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Functional Interactions between Nuclear Receptors Recognizing a Common Sequence Element, the Direct Repeat Motif Spaced by One Nucleotide (DR-1)[1]

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Direct repeat motifs composed of two hexamer half-sites spaced by a single nucleotide (DR-1) are recognized by several members of the nuclear hormone receptor superfamily. We examined, by means of gene transfection assays, the interplay between the DR-1-binding nuclear receptors commonly expressed in liver, peroxisome proliferator-activated receptor α (PPARα), hepatocyte nuclear factor-4 (HNF-4), and chicken ovalbumin upstream transcription factor I (COUP-TFI). Both PPARα and HNF-4 efficiently bound to the acyl-CoA oxidase gene enhancer element, but PPARα exhibited much stronger transactivation than HNF-4. As a result, HNF-4 suppressed the gene-activating function of PPARα, when they were expressed together, due to competition for a common binding site. On the other hand, HNF-4, but not PPARα, effectively bound to the apolipoprotein CIII gene element, and activated gene transcription. PPARα had no effect even when co-expressed with HNF-4. COUP-TFI bound to both elements, and suppressed the gene activation by PPARα and HNF-4. Thus, these nuclear receptors have individual functions in gene regulation, and exhibit complex compound effects when they co-exist.

Key words: chicken ovalbumin gene upstream promoter transcription factor, hepatocyte nuclear factor-4, nuclear receptor, peroxisome proliferator, peroxisome proliferator-activated receptor.

Proteins of the nuclear hormone receptor superfamily regulate the transcription of target genes, upon recognizing the cognate binding site sequences (*1*). Non-steroid receptors, such as retinoic acid receptor, thyroid hormone receptor, vitamin D receptor, and peroxisome proliferator-activated receptor (PPAR), form heterodimers with retinoid X receptor (RXR), and bind to direct repeat (DR) sequences composed of two half-site motifs, AGGTCA or related sequences (*1*). The spacing between the half-sites is a critical determinant of the binding specificity (*2*). The PPAR/RXR heterodimer recognizes the DR-1 sequence, in which the half-sites are spaced by a single nucleotide (*3*). An extended half-site of 10 residues, not a simple hexamer half-site, is required for the efficient binding of PPAR (*4-6*). Certain orphan nuclear receptors, including hepatocyte nuclear factor-4 (HNF-4) (*7*) and chicken ovalbumin upstream promoter-transcription factor I (COUP-TFI) (*8*), also bind to DR-1 motifs.

PPAR forms a subfamily within the nuclear hormone receptor superfamily, governing body lipid homeostasis (*9*). PPARα is most abundantly expressed in liver (*10*), and was suggested to regulate the genes coding for fatty acid-metabolizing enzymes, including peroxisomal acyl-CoA oxidase (AOX), as well as proteins involved in lipid circulation (*11*). A diverse array of compounds, such as the fibrate class of peroxisome proliferators, long-chain fatty acids, and arachidonic acid metabolites, activate PPARα, as ligands (*12-15*). HNF-4 is also expressed preferentially in liver, and activates the transcription of many liver-specific genes, including those of apolipoproteins, in an apparently constitutive manner (*16*). On the other hand, COUP-TFI is expressed in a variety of tissues, and acts on many genes, negatively (*8*). These observations raise the question of how these nuclear receptors interplay on a given gene promoter carrying a DR-1 sequence, when their expression in the same cell type, *e.g.* hepatocytes, is considered.

In this paper, we describe the actions of PPARα, HNF-4, and COUP-TFI on the DR-1 motifs in the AOX and apolipoprotein CIII (apoCIII) gene promoters. These nuclear receptors exhibit different functions on the respective DR-1s, and hence, when expressed together, have complex compound effects on gene transcription.

