HD- PPRE: 5′-gatCCTCTCCTT*TGACCTATTGAACTATTACCT*ACATTTGA and its complement 5′-gatcTCAAATGTAGGTAATAGTTCAATAGGTCAAAGGAGAG; AOx-PPRE: 5′-gatCCTTTCCCGAACG*TGACCTTTGTCCTGGT*CCCCTTTTGCTa and its complement 5′-gatctAGCAAAAGGGGACCAGGACAAAGGTCACGTTCGGGAAAG; M4: 5′-gatCCTCTCCTTattttaAT*TGAACTATTACCT*ACATTTGA and its complement 5′-gatcTCAAATGTAGGTAATAGTTCAATtaaaatAAGGAGAG (Zhang et al., 1993; Miyata et al., 1993). The italicised nucleotides denote the TGACCT-like direct repeats. Nucleotides shown in lower case at the ends of the oligonucleotides were added to provide cohesive *Bam*HI-*Bgl*II ends at the 5′ and 3′ termini, respectively. M4 is a mutant version of the wild-type HD-PPRE in which the first repeat was mutated, as indicated by the nucleotides in lower case. The double-stranded probes were end-labeled with the Klenow fragment of DNA polymerase I and [α-^{32}P]dATP and were purified from polyacrylamide gels prior to use in gel retardation assays. In binding reactions carried out with in vitro synthesized proteins, 2–3 μl of translation mixture was incubated with labeled probe in a final reaction volume of 15 μl. The total amount of reticulocyte lysate in each reaction was kept constant by the addition of unprogrammed lysate. Where indicated, 1 μl of preimmune serum or 1 μl of anti-rat HNF-4 serum was added to the binding reactions, which were then preincubated for 5 min prior to the addition of probe. Binding reactions were analyzed by electrophoresis at 4°C on prerun 3.5% polyacrylamide gels (30:1 acrylamide:*N*′,*N*′-methylenebisacrylamide weight ratio) with 22 mM Tris base/22 mM boric acid/1 mM EDTA as running buffer.

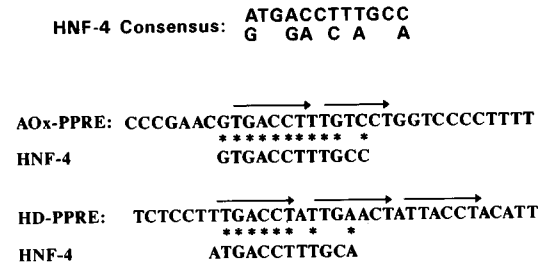

FIG. 1. Comparison of the AOx-PPRE and the HD-PPRE to the consensus HNF-4 binding site. The consensus HNF-4 binding site (Sladek et al., 1990) is shown at the top and is compared to the AOx- and HD-PPREs. The arrows indicate the TGACCT-related repeat motifs.

RESULTS

HNF-4 Interacts With the AOx- and HD-PPREs

Both the AOx-PPRE and the HD-PPRE contain two direct repeats of the TGACCT-related half-site separated by a single nucleotide (denoted DR1) (Osumi et al., 1992; Dreyer et al., 1992; Zhang et al., 1993). The HD-PPRE contains an additional upstream direct repeat separated by a two nucleotide spacing (DR2) that is necessary for peroxisome proliferator responsiveness (Miyata et al., 1993). Figure 1 compares the sequences of the AOx- and HD-PPREs with the HNF-4 consensus binding site (Sladek

