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Human and Rat Peroxisome Proliferator Activated Receptors (PPARs) Demonstrate Similar Tissue Distribution but Different Responsiveness to PPAR Activators

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We have isolated a human peroxisomal proliferator activated receptor (hPPAR) from a human liver cDNA library. Based on sequence analysis, we have determined that this cDNA encodes the human PPARα. When assayed in a reconstituted hPPAR responsive transcription system in mammalian CV-1 cells, this receptor was shown to be transcriptionally activated by hypolipidemic agents like clofibric acid, and ETYA (5,8,11,14-eicosatetraynoic acid; a synthetic arachidonic acid homolog). When analyzed in CV-1 cells, the rat PPARα was similarly transcriptionally regulated. However, when assayed in a human liver cell line (HepG2) we noticed that ETYA was a more efficient activator of hPPARα than rPPARα. Thus, factors other than the receptor are important in determining the cellular responsiveness to this class of compounds. Interestingly, WY-14,643, another peroxisome proliferator, was a much more potent activator of rPPARα than human PPARα when assayed in both cell lines. This may explain in part why certain fibrates are potent hepatocarcinogens in rodents. Northern analysis indicates that hPPARα and rPPARα are well expressed in heart, kidney and liver. We further demonstrate that hPPARα and human retinoid X receptorα synergistically interact to bind and transactivate through a peroxisomal proliferator response element. Thus in a similar cell and promoter context the rat and human PPARs show a differential response to certain activators. Cumulatively these data suggest that differential ligand responsiveness does not provide a complete explanation for the different biological effects exhibited by hypolipidemic drugs when administered to humans and rats.

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INTRODUCTION

Fibric acid drugs function to lower serum cholesterol and triglycerides [1, 2]. These drugs along with herbicides, industrial plasticizers, leukotriene antagonists and other xenobiotics form a group of compounds called peroxisome proliferators. There is no apparent structural similarity in these compounds other than the presence of a carboxylic acid functional group. This group may be initially present or may be derived from alcohols or aldehydes by metabolic oxidation [3, 4].

Peroxisomes are subcellular organelles found in animals and plants, and contain enzymes for respiration, cholesterol and lipid metabolism. A variety of chemical agents including hypolipidemic drugs like clofibrates cause proliferation of peroxisomes in rats, mice and hamsters [5]. In addition, many of these agents transcriptionally upregulate expression of genes coding for enzymes involved in the β-oxidation of long chain fatty acids (acyl coenzyme A oxidase, bifunctional enzyme and thiolase) as well as genes in the cytochrome *P*450 IV family [6–9]. Most hypolipidemic drugs and chemicals that induce peroxisome proliferation cause hepatomegaly and hepatocarcinomas in rats and mice [3, 5] but not in humans [10, 11]. However, the fact that hypolipidemic drugs cause hepatocarcinomas in rats creates regulatory concerns with respect to the use of such drugs in the treatment of humans. Peroxisome proliferators are non-genotoxic carcinogens; they do not damage DNA directly [12]. However, a recent report [13] suggesting genotoxic effects of certain peroxisome

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proliferators necessitates further investigation on this point.

Two hypotheses have been put forward to explain the mechanism of peroxisome proliferation. The first is the "lipid overload hypothesis" whereby an increase in the intracellular concentration of fatty acids is the main stimulus for peroxisome proliferation [14, 15]. The second hypothesis postulates a receptor mediated mechanism and an as yet unidentified ligand [3]. In keeping with the second postulate, peroxisome proliferator activated receptors (PPARs) for various species have been cloned [16–20]. These receptors can bind to specific DNA response elements (PPREs) in the regulatory regions of target genes and thereby alter their rate of transcription in response to a variety of classical peroxisome proliferators and hypolipidemic agents [16–20]. It is thought that activation of the PPARs leads to alterations in the expression of a network of genes required for the processes of peroxisome proliferation and lowering of cholesterol and lipids.

We are interested in understanding the differential sensitivities of rodents and humans to fibric acid derived drugs, and specifically whether or not this could be reconciled through PPAR expression and activation. To that end we have cloned a human and rat peroxisome proliferator activated receptor homologous to the mouse PPAR (mPPARα) and have used these cDNAs to reconstitute a receptor-responsive transcription system in mammalian cells in culture.