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Abbreviations: AOX, acyl-CoA oxidase; ApoCIII, apolipoprotein CIII; COUP-TFI, chicken ovalbumin upstream transcription factor I; DR-1, direct repeat motif spaced by a single nucleotide; EMSA, electrophoretic mobility-shift assay; HNF-4, hepatocyte nuclear factor-4; luc, luciferase; PPAR, peroxisome proliferator-activated receptor; PPRE, peroxisome proliferator-response element; RXR, retinoid X receptor; tk, thymidine kinase.

MATERIALS AND METHODS

Plasmids—Luciferase (luc) was used as a reporter throughout this study. The luc gene was driven by the Herpes simplex virus thymidine kinase (tk) promoter. The reporter vector was constructed as follows: A tk promoter-driven chloramphenicol acetyltransferase reporter vector, pBLcat5 (*17*), was cleaved with *Bgl*II and *Pfl*MI, to remove the cat gene portion. Between these sites was inserted a luc gene fragment cleaved from pGV-B (Toyo Ink) with the same restriction enzymes. The resulting plasmid, pBLluc, was used as the starter for constructing the test plasmids.

pBLPPRE2luc contained two copies of the PPAR/RXR binding site [peroxisome proliferator-response element (PPRE)] of the rat AOX gene (*5*), upstream of the tk promoter. For construction of the plasmid, a double strand oligonucleotide composed of 5′-CGAACGTGACCTTTGT-CCTGGTCCCCTTTTGCTCC-3′ and 5′-TCGGGAGCAA-AAGGGGACCAGGACAAAGGTCACGT-3′ was used. The oligonucleotide was ligated into a dimer, blunt-ended with Klenow fragment, and inserted in pBluescript II KS(−) at the *Hin*cII site. A correctly ligated dimer, as verified by nucleotide sequencing, was cleaved out with *Hin*dIII and *Bam*HI, and inserted between the *Hin*dIII and *Bam*HI sites of pBLluc. The resulting plasmid contained two copies of the PPRE sequence in the same orientation as in the natural AOX gene.

A reporter construct containing the HNF-4 binding site of the apoCIII gene [C3P (*18*)] was constructed as follows: A double strand oligonucleotide, composed of 5′-TCGACA-GGGCGCTGGGCAAAGGTCACCTGCT-3′ and 5′-TCGA-AGCAGGTGACCTTTGCCCAGCGCCCTG-3′, was ligated into the dimer oriented in the same direction. The resulting dimer was inserted into pBLluc at its *Sal*I site, giving a construct, pBLC3P2luc, which contained two copies of the C3P element, upstream of the tk promoter.

cDNAs of PPARα (*5*) and HNF-4 [HNF-4CL (*19*)] were obtained, as described. cDNA of COUP-TFI was provided by Dr. M. Takiguchi. The expression vectors of these nuclear receptors were all constructed with the plasmid, pCMX (*2*; a gift from Drs. K. Umesono and R.M. Evans), which contains the human cytomegalovirus enhancer and promoter.

Transfection Experiments—A rat hepatoma line, HepG2, was used throughout this study. For each transfection, 6×10^5 cells were seeded into every 60 mm dish. After culturing overnight, the cells were transfected by the calcium phosphate method (*20*) with 12 μg of DNA containing 8 μg of a reporter plasmid, 1 μg of a β-galactosidase expression vector, pCMVβ (*21*), as a reference, and varying amounts of expression vectors of appropriate nuclear receptors. The total amount of the DNA was kept constant for each dish by adding an appropriate amount of the empty vector, pCMX. Other conditions for the transfection and luciferase assays were as described (*5*). The reporter gene expression was represented by the luciferase activity, which was normalized as to the efficiency of transfection based on the β-galactosidase activity. Most transfection experiments were performed twice independently or in duplicate, the mean luciferase activities being presented. Where indicated, mean$\pm$SD values obtained for three independent assays are given.

Electrophoretic Mobility-Shift Assay (EMSA)—The oligonucleotides of AOX-PPRE and C3P described above were used as probes. A rat liver nuclear extract was prepared as described (*22*). Nuclear receptors were also prepared by *in vitro* transcription/translation of the respective cDNAs with rabbit reticulocyte lysate (Promega), as recommended by the manufacturer. Other assay conditions were as described (*5*).