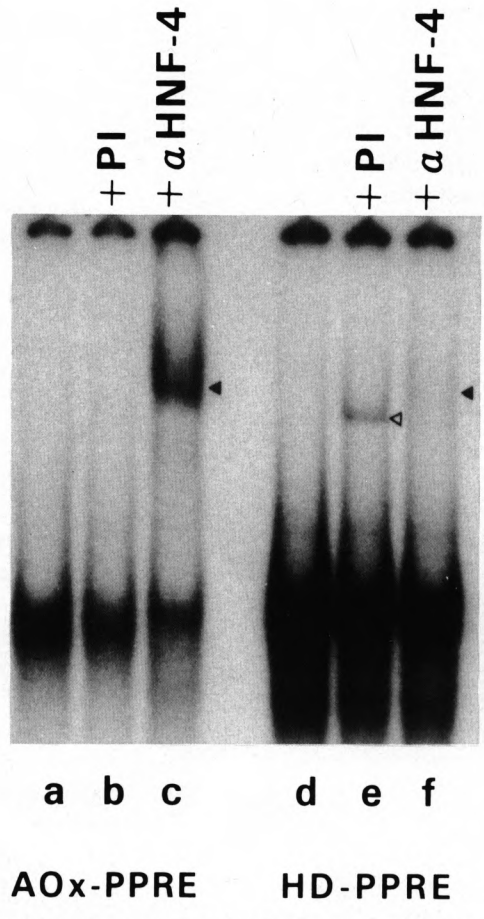

FIG. 2. Endogenous HNF-4-related proteins of rat hepatoma H4IIEC3 cells bind to the AOx- and HD-PPREs. Electrophoretic mobility shift assays were performed with ^{32}P-labeled AOx-PPRE probe or HD-PPRE probe, as indicated at the bottom. Probes were incubated with rat hepatoma H4IIEC3 nuclear extract alone (lanes a and d) or with nuclear extract supplemented with preimmune serum (+PI, lanes b and e) or anti-rat HNF-4 serum (+αHNF-4, lanes c and f). The arrowheads in lanes c and f indicate the supershifted complexes observed with anti-HNF-4 serum. This supershifted complex is readily evident with the AOx-PPRE probe (lane c) but is barely visible with the HD-PPRE probe (lane f). The band indicated by the open arrowhead in lane 3 is a nonspecific complex generated by a factor present in the preimmune serum that has a higher affinity for the HD-PPRE probe than for competitor DNA or for the AOx-PPRE probe.

et al., 1990). The HNF-4 consensus binding sequence matches the AOx-PPRE in 11 of 12 positions, almost overlapping the DR1 repeat. The best match of the HNF-4 consensus binding sequence within the HD-PPRE is 8 of 12 nucleotides encompassing the entire first repeat and part of the second repeat of the HD-PPRE. This high degree of similarity led us to examine whether HNF-4 is among the rat liver nuclear proteins that bind to the AOx- and HD-PPREs.

As shown in Figure 2, incubation of labeled AOx-PPRE (lane a) and HD-PPRE (lane d) probes with nuclear extracts prepared from H4IIEC3 cells, a rat hepatoma cell line responsive to peroxisome proliferators, resulted in the formation of a major protein/DNA complex in each case. We have previously demonstrated that PPAR, RXR, and COUP-TF are among the repertoire of DNA binding proteins present in hepatoma cells that are capable of binding to both the AOx- and HD-PPREs (Miyata et al., 1993). We used antiserum to rat HNF-4 to determine whether HNF-4-like proteins were also present in these protein/DNA complexes. As shown in Fig. 2, inclusion of antiserum specific to HNF-4 (lane c) but not preimmune serum (lane b) led to the formation of a supershifted complex (arrowhead) and a corresponding decrease in the signal intensity of the major protein/DNA complex formed with the AOx-