MATERIALS AND METHODS

Reagents

ETYA (5,8,11,14-eicosatetraynoic acid) and clofibric acid was purchased from Sigma (St Louis, MO), and WY-14,643 ([4-chloro-6-(2,3-xylidino)-2-pyrmidinylthio]acetic acid) from Chemsyn Science Labs (Lenexa, KS). Stock solutions of these compounds were made in ethanol. 9-*cis*-retinoic acid was made and purified at Ligand Pharmaceuticals and stock solutions made in dimethyl sulfoxide.

Isolation of rat PPARα cDNA

Regenerating liver tissue was isolated from 10–12 week old Lewis rats (Harlan Sprague–Dawley Inc.) as described previously [21]. Following partial hepatectomy of the median and left lateral lobes of the liver (ca. two thirds of the liver), animals were housed individually with free access to food and water. Animals were sacrificed after 7 days, a time previously determined to regenerate approximately one half the final liver mass. Livers were removed and snap frozen in liquid nitrogen. RNA from 7-day-old regenerating livers was isolated using a modified guanidine isothiocyanate method [22], and poly A+ RNA species selected by standard procedures [23]. Poly A+ RNA was used to synthesize cDNA and construct a cDNA library into the λgt10 vector as described by Huynh *et al.* [24]. A library of approx. 2×10^6 plaques was plated and screened under low stringency conditions (35% formamide, 5×SSC, 37°C) using a nick translated DNA binding domain fragment isolated from a human estrogen receptor cDNA clone [25]. Hybridization and wash conditions were as described previously [23]. The 2.5-kb rPPAR cDNA insert was subcloned into the *Eco*R1 site of the Bluescript KS+ vector and sequenced by the dideoxy chain termination methodology of Sanger [26]. The coding region was identical to that of the published rat PPAR sequence [17].

Isolation of human PPARα cDNA

A human homolog of rat PPARα was isolated from a human liver 5'-stretch λgt10 cDNA library (Clontech). The library was screened at medium stringency (40% formamide, 5×SSC at 37°C), with a rPPAR nick translated DNA fragment specific to the A/B and DNA binding domain (DBD; from the *Eco*R1 to the the *Bgl*II site, nucleotides 450–909) [17]. Positive clones were isolated and subcloned into the Bluescript KS vector (Stratagene) for sequencing.

Northern blot analysis

A human multiple tissue Northern blot was purchased from Clontech. Hybridization was done following the manufacturer's protocol. The blot was prehybridized in 5×SSPE, 10×Denhardt's solution, 100 μg/ml of freshly denatured salmon sperm DNA, 50% formamide and 2% sodium deodecyl sulfate (SDS) for 3 h at 42°C. DNA from the *Eco*R1 site at position 1025 of the coding region of hPPARα to the end of the cloned gene was used as probe. This DNA was labeled by random priming and added at a concentration of 10^6 cpm/ml of prehybridization solution. Hybridization was carried out for 13 h at 42°C. The blot was then washed in 2×SSC, 0.05% SDS at room temperature followed by two washes in 0.1×SSC, 0.1% SDS at 50°C. and exposed to X-ray film. To control for differences in the amount of RNA during loading and transfer, the blot was also hybridized with a random-primed 2-kb human β-actin probe supplied by Clontech.

RNAse protection assay

A 147-bp *Pst*1/*Bgl*II DNA fragment located at the 3' end of the rPPARα DBD was subcloned into the *Pst*1/*Bam*HI sites of the pGem4 vector (Promega). T7 RNA polymerase was used to synthesize the cRNA probe and was used in liquid hybridization with 10 μg of total RNA from the tissues indicated. Buffers and reaction conditions for the assay have been described previously [27].

Receptor expression and reporter constructs

For expression in mammalian cells, the rPPARα cDNA was subcloned into the *Kpn*1/*Hpa*1 sites of the pBKCMV vector (Stratagene) to give pCMVrPPARα. The hPPARα cDNA was cloned into the *Not*I site of pBKCMV yielding pCMVhPPARα.