Anti-PPARα antibodies were raised by injecting recombinant PPARα expressed in *Escherichia coli*. A fusion protein comprising mouse PPARα and maltose binding protein was expressed, purified by affinity chromatography, and digested with factor Xa. The PPARα portion was purified by SDS-PAGE, and used for immunization. Anti-HNF-4 antibodies raised against the carboxyl-terminal peptide were a gift from Dr. M. Takiguchi.

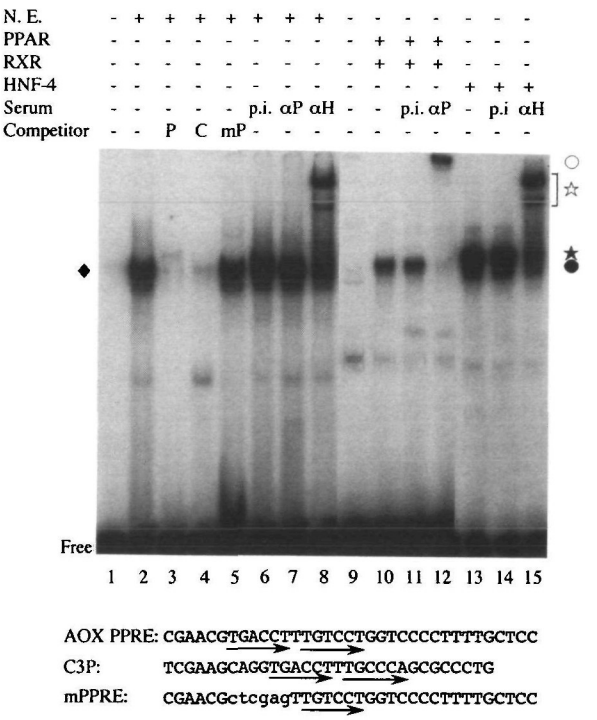


Fig. 1. **EMSA of a rat liver nuclear extract (N.E.) and *in vitro*-synthesized nuclear receptors with the AOX PPRE probe.** A nuclear extract (9 μg protein; lanes 2-8), PPARα and RXRα (lanes 10-12), and HNF-4 (lanes 13-15) were mixed with 0.2 pmol of the ^{32}P-labeled AOX PPRE probe (*ca.* 2×10^4 cpm), and then subjected to the gel retardation assay. The nuclear receptors were synthesized with a rabbit reticulocyte lysate, and 1.5 μl of the translation mixture was used for each receptor. Other conditions were as described (*5*). In lane 1, no protein was added, whereas in lane 9, unprogrammed reticulocyte lysate was added. Competitors AOX PPRE (P), C3P (C), and mutated PPRE (mP) were added at a 20-fold molar excess over the probe, where indicated. Preimmune (p.i.), anti-PPARα (αP), and anti-HNF-4 (αH) sera (1 μl each) were added to the lanes specified. The filled diamond, circle, and star indicate the bands resulting on the binding of hepatic nuclear proteins, PPAR/RXR, and HNF-4, respectively, whereas the open circle and star indicate the supershifted bands due to anti-PPAR and anti-HNF-4 antibodies, respectively. The nucleotide sequences of the three oligonucleotides used as probes and competitors are given, in the direction corresponding to the natural orientation of AOX PPRE. The two half-sites are indicated by horizontal arrows, and mutated residues are written in lower case.