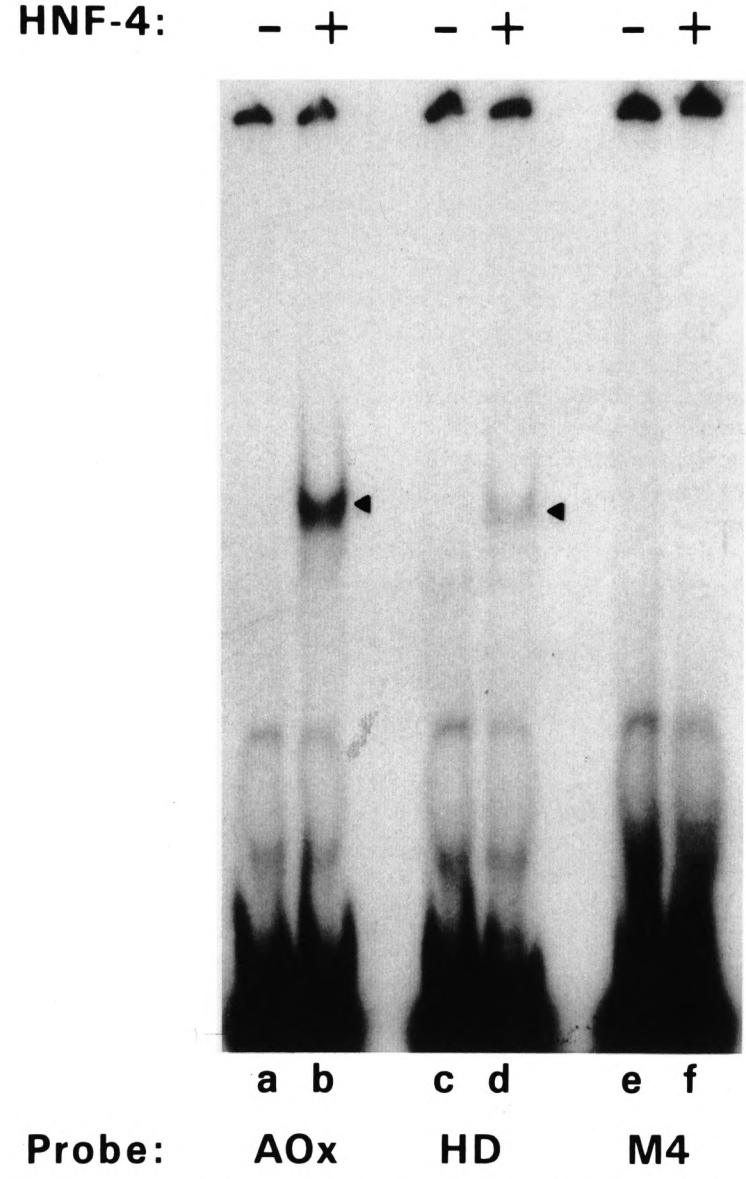

FIG. 3. HNF-4 synthesized in vitro binds to the AOx- and HD-PPREs. Electrophoretic mobility shift assays were carried out with the indicated labeled probes in the absence (−) or presence (+) of in vitro-synthesized rat HNF-4. The arrowheads in lanes b and d indicate the HNF-4 protein/DNA complexes. The M4 probe used in lanes e and f is a mutant HD-PPRE in which the first TGACCT-like repeat is disrupted.

PPRE probe, indicating that HNF-4, or HNF-4-related proteins, present in this extract interact with the AOx-PPRE. A significant fraction of the AOx-PPRE/protein complex could be supershifted with the anti-HNF-4 serum, indicating that HNF-4 is a major component of the protein complexes formed on this element. In similar experiments carried out with the HD-PPRE, the generation of a supershifted complex with anti-HNF-4 serum was at the limit of detection (arrowhead, lane f). The above results suggest that HNF-4 or HNF-4-related proteins present in extracts of rat liver hepatoma cells bind to the AOx-PPRE and, with much lower affinity, to the HD-PPRE. The differences in affinities correlate with the degree of relatedness of the respective PPREs to the HNF-4 consensus binding sequence.

To determine if HNF-4 could bind directly to the AOx- and HD-PPREs, we transcribed and translated rat HNF-4 in vitro from its cDNA and used the in vitro-synthesized protein in mobility shift assays. As shown in Fig. 3, in vitro-synthesized HNF-4 bound strongly to the AOx-PPRE probe (lane b) and more