The reporter plasmid pPPREA3-tk-luc was generated by inserting three copies of the synthetic oligonucleotide (5'-CCCGAACGTGACCTTTGTCCTGGTCC-3') containing the "A" site of the acyl-coenzyme A oxidase (AOX) gene regulatory sequence [29] into the *Xho*I site 5' of the thymidine kinase (tk) promoter in the previously described pBLtk-luciferase vector [30].

Co-transfection assay

CV-1 (an African green monkey kidney cell line), a kind gift from Dr Ron Evans, and HepG2 (a human hepatocarcinoma cell line) was obtained from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%(v/v) fetal bovine serum (Hyclone), 2 mM L-glutamine, and 55 μg/ml gentamicin (BioWhittaker). H4IIEC3 (a rat hepatoma cell line from ATCC) was grown in DMEM containing 10% horse serum, 5% fetal bovine serum plus 0.1 mM non-essential amino acids and the above supplements. Cells were plated at a density of 6×10^4 cells per well for CV-1, 2×10^5 cells for HepG2 and 1.5×10^5 for H4IIEC3 in 12 well cell culture dishes (Costar). Fourteen hours later DNA was added by the calcium phosphate co-precipitation technique [28]. Typically, 0.1 μg of PPAR expression plasmid, 0.5 μg of the β-galactosidase expression plasmid pCH110, 0.5 μg of reporter plasmid and 0.9 μg of pGEM carrier DNA were added to each well. Titration experiments indicated that these concentrations of expression plasmids for the two PPARs gave the maximal activation in each case. After 6 h the cells were washed with 1×phosphate buffered saline (PBS) and fresh media added. At this stage DMEM with 10% charcoal stripped fetal bovine serum (Hyclone) plus the above supplements was used for CV-1 and HepG2 cells. Peroxisome proliferators were added to the final concentrations indicated. These compounds were toxic at concentrations higher than the highest used in these assays. Control cells received ethanol and/or dimethyl sulfoxide (vehicle). After 36 h the cells were harvested and the luciferase and β-galactosidase activities quantified on a Dynatech ML 1000 luminometer and a Beckman Biomek 1000 workstation, respectively. The normalized response is the luciferase activity of the extract divided by the β-galactosidase activity of the same. Each data point represents the mean of triplicate transfections. Error bars represent the standard deviation from the mean.

Gel retardation assays

COS cells were transfected with 5 μg of pCMVhPPARα or pRShuman retinoid X receptorα (pRShRXRα) [35] per 100 mm dish for 48 h. Whole cell extracts were made by four cycles of freeze–thawing in 0.4 M KCl containing buffer followed by centrifugation. Gel retardations were performed by incubating 5 μg cell extract in buffer containing 10 mM Hepes (7.8), 50 mM KCl, 1 mM dithiothreitol, 2.5 mM $MgCl_2$, 2μg/ml dIdC and 20% glycerol at 4°C for 5 min. About 100,000 cpm of ^{32}P-end-labeled probe was then added and incubated at 25°C for another 5 min. Protein–DNA complexes were resolved by electrophoresis on 5% polyacrylamide gels in 0.5×TBE. The PPRE sequence from the AOX gene used as probe is 5'-CTAGCGATATCATGACCTTTGTCCTAGGCCTC-3' (upper strand) and 5'-CTAGGAGGCCTAGGACAAAGGTCATGATATCG-3' (lower strand).

RESULTS

Identification of hPPARα and rPPARα

We isolated the rPPARα cDNA from a regenerating rat liver cDNA library using a human estrogen receptor DBD probe. The sequence of this cDNA is identical to the published rPPAR [17]. Since this sequence has 98% identity to the mPPARα [31] it most likely represents the rat homolog of mPPARα. We then used a rat cDNA probe corresponding to the highly conserved DBD of rPPARα to identify homologs from a human liver cDNA library. Using this approach we identified a cDNA which displayed the sequence characteristics of an authentic human PPAR. Figure 1 shows the nucleotide and predicted amino acid sequence of the cloned hPPARα cDNA. Analysis of this cDNA sequence indicated that there is an open reading frame of 468 amino acids initiating at an ATG located at nucleotide 124. This methionine is contained within a Kozac translation initiation consensus sequence. Upstream of this open reading frame, there is an inframe translation stop codon at nucleotide 76.