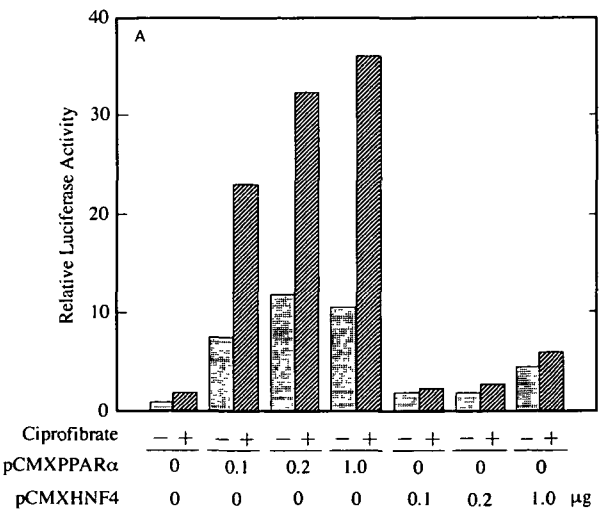
RESULTS

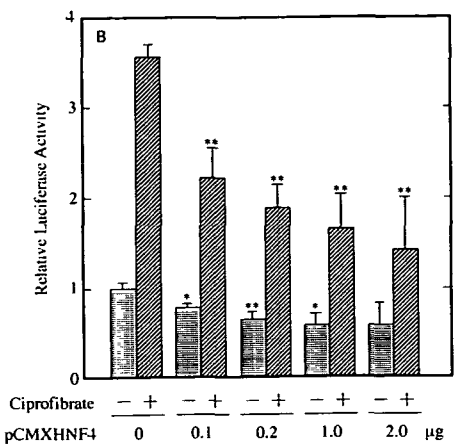
Identification of HNF-4 as a Major Hepatic Binding Activity as to PPRE—The reported overlapping binding specificities of PPARα and HNF-4 prompted us to examine the major hepatic binding activity as to AOX PPRE. When the PPRE probe was mixed with a rat liver nuclear extract and then subjected to EMSA, a strong band shift was observed (Fig. 1, lane 2). This band was entirely dependent on the nuclear extract, because the probe itself did not give a band at the same position (lane 1). The addition of anti-HNF-4 antiserum to the binding mixture resulted in a significant supershift, and the uppermost part of the band decreased (lane 8). The anti-PPARα antibodies, however, caused no detectable supershift (lane 7), even when *in vitro* synthesized RXRα was added to the binding reaction mixture (data not shown). As described in detail in a later section, both *in vitro* synthesized PPAR/RXR and HNF-4 bound to the PPRE probe (lanes 10 and 13; see also Fig. 4A). The positions of the shifted and supershifted bands as well as the effectiveness of the antisera (lanes 10-15) were all consistent with the results obtained with the liver nuclear extract.

These results indicate that HNF-4 significantly contributes to the hepatic binding activity as to AOX PPRE, whereas endogenous PPARα exhibits only a minor capacity for the binding, under these experimental conditions. The result also suggests that other binding activities as to AOX PPRE are present in the hepatic nuclear extract. These binding activities were effectively competed with by a 20-fold excess of unlabeled oligonucleotides of AOX PPRE and C3P, the binding site of HNF-4 of the apoCIII gene (*18*) (Fig. 1, lanes 3 and 4). On the other hand, they were not competed with by a mutant PPRE oligonucleotide (mPPRE) in which the first half-site of AOX PPRE was changed (lane 5). Thus, these binding activities are dependent on the DR-1 sequence, but were not characterized further in this study.

Transfection Assays with a PPRE-Driven Reporter Gene—The above results raised the question of the relative importance of PPARα and HNF-4 in AOX gene expression. Accordingly, we performed transcription assays with a luciferase reporter construct, pBLPPRE2luc, containing two tandem copies of AOX PPRE upstream of the tk promoter (Fig. 2A). When HepG2 cells were transfected with the reporter, together with increasing amounts of the PPARα expression vector, the reporter expression increased dose-dependently, in both the presence and absence of ciprofibrate. Two- to threefold enhancement of the expression was observed upon the addition of ciprofibrate. HNF-4 also activated PPRE-dependent transcription, but the extent of the activation was much lower than that by PPARα, and not changed by ciprofibrate, significantly.

The differential transcriptional activating effects of PPARα and HNF-4 on AOX PPRE led us to perform an experiment on the compound effect of the two receptors expressed together. When increasing amounts of the HNF-4 expression vector were co-transfected with a fixed amount of the PPARα expression vector, the luciferase expression decreased (Fig. 2B). The effect of HNF-4 was dose-dependent, but nearly saturated with higher concentrations of the vector. This was particularly true in the absence of ciprofibrate.