weakly to the HD-PPRE probe (lane d). The binding observed with both the AOx- and HD-PPREs was sequence specific, as determined by using mutant oligonucleotides as probes and competitors. For example, HNF-4 was unable to bind to a mutant HD-PPRE probe (M4) in which the first TGACCT repeat was mutated [Fig. 3, compare M4 (lane f) to HD (lane d)], indicating that the first direct repeat in the HD-PPRE, which encompasses the HNF-4 consensus sequence, is required for binding to HNF-4. We have previously shown that luciferase reporter constructs containing the M4 mutant HD-PPRE could not be activated by peroxisome proliferators in vivo, yet PPAR/RXR heterodimers could still efficiently bind to this element in vitro (Miyata et al., 1993). HNF-4 and PPAR/RXR may therefore have overlapping recognition sequences on the HD-PPRE, similar to what is observed with COUP-TF (Miyata et al., 1993). This finding would be consistent with the overlapping sequence identity between the first two repeats of the HD-PPRE and the HNF-4 consensus binding site (Fig. 1). It is not yet known whether all three receptors can occupy this site simultaneously.

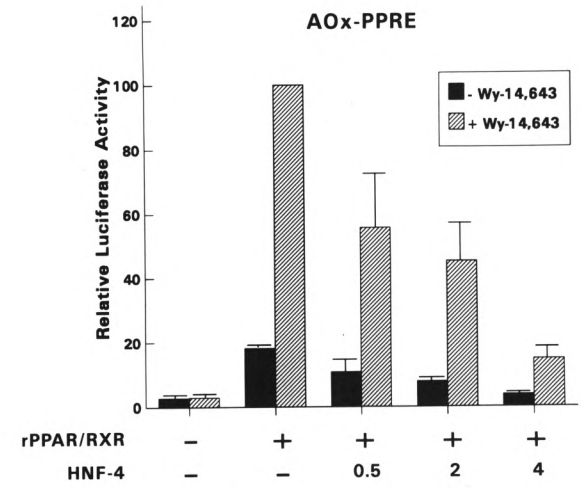

FIG. 4. HNF-4 antagonizes rPPAR-mediated induction of an AOx-PPRE reporter gene. pAOx(X2)*luc* was transfected into BSC40 cells in the presence or absence of the peroxisome proliferator Wy-14,643, along with a constant amount of rPPAR and RXRα expression plasmids and increasing amounts of rat HNF-4 expression plasmid (in μg), as indicated. Transfections were carried out in duplicate and repeated a minimum of three times. The values shown (± SEM) are normalized to the value obtained from Wy-14,643-treated cells cotransfected with rPPAR and RXRα expression plasmids, which was taken as 100%.

HNF-4 Represses rPPAR-Dependent Induction From the AOx-PPRE Both in the Presence and Absence of a Potent Peroxisome Proliferator

To examine the consequences of ectopic expression of HNF-4 on transcription from the AOx- and HD-PPREs, we carried out transient transfection assays with luciferase reporter gene constructs containing the minimal carbamoyl phosphate synthetase promoter linked to either two tandem copies of the minimal AOx-PPRE [pAOx(X2)*luc*] or three tandem copies of the minimal HD-PPRE [pHD(X3)*luc*] (Zhang et al., 1993). The effector plasmids that were used expressed full-length cDNAs encoding rPPAR, human RXRα, and rat HNF-4. BSC40 cells were used in the transient transfection assays, because efficient induction by peroxisome proliferators in this cell line requires the cotransfection of both PPAR and RXR (Miyata et al., 1993).

Cotransfection of pAOx(X2)*luc* with rPPAR and RXRα expression vectors resulted in a 5- to 10-fold induction in activity of the reporter gene, as we have previously demonstrated (Fig. 4) (Marcus et al., 1993). This rPPAR-mediated induction is independent of exogenously added peroxisome proliferators and likely results from the presence of endogenous PPAR and/or RXR activators present in these cells. Addition of the potent peroxisome proliferator Wy-14,643 resulted in a 50-fold stimulation of activity over basal levels. This stimulation required the presence of both rPPAR and RXRα. Expression of HNF-4 during the transient transfection resulted in inhibition of both the rPPAR-dependent/Wy-14,643-independent induction and the rPPAR-dependent/Wy-14,643-dependent induction. Inhibition was dose dependent under both circumstances. Inclusion of 4 μg of the HNF-4 expression plasmid reduced rPPAR-dependent activation to the basal levels observed with pAOx(X2)*luc* alone. Similarly, peroxisome proliferator-dependent induction was almost completely inhibited in the presence of 4 μg of HNF-4 expression plasmid. HNF-4 dependent inhibition was specific for the rPPAR-mediated response, because the basal level expression of pAOx(X2)*luc* was not significantly affected by cotransfection with increasing amounts of HNF-4 expression plasmid (data not presented). These findings demonstrate that HNF-4 antagonizes induction from the AOx-PPRE by rPPAR, both in the absence and in the presence of an exogenously added peroxisome proliferator.