PPARs are members of the steroid receptor superfamily. Each receptor can be divided into six domains based on sequence homology (Fig. 2). The ligand binding domain (LBD) and DBD of hPPARα are indicated in Fig. 2. The predicted amino acid sequence reveals a characteristic cysteine rich region that contains two zinc fingers required for DNA binding. All the PPARs cloned thus far demonstrate a unique structure in this region, containing three amino acids between the two cysteines in the D-box. This is a distinguishing feature of all known PPARs since all the other receptors have five amino acids in their D-boxes [32]. The three amino acids between the two cysteines in the D-box of hPPARα are underlined (Fig. 1).

Figure 2 shows the amino acid sequence comparision between hPPARα and the known PPARs from other

species. As expected, the DBD is the most conserved. The DBD of hPPARα has 100 and 99% identity with mPPARα and rPPARα, respectively. Similarly, the LBD of the hPPARα has 94% identity with the corresponding domain of mPPARα and rPPARα. Comparing hPPARα with the *Xenopus* PPARα, there is 88 and 89% identity in the DBD and LBD, respectively. The identity is less with the *Xenopus* β and γ subtypes as indicated (Fig. 2). Sequence comparision with the thyroid hormone receptor (hTRβ1) another member of the steroid receptor family reveals 59 and 29% homology in the DBD and LBD, respectively. With hNUC1 [34], the homology in the DBD and LBD is 86 and 70%, respectively.

The overall homology of the hPPARα to the cloned mouse and rat PPAR is very high. There are only 36 amino acids that are different between the hPPARα and rPPARα. These differences are distributed over the entire receptor molecule. This translates to 93% identity between the amino acid sequences of these two proteins.

Tissue distribution of rat and human PPARα mRNA

Peroxisome proliferators are potent hepatocarcinogens in rats, but limited primate and human studies suggest there is no such relationship in higher species [10, 11]. It is unclear whether this results from pharmacokinetic differences in the way these two species treat these compounds or if there is a specific molecular explanation. One hypothesis suggests that this species specific difference may relate to differences in the expression of rat and human PPAR proteins. Unfortunately, as yet there is no adequate method to examine PPAR protein expression in the two species. As an alternative we have examined the expression of PPARα mRNA from the corresponding genes in various tissues of the two species.

To detect the expression of hPPARα, a Northern blot of mRNA from various human tissues was probed with a hPPARα specific LBD fragment [Fig. 3(A)]. The hPPARα gene gives rise to a 10 kb transcript that is highly expressed in skeletal muscle, heart, liver and kidney [Fig. 3(A)] and expressed at low levels in the brain and lung. Figure 3(B) shows the same blot hybridized to a human actin probe.

An RNAse protection assay using rat RNA and a probe specific to the LBD of rPPARα was used to quantitate rPPARα mRNA expression in rat tissues. This study indicated that the rPPARα gene was also expressed in heart, kidney, liver and muscle. It was expressed at low levels in bone, brain and lung, and was undetectable in spleen and testis (Fig. 4).

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   1 GTTCTGGAGGCTGGGAAGTTCAAGATCAAAGTGCCAGCAGATTCAGTGTCATGTGAGGACGTGCTTCCTGCTTCATAGATAAGAGCTTGGAGCTCGGCGCACAACCAGCACCATCTGGTC

 121 GCGATGGTGGACACGGAAAGCCCACTCTGCCCCCTCTCCCCACTCGAGGCCGGCGATCTAGAGAGCCCGTTATCTGAAGAGTTCCTGCAAGAAATGGGAAACATCCAAGAGATTTCGCAA
   1     M  V  D  T  E  S  P  L  C  P  L  S  P  L  E  A  G  D  L  E  S  P  L  S  E  E  F  L  Q  E  M  G  N  I  Q  E  I  S  Q

 241 TCCATCGGCGAGGATAGTTCTGGAAGCTTTGGCTTTACGGAATACCAGTATTTAGGAAGCTGTCCTGGCTCAGATGGCTCGGTCATCACGGACACGCTTTCACCAGCTTCGAGCCCCTCC
  39  S  I  G  E  D  S  S  G  S  F  G  F  T  E  Y  Q  Y  L  G  S  C  P  G  S  D  G  S  V  I  T  D  T  L  S  P  A  S  S  P  S