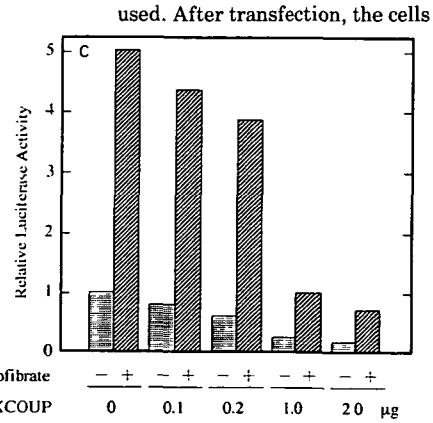


Fig. 2. **Functions of PPARα, HNF-4, and COUP-TFI in transcriptional activation of the AOX PPRE-carrying reporter gene.** A, effects of the individually expressed receptors; B, effects of varying amounts of the HNF-4 expression vector (pCMXHNF4), when co-transfected with 0.1 μg of the PPARα expression vector (pCMXPPARα); C, effects of varying amounts of the COUP-TFI expression vector (pCMXCOUP), co-transfected with 0.1 μg of pCMXPPARα. The reporter plasmid, pBLPPRE2luc, was transfected into HepG2 cells, together with the expression vectors indicated. The numbers on abscissa show the amounts of the expression vectors used. After transfection, the cells were cultured in the presence (shadowed bars) or absence (stippled bars) of 0.1 mM ciprofibrate. Relative luciferase activities are given, taking as one the activity in the absence of ciprofibrate and any expression vector (A), pCMXHNF4 (B), and pCMXCOUP (C), respectively. In B, asterisks indicate that the differences from the value obtained without HNF-4 (comparison was performed for each set of measurements with or without ciprofibrate) are significant based on Student's t test: $^{*}p<0.05$; $^{**}p<0.01$. Other experimental conditions were as described under "MATERIALS AND METHODS," and in Ref. *5*.

We also examined the effect of COUP-TFI, another DR-1-recognizing nuclear receptor, on the expression of AOX PPRE reporter. Increasing amounts of the COUP-TFI expression vector were co-transfected with a fixed amount of the PPARα expression vector. The luciferase expression decreased in a dose-dependent manner, in both the presence and absence of ciprofibrate (Fig. 2C). The repressing activity was more dramatic than that of HNF-4 with higher vector concentrations. Thus, COUP-TFI acts negatively on AOX gene expression. In the absence of the PPARα expression vector, COUP-TFI did not support the luciferase expression by itself, even with the highest vector concentration tested (2 μg; data not shown).

Transfection Assays with a Reporter Driven by the C3P Element—HNF-4 activates the transcription of many liver-specific genes. Having the above data on the functional interplay between PPAR and HNF-4 on AOX PPRE, we next examined the effects of these receptors on the gene expression driven by the C3P element of the apoCIII gene, which was reported to be a representative target of HNF-4 (*18*). In HepG2 cells, luciferase expression is enhanced on the co-transfection of increasing amounts of the HNF-4 expression vector (Fig. 3A). Substantial luciferase activity was observed in the absence of the HNF-4 expression vector, possibly due to endogenous HNF-4. It was reported that HepG2 cells contain a considerable level of HNF-4 (*23*), though we did not observe significant binding activity as to the PPRE or C3P probe in a HepG2 nuclear extract on EMSA (data not shown). PPARα did not activate reporter gene expression in the range of amounts tested (up to 2 μg; Fig. 3A and data not shown). When increasing amounts of the PPARα expression vector were transfected together with a fixed amount of the HNF-4 expression vector, no significant change in the luciferase expression was observed (Fig. 3B).

On the other hand, we observed that COUP-TFI antagonized the gene-activating function of HNF-4 on the C3P element, when they were co-expressed (data not shown), confirming the published results (*18*).