HNF-4 Potentiates Activity of rPPAR on the HD-PPRE in the Presence of a Peroxisome Proliferator

Transfection experiments like those carried out with the AOx-PPRE reporter gene construct were carried out with the HD-PPRE reporter gene con-

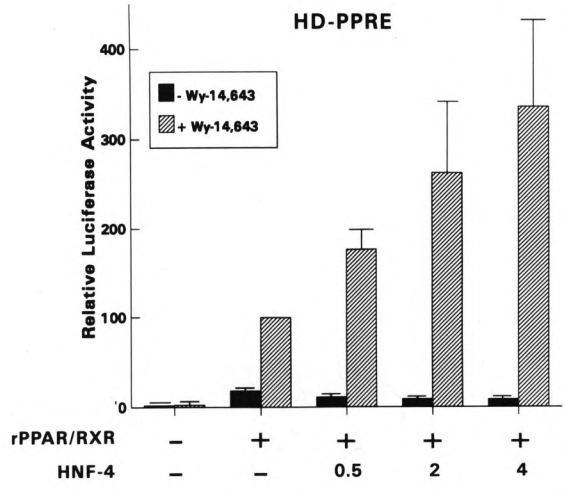

FIG. 5. HNF-4 differentially modulates the function of rPPAR on the HD-PPRE. Cotransfection of pHD(X3)*luc* with various expression plasmids into BSC40 cells in the presence or absence of Wy-14,643 and luciferase activity measurements were performed as described in Fig. 4. The results demonstrate that HNF-4 antagonizes rPPAR function in the absence of Wy-14,643 but potentiates rPPAR activity in the presence of Wy-14,643.

struct. Cotransfection of pHD(X3)*luc* with both rPPAR and RXRα expression plasmids gave a fivefold peroxisome proliferator-independent induction and a 20-fold peroxisome proliferator-dependent induction of reporter gene activity over basal levels (Fig. 5). As seen with the AOx-PPRE reporter gene construct, inclusion of HNF-4 during transient transfection resulted in inhibition of the peroxisome proliferator-independent activation mediated by rPPAR/RXRα from the HD-PPRE. However, HNF-4 mediated inhibition was less efficient with the HD-PPRE than with the AOx-PPRE, causing only a 50% inhibition of activation with 4 μg of HNF-4 expression plasmid compared to a control transfection in the absence of HNF-4. Surprisingly, when Wy-14,643 was included in the transfections, HNF-4 significantly stimulated the induction response of the HD-PPRE. Cotransfection with 4 μg of HNF-4 expression plasmid resulted in a two- to threefold potentiation of the rPPAR-mediated response to the presence of this peroxisome proliferator. Both repression and stimulation by HNF-4 required the presence of rPPAR and RXRα, because HNF-4 alone had no significant effects on the basal level expression of the HD-PPRE reporter gene construct (data not shown). Therefore, in contrast to the results seen with the AOx-PPRE, HNF-4 cooperates with rPPAR to stimulate transcription of the HD-PPRE reporter gene construct, but only in the presence of a peroxisome proliferator. Because HNF-4 both repressed the rPPAR-mediated response in the absence of drug and increased the response in the presence of drug, the peroxisome proliferator-dependent induction ratio was effectively increased from fivefold to approximately 35-fold.