 361 TCGGTGACTTATCCTGTGGTCCCCGGCAGCGTGGACGAGTCTCCCAGTGGAGCATTGAACATCGAATGTAGAATCTGCGGGGACAAGGCCTCAGGCTATCATTACGGAGTCCACGCGTGT
  79  S  V  T  Y  P  V  V  P  G  S  V  D  E  S  P  S  G  A  L  N  I  E  C  R  I  C  G  D  K  A  S  G  Y  H  Y  G  V  H  A  C

 481 GAAGGCTGCAAGGGCTTCTTTCGGCGAACGATTCGACTCAAGCTGGTGTATGACAAGTGCGACCGCAGCTGCAAGATCCAGAAAAAGAACAGAAACAAATGCCAGTATTGTCGATTTCAC
 119  E  G  C  K  G  F  F  R  R  T  I  R  L  K  L  V  Y  D  K  C  D  R  S  C  K  I  Q  K  K  N  R  N  K  C  Q  Y  C  R  F  H

 601 AAGTGCCTTTCTGTCGGGATGTCACACAACGCGATTCGTTTTGGACGAATGCCAAGATCTGAGAAAGCAAAACTGAAAGCAGAAATTCTTACCTGTGAACATGACATAGAAGATTCTGAA
 159  K  C  L  S  V  G  M  S  H  N  A  I  R  F  G  R  M  P  R  S  E  K  A  K  L  K  A  E  I  L  T  C  E  H  D  I  E  D  S  E

 721 ACTGCAGATCTCAAATCTCTGGCCAAGAGAATCTACGAGGCCTACTTGAAGAACTTCAACATGAACAAGGTCAAAGCCCGGGTCATCCTCTCAGGAAAGGCCAGTAACAATCCACCTTTT
 199  T  A  D  L  K  S  L  A  K  R  I  Y  E  A  Y  L  K  N  F  N  M  N  K  V  K  A  R  V  I  L  S  G  K  A  S  N  N  P  P  F

 841 GTCATACATGATATGGAGACACTGTGTATGGCTGAGAAGACGCTGGTGGCCAAGCTGGTGGCCAATGGCATCCAGAACAAGGAGGCGGAGGTCCGCATCTTTCACTGCTGCCAGTGCACG
 239  V  I  H  D  M  E  T  L  C  M  A  E  K  T  L  V  A  K  L  V  A  N  G  I  Q  N  K  E  A  E  V  R  I  F  H  C  C  Q  C  T

 961 TCAGTGGAGACCGTCACGGAGCTCACGGAATTCGCCAAGGCCATCCCAGGCTTCGCAAACTTGGACCTGAACGATCAAGTGACATTGCTAAAATACGGAGTTTATGAGGCCATATTCGCC
 279  S  V  E  T  V  T  E  L  T  E  F  A  K  A  I  P  G  F  A  N  L  D  L  N  D  Q  V  T  L  L  K  Y  G  V  Y  E  A  I  F  A

1081 ATGCTGTCTTCTGTGATGAACAAAGACGGGATGCTGGTAGCGTATGGAAATGGGTTTATAACTCGTGAATTCCTAAAAAGCCTAAGGAAACCGTTCTGTGATATCATGGAACCCAAGTTT
 319  M  L  S  S  V  M  N  K  D  G  M  L  V  A  Y  G  N  G  F  I  T  R  E  F  L  K  S  L  R  K  P  F  C  D  I  M  E  P  K  F

1201 GATTTTGCCATGAAGTTCAATGCACTGGAACTGGATGACAGTGATATCTCCCTTTTTGTGGCTGCTATCATTTGCTGTGGAGATCGTCCTGGCCTTCTAAACGTAGGACACATTGAAAAA
 359  D  F  A  M  K  F  N  A  L  E  L  D  D  S  D  I  S  L  F  V  A  A  I  I  C  C  G  D  R  P  G  L  L  N  V  G  H  I  E  K