Binding of Nuclear Receptors to the PPRE and C3P Sites—To examine the relationship between the transcriptional activating function and the binding ability of each receptor on the PPRE and C3P sites, EMSA was carried out using *in vitro* synthesized receptors. Each receptor was first synthesized in the presence of [^{35}S]-methionine, to estimate the relative amounts of receptors synthesized under the experimental conditions. For EMSA, the proteins were then synthesized without radiolabeling. Approximately comparable amounts of PPARα, RXRα, and HNF-4 were used for the assays, as estimated by the incorporation of radioactivity into each protein in the first synthesis. However, COUP-TFI was synthesized rather inefficiently, which forced us to use a significantly smaller amount of COUP-TFI than those of the others.

PPARα bound to the AOX PPRE probe only when RXRα co-existed (Fig. 4A, lane 5), as described previously (*24*). This binding was effectively competed with by PPRE, much less efficiently by C3P, and not at all by mPPRE oligonucleotides (lanes 6-8). HNF-4 also bound to this probe efficiently (Fig. 1, lane 13), and the binding was competed with by both the PPRE and C3P oligonucleotides, to similar extents (data not shown). The bands of PPAR/RXR and HNF-4 were supershifted with the respective antisera (Fig.

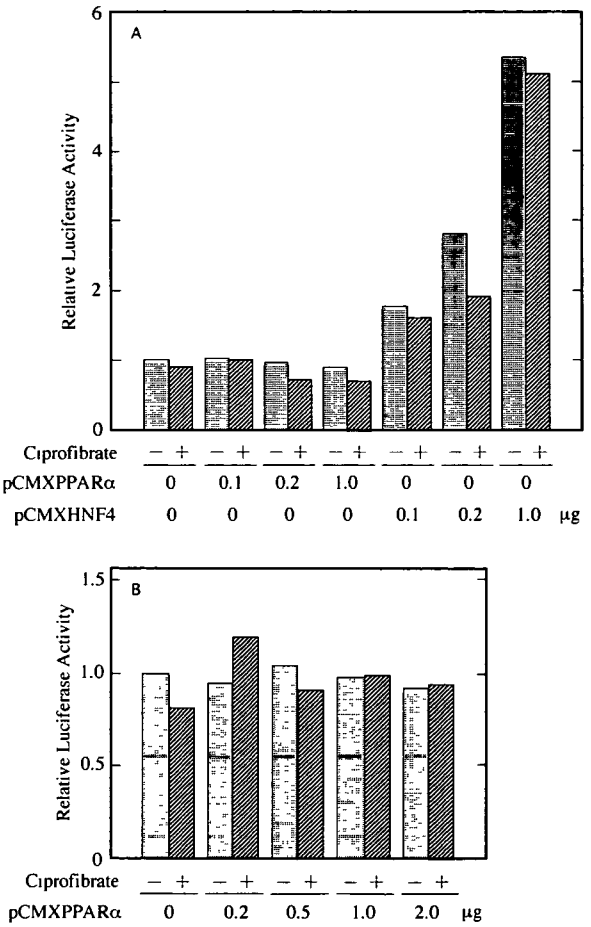


Fig. 3. **Transcriptional activation by HNF-4, but not PPARα, of the C3P element-carrying reporter gene.** A, effects of separate expression of PPARα and HNF-4; B, effects of varying amounts of pCMXPPARα in the presence of 0.2 μg of pCMXHNF4. Data are given taking as one the luciferase activity in the absence of ciprofibrate and any expression vector (A), and pCMXPPARα (B), respectively.

1, lanes 12 and 15). Anti-PPARα antibodies trapped all of the complex at the top of the gel, probably due to the presence of multiple epitopes on the PPARα protein. We also verified the specificities of the antisera; that is, preimmune serum did not cause a detectable supershift (lanes 11 and 14), and anti-PPARα did not supershift the band of HNF-4, and *vice versa* (data not shown). PPARα and RXRα bound only very weakly to the C3P probe as a heterodimer (Fig. 4B, lanes 5-7), consistent with the weak competition by C3P oligonucleotide for the binding to PPRE. As expected, HNF-4 gave a clear retarded band that was supershifted with the anti-HNF-4 antiserum (lanes 8-10). COUP-TFI bound to both probes (Fig. 4C). The apparently weak binding was due to the smaller amount of protein synthesized in the reticulocyte lysate.