HNF-4 Does Not Cooperate With RXRα or rPPAR for DNA Binding

rPPAR heterodimerizes with RXRα and binds cooperatively to both the AOx- and HD-PPREs in vitro (Fig. 6, compare lanes b and c with lanes e) (Marcus et al., 1993). In contrast, HNF-4 has been shown to bind to cognate response elements exclusively as a homodimer, and there is no evidence that HNF-4 heterodimerizes with other members of the nuclear hormone receptor family (Sladek et al., 1990). Nevertheless, we explored the possibility that the effects of HNF-4 on rPPAR-dependent induction of transcription in vivo was mitigated by cooperative interactions with PPAR and/or RXRα by mobility shift analyses with in vitro-translated receptor proteins. As shown in Fig. 6, the binding of HNF-4 to the AOx-PPRE or the HD-PPRE was not affected by the presence of RXRα or rPPAR and vice versa (compare lanes d to lanes f and g, respectively). Therefore, the ability of HNF-4 to modulate PPAR function apparently is not the result of cooperative DNA binding by HNF-4 with either RXRα or rPPAR.

DISCUSSION

The results presented herein demonstrate that HNF-4 differentially modulates the functioning of the peroxisome proliferator-activated receptor on specific PPREs, suggesting that this nuclear hormone receptor plays a role in the regulation of expression of genes encoding peroxisomal β-oxidation enzymes. HNF-4 has been shown to act as a cell-restricted, positive regulator of genes for which the products are involved in several metabolic pathways (Sladek et al., 1990; Ladias et al., 1992; Chen et al., 1994). Our findings are the first demonstration that HNF-4 can both activate and repress specific target genes involved in lipid metabolism through a common response element.

HNF-4 was a major component of the protein/DNA complex formed between the AOx-PPRE and rat liver hepatoma nuclear extracts, and HNF-4 synthesized in vitro interacted strongly with the AOx-PPRE. In transient transfection assays, HNF-4 was capable of competitively inhibiting rPPAR-dependent transactivation of a reporter gene linked to the AOx-PPRE, both in the presence or the absence of