1321 ATGCAGGAGGGTATTGTACATGTGCTCAGACTCCACCTGCAGAGCAACCACCCGGACGATATCTTTCTCTTCCCAAAACTTCTTCAAAAAATGGCAGACCTCCGGCAGCTGGTGACGGAG
 399  M  Q  E  G  I  V  H  V  L  R  L  H  L  Q  S  N  H  P  D  D  I  F  L  F  P  K  L  L  Q  K  M  A  D  L  R  Q  L  V  T  E

1441 CATGCGCAGCTGGTGCAGATCATCAAGAAGACGGAGTCGGATGCTGCGCTGCACCCGCTACTGCAGGAGATCTACAGGGACATGTACTGAGTTCCTTCAGATCAGCCACACCTTTTCCAG
 439  H  A  Q  L  V  Q  I  I  K  K  T  E  S  D  A  A  L  H  P  L  L  Q  E  I  Y  R  D  M  Y  *

1561 GAGTTCTGAAGCTGACAGCACTACAAAGGAGACGGGGGAGCAGCACGATTTTGCACAAATATCCACCACTTTAACCTTAGAGCTTGGACAGTCTGAGCTGTAGGTAACCGGCATATTATT

1681 CCATATCTTTGTTTTAACCAGTACTTCTAAGAGCATAGAACTCAAATGCTG
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Fig. 1. Nucleotide sequence and predicted amino acid sequence of the hPPARα clone. The three amino acids between the cysteines in the second zinc finger are underlined and the inframe stop codon upstream of the predicted first methionine is shown by a line on top. The translation termination codon is indicated by a star. The numbers shown on the side are positions in the nucleotide and amino acid sequence.

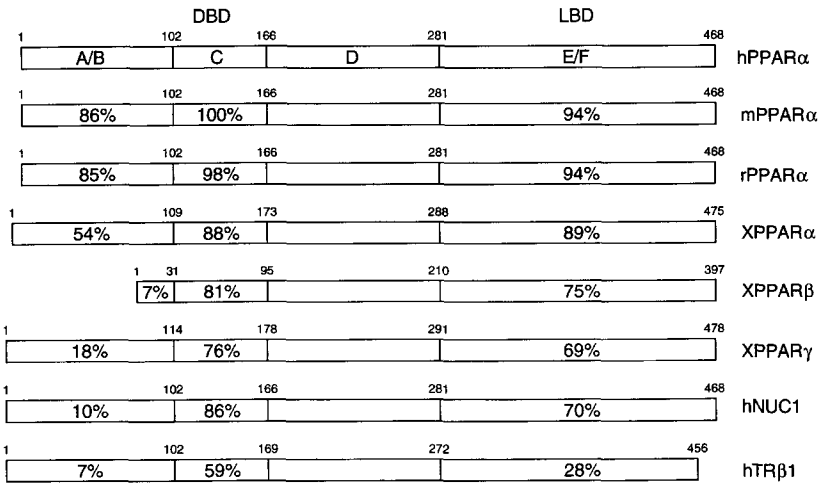

Fig. 2. Comparision of the amino acid sequence of PPARs from various species. The structure of the various PPARs are shown schematically divided into domains A–F [40]. Alignments were done with the Geneworks protein alignment program, Intelligenetics, Inc. mPPARα, mouse peroxisome proliferator activated receptor [16, 31]; rPPARα, rat peroxisome proliferator activated receptor [17], and xPPARα,β,γ, *Xenopus* peroxisome proliferator activated receptors α, β and γ, respectively [19]; hNUC1 [34]; and hTRβ1, human thyroid hormone receptor-β [41]. The numbers within the boxes represent the percentage amino acid identity of that domain between hPPARα and the other PPARs. The numbers on top of the boxes denote how the domains are defined in each receptor.

Thus hPPARα and rPPARα demonstrate qualitatively similar patterns of mRNA expression in various tissues.

Rat and human PPARs are differentially activated by fibrates and fatty acids

To permit examination of the *in vitro* transcriptional activity of the fibrates and fatty acids as PPAR modulators, we reconstituted a PPAR responsive *cis–trans* assay in CV-1, HepG2 and H4IIEC3 cells. We tested the effects of a series of peroxisome proliferators on PPAR transcriptional activity (Fig. 5). Both hPPARα and rPPARα have similar activation profiles with clofibric acid (CFA) [Fig. 5(A)]. The activation of the reporter gene observed in the absence of any transfected receptor by CFA is probably due to activation of endogenous PPARs present in CV-1 cells. We have tested these rat and human PPARs over a range of input DNA concentrations and have obtained similar results.