DISCUSSION

We observed differential functions of PPARα, HNF-4, and COUP-TFI, on the AOX PPRE and apoCIII C3P elements. PPARα activated AOX PPRE-driven gene expression, but did not activate the apoCIII promoter, because of the poor

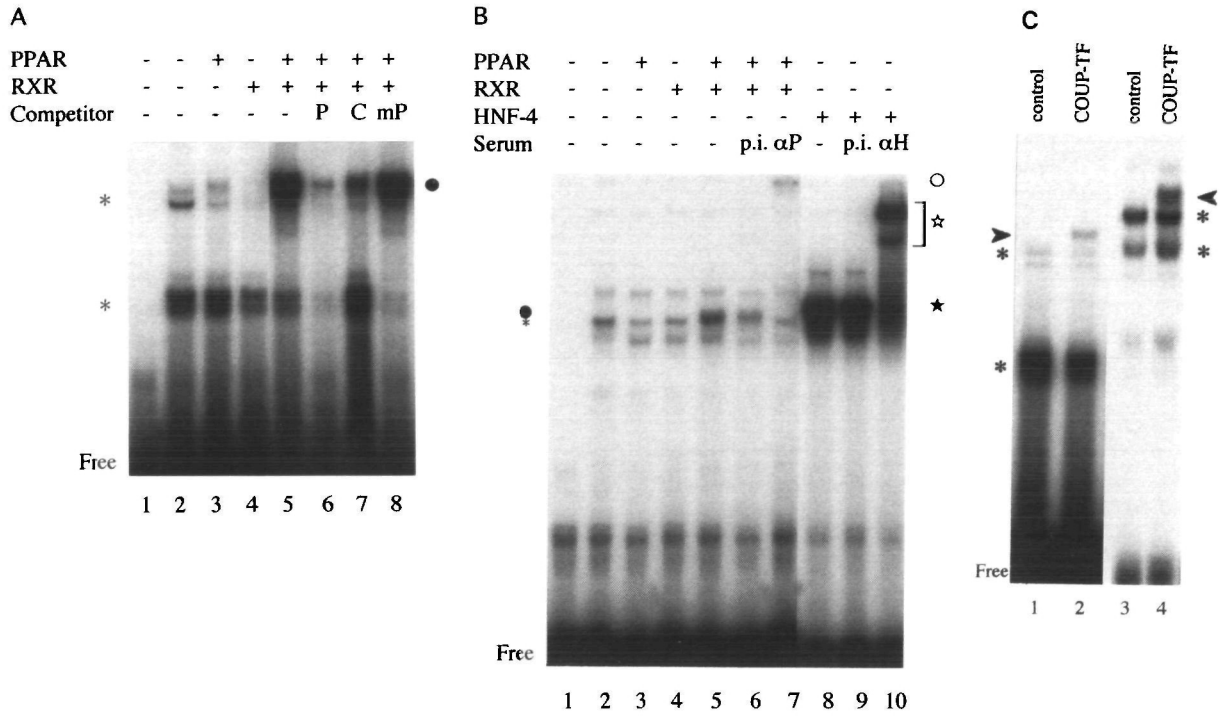


Fig. 4. **EMSA of the binding of PPARα, HNF-4, and COUP-TFI to the AOX PPRE and C3P probes.** A, binding of the PPAR/RXR heterodimer to AOX-PPRE; B, binding of PPARα and HNF-4 to C3P; C, binding of COUP-TFI to AOX PPRE (lanes 1 and 2) and C3P (lanes 3 and 4). The probes were incubated with the receptors translated in the rabbit reticulocyte lysate or unprogrammed reticulocyte lysate (lane 2 in A and B; lanes 1 and 3 in C). In A, competitors AOX PPRE (P), C3P (C), and mutant PPRE (mP) were added at a 20-fold excess, as indicated. Anti-PPARα (αP) and anti-HNF-4 (αH) antisera or preimmune serum (p.i.) were also added to some binding reactions in B, as indicated. The filled circles, star, and arrowheads show the bands due to the binding of PPAR/RXR, HNF-4, and COUP-TFI, respectively. The open circle and star indicate the supershifted bands of PPAR/RXR and HNF-4, respectively, whereas the asterisks show non-specific binding. The sequences of the probes and competitors were as in Fig. 1.

binding activity as to the C3P element. In addition, PPARα did not significantly affect the function of HNF-4, when both receptors were expressed together. Thus, PPARα did not have a squelching effect on HNF-4, either. Hertz *et al.* (*23*) reported that the PPAR/RXR heterodimer bound to the C3P site, and repressed the transcription of the apoCIII gene. It is not clear why our results and theirs apparently differ, although possible reasons might be the differences in the conditions for EMSA, the lengths of the PPRE element used as the probe and enhancer, *etc.* In any case, we would like to point out that the sequence just downstream of the DR-1 motif in the C3P element does not conform to the extended half-site consensus for PPARα/RXRα binding that we reported recently (*5*). That is, when the C3P DR-1 sequence is written in the direction corresponding to that of AOX PPRE, the sequence just downstream of the second half-site is GCGC. In contrast, the consensus sequence is AG(A/T)T, in which the third nucleotide must exclusively be A or T for efficient binding. Hence, the C3P sequence should be less favorable for PPAR/RXR binding.