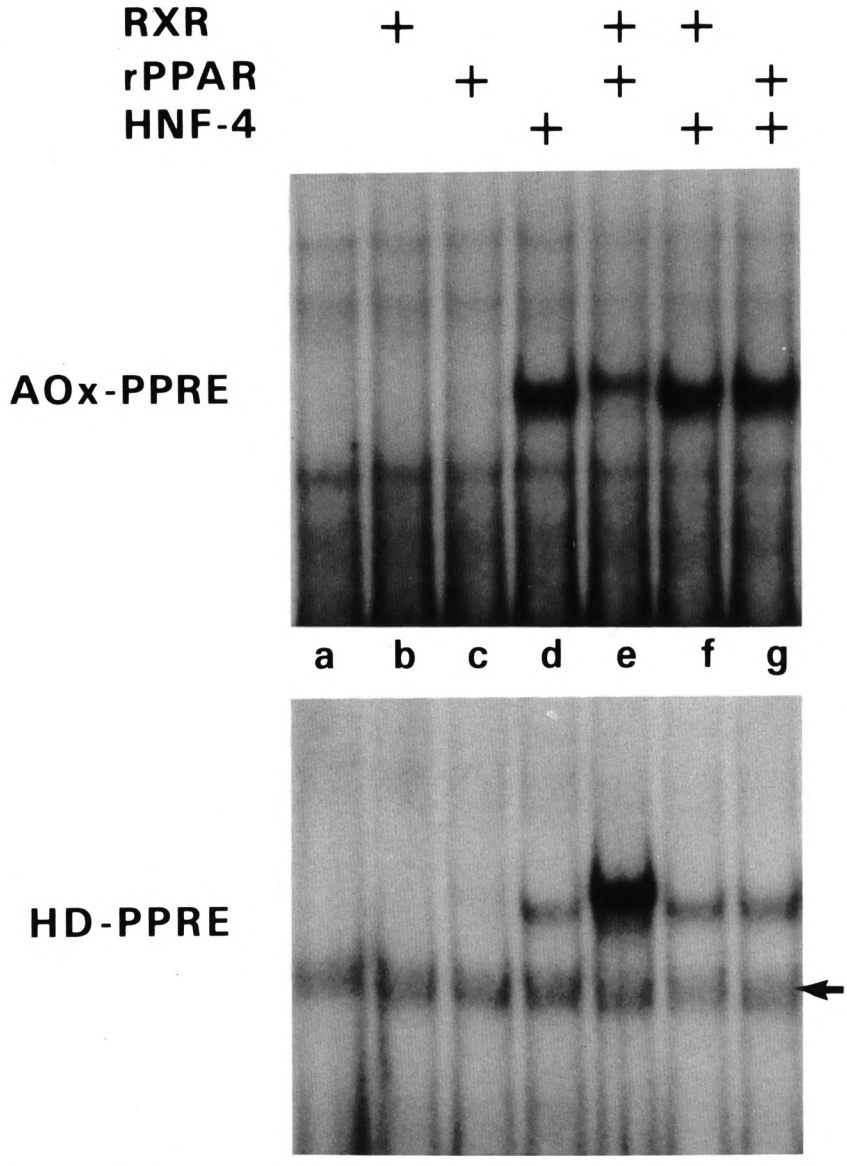

FIG. 6. HNF-4 does not cooperate with rPPAR or RXRα for DNA binding. In vitro-translated human RXRα, rPPAR, and rat HNF-4 were incubated singly or in pairwise combinations, as indicated, with labeled AOx-PPRE probe (upper panel) or HD-PPRE probe (lower panel) and analyzed by electrophoretic mobility shift assay. Lanes a contained probes incubated with unprogrammed reticulocyte lysate. Arrow: nonspecific complex formed between the HD-PPRE probe and reticulocyte lysate.

the peroxisome proliferator, Wy-14,643. The mechanism by which HNF-4 antagonizes rPPAR functioning is not known at present, but one scenario is that HNF-4 can compete directly with rPPAR/RXR heterodimers for binding to the AOx-PPRE. This explanation would be consistent with the near-perfect match between the consensus HNF-4 binding site and part of the AOx-PPRE and with the fact that the region of homology between the two sequences almost completely overlaps the two TGACCT DR1 repeats that are essential for rPPAR/RXRα binding to the AOx-PPRE. HNF-4 was unable to bind cooperatively with either rPPAR or RXRα, implying that competition for heterodimerization partners is probably not involved in the observed inhibition of activation from the AOx-PPRE. However, it remains possible that under physiological conditions, non-DNA binding heteromers could form between HNF-4 and rPPAR or RXRα, thereby leading to inhibition of the rPPAR-mediated response via indirect mechanisms.

Inhibition of activation from the AOx-PPRE by HNF-4 could result from the absence of an appropriate ligand for HNF-4 in the BSC40 cells used in

the transfection assays. It has been shown that, depending on the nature of the response element, the thyroid receptor can function as a repressor in the unliganded state but as an activator when associated with its cognate hormone (Graupner et al., 1989; Sap et al., 1989; Damm et al., 1989). The repression observed with the thyroid receptor in the absence of its ligand is thought to be mediated via an active intrinsic silencing domain that may directly inhibit the formation of the functional preinitiation complex (Baniahmad et al., 1992; Fondell et al., 1993). There is no evidence to date that HNF-4 possesses an active transcriptional silencing function or even that a ligand exists for this receptor.

The results obtained with the HD-PPRE are even more intriguing than those obtained with the AOx-PPRE. HNF-4 had a repressive effect (50%) on the rPPAR-dependent activation of the HD-PPRE in the absence of exogenously added peroxisome proliferator. However, in the presence of Wy-14,643, HNF-4 cooperated with rPPAR/RXRα to provoke a significant stimulation of transcription. Therefore, under conditions of peroxisome proliferator administration, HNF-4 had diametric effects on the rPPAR-mediated, peroxisome proliferator responsiveness of the AOx-PPRE vis-à-vis the HD-PPRE. It is unclear whether the effects of HNF-4 on activation from the HD-PPRE are mitigated directly through binding to the HD-PPRE or indirectly, for example, through interaction with, or modulation of, auxiliary coregulators involved in peroxisome proliferator signaling or transcriptional control.