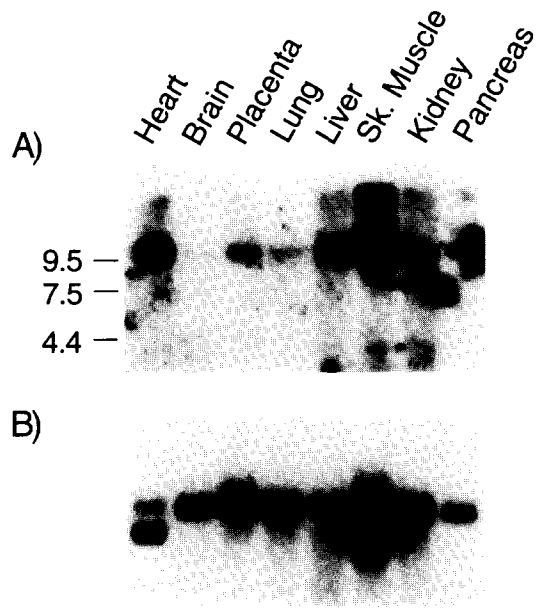

Fig. 3. Distribution of hPPARα in adult human tissues. Northern blots were prepared with 2 mg of polyA$^+$ RNA from the various human tissues indicated. (A) The Northern blot was probed with a hPPARα LBD specific probe. (B) The same blot was probed with the human β-actin cDNA probe.

We extended our analysis to examine the transcriptional effects of another peroxisome proliferator WY-14,643 on PPAR function [Fig. 5(B)]. Using the *cis–trans* assay in CV-1 cells we observed that WY-14,643 was a more potent activator of rPPARα compared to hPPARα.

Recently, it has been shown that a synthetic arachidonic acid analog ETYA (5,8,11,14-eicosatetraynoic acid) was 100-fold more potent than WY-14,643 as a transcriptional modulator of xPPARα [19]. We decided, therefore, to test the responsiveness of rat and human PPARs to this compound. The results of this analysis are shown in Fig. 5(C). Both hPPARα and rPPARα show similar profiles of activation by ETYA in CV-1 cells. However, a noticeable difference in response to ETYA was observed in HepG2 cells.

In HepG2 cells, ETYA is a less potent activator of rPPARα compared to hPPARα [Fig. 6(A)]. These results support the hypothesis that the biological impact of the peroxisome proliferators on transcriptional regulation is determined in part by cell context and that differences in the biological activity of ETYA may be attributed to processes independent of receptor. We are currently investigating whether metabolism is a possible explanation for the observed cell-specific activation.

In CV-1 cells we observed that WY-14,643 is a more

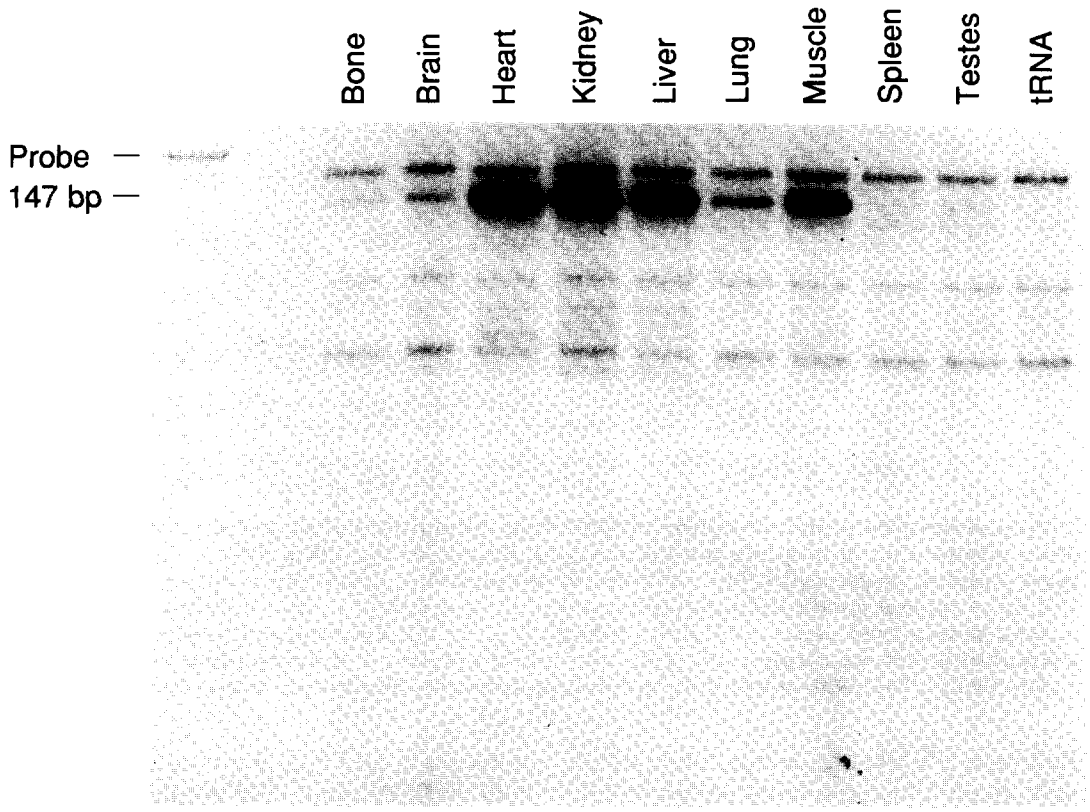

Fig. 4. Tissue distribution of rPPARα RNA. RNAse protection assay was performed with total RNA (10 μg) isolated from a variety of rat tissues as indicated and a 147 bp cRNA probe from the rPPARα LBD. Undigested probe and tRNA controls are as indicated. RNA quantities were quantitated and equilibrated using OD 260 nm and ethidium bromide staining of rRNA species.

potent activator of rPPARα than hPPARα [Fig. 5(B)]. In HepG2 cells we observe the same relationship. However, the sensitivity of rPPARα to WY-14,643 in this cell line is much higher [Fig. 6(B)]. In a rat hepatoma cell line (H4IIEC3), rPPARα is activated by WY-14,643 but hPPARα is hardly activated [Fig. 6(C)]. Thus in both human and rat hepatoma cell lines, rPPARα shows a significantly better response than hPPARα to WY-14,643. This may explain in part why such compounds are hepatocarcinogens in rats but not in humans [3, 5, 10, 11]. However, this is not the only explanation since the activation profile with CFA is similar for both receptors in HepG2 cells (data not shown).

Thus, in a similar cell and promoter context, the rat and human PPARs show a differential responsiveness to certain but not all effectors, suggesting that the receptors are at least partially involved in discriminating among peroxisome proliferators.

Synergistic activation by hPPARα and hRXRα through a PPRE

The PPRE located in the rat AOX gene is similar to the retinoid X response element (RXRE) [37, 38]. This reporter is activated by hPPARα in the presence of CFA. Co-transfection of hRXRα expression plasmid in CV-1 cells also resulted in activation of the PPREA3-luciferase reporter in the presence of 9-*cis*-retinoic acid (Fig. 7). However, we observe a greater than additive activation of the reporter with hPPARα and hRXRα in the presence of CFA and 9-*cis*-retinoic acid. Similar co-operative transcription activation was observed for rPPARα [35], mPPARα [31], xPPARα [39] and RXRs. Thus, hPPARα and hRXRα synergistically activate gene transcription through a PPRE. These results suggest that other than PPAR ligands, retinoids can have an impact on the biological function of PPARs.

Co-operative binding of hPPARα and hRXRα to a PPRE

One mechanism by which hRXRα can stimulate transcription of hPPARα through a PPRE is by stimulating binding of the PPAR to the PPRE. Accordingly, gel retardation assays were performed with whole cell extracts from COS cells transfected with hPPARα or hRXRα. A weak band is seen with extracts from cells transfected with hPPARα or hRXRα alone (Fig. 8, lanes 2 and 5) or hPPARα and mock transfected COS cell extract (lane 4). However, hPPARα and hRXRα extracts together give a stronger retarded band (lane 3). Binding to DNA occurred in the absence of CFA or 9-*cis*-retinoic acid. This suggests that hRXRα