HNF-4 also binds to the AOX element, but apparently suppresses the gene activation by PPARα, when they are co-expressed. These results can be interpreted in terms of competition between the two receptors in the binding to a limited amount of template. HNF-4 has an activating effect when bound to the PPRE, but it is much lower than that of PPARα, and not enhanced by ciprofibrate. Hence, the compound transcriptional activating effect of the two receptors is lower than that of PPARα itself. At higher HNF-4 concentrations, however, the relative contribution of HNF-4 to the luciferase expression gradually increases, particularly in the absence of ciprofibrate, leading to the saturation of suppression (Fig. 2B). It is an intriguing issue as to whether or not HNF-4 always has a lower gene-activating function than the PPARα/RXRα heterodimer, on any binding element that is commonly recognized by these nuclear receptors. The activating function might be affected by the respective sequence contexts of the binding site, and thus HNF-4 might be more active in gene activation than PPARα/RXRα in certain favorable contexts.

COUP-TFI suppressed the reporter expression dependent on both the AOX and C3P elements. This is most probably because COUP-TFI competes with PPAR/RXR and HNF-4, respectively, in the binding to DNA elements, and lacks a gene-activating function. The suppressive effects of COUP-TFI on PPAR (*25, 26*), HNF-4 (*18, 27-29*), and other nuclear receptors (for a review, see Ref. *7*) have been observed for many genes. It is possible that this effect is simply due to the binding competition, and COUP-TFI itself is functionally inert. Also possible would be that COUP-TFI causes active repression upon binding to a DNA element. The latter alternative has been supported by the recent observation that COUP-TFI recruits transcriptional co-repressors (*30*).

In liver, the content of HNF-4 seems far higher than that of PPAR, as indicated by the relative binding activities as to AOX PPRE (Fig. 1). Thus, *in vivo*, the expression of the AOX gene seems to be rather suppressed due to the

presence of excess HNF-4. It is also likely that HNF-4 contributes to a considerable extent to the AOX gene expression, especially in the absence of a peroxisome proliferator. It became clear with recent investigations that the level of eukaryotic gene expression is modified by many transcription factors binding to a complex array of sequence elements. Individual nuclear receptors have different characteristics as to gene activation or suppression, as described above. Thus, functional interplay between the transcription factors recognizing common binding elements must be an important mechanism of gene regulation.

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