HNF-4 synthesized in vitro or present endogenously in rat hepatoma cells had only a weak affinity for the HD-PPRE, although its binding to the HD-PPRE was specific for the sequence of this element. The structure of the HD-PPRE is more complex than that of the AOx-PPRE. The HD-PPRE consists of three TGACCT-related half-sites separated by two nucleotides and one nucleotide, respectively. The integrity of all three repeats, as well as the spacing between them, is necessary for efficient peroxisome proliferator responsiveness in vivo; however, only the DR1 repeats are necessary for rPPAR/RXRα binding in vitro (Miyata et al., 1993). The region within the HD-PPRE that most closely matches the HNF-4 consensus sequence contains the first direct repeat. We have shown that this repeat is necessary for HNF-4 binding (Fig. 3). It is conceivable that binding of HNF-4 to the first repeat might interfere with binding of rPPAR/RXR to the HD-PPRE, leading to the inhibitory effects on activation observed in transient transfections. Activation of rPPAR by exogenously added peroxisome proliferators may serve to alter the binding affinity or conformation of the rPPAR/RXRα heterodimer specifically on the HD-PPRE, thereby allowing cooperative interactions with HNF-4 and consequently potentiation of induction. We have previously shown that the first repeat in the HD-PPRE is also required for binding to COUP-TF1, an orphan receptor that antagonizes peroxisome proliferator responsiveness in vivo (Miyata et al., 1993). Therefore, the additional repeat element present in the HD-PPRE compared to the AOx-PPRE may allow more complex strategies of regulation for the HD gene.

It is becoming increasingly apparent that PPARs play a central role in regulating lipid metabolic pathways. More recently, PPARs have been implicated in cellular differentiation and proliferation (Chawla and Lazar, 1994). Our findings suggest that HNF-4 can modulate PPAR function both positively and negatively, consequently resulting in differential regulation of at least two distinct peroxisome proliferator-responsive genes. These differential effects are conditional upon the structure of a particular target PPRE and the presence or absence of PPAR activators. This finding is interesting in light of the recent observation that HNF-4 activates transcription of the medium-chain acyl-CoA dehydrogenase gene (Carter et al., 1993). Medium-chain acyl-CoA dehydrogenase is the initial and rate-limiting enzyme of the mitochondrial fatty acid β-oxidation pathway, which metabolizes short- and medium-chain length fatty acids in response to energy demands of the cell. In contrast, the peroxisomal β-oxidation pathway preferentially uses long-chain fatty acids as substrates, and acyl-CoA oxidase is the initial and rate-limiting step of this pathway. Our finding that HNF-4 represses PPAR-mediated induction of the AOx gene suggests that HNF-4 may play a key role in coordinating and integrating the mitochondrial and peroxisomal β-oxidation pathways through the bidirectional regulation of the respective rate-limiting enzymes. Similarly, the HNF-4-mediated, peroxisome proliferator-dependent upregulation of the activity of the HD gene, for which the product catalyzes the second step in the peroxisomal β-oxidation pathway, may represent an adaptive cellular response that primes the peroxisomal β-oxidation pathway to respond rapidly to cellular oxidative demands under physiological conditions where repression of the AOx gene is alleviated. HNF-4/PPAR cooperativity on the HD-PPRE may be relevant to the observation that HD accumulates to higher levels in the liver compared to the other two enzymes of the peroxisomal β-oxidation pathway following administration of peroxisome proliferators (Reddy et al., 1986). Our findings add an additional layer of complexity to peroxisome proliferator-signaling path-

ways and may begin to provide insight into how combinatorial interactions among distinct nuclear receptors and convergence of multiple signaling pathways integrate complex metabolic regulatory networks.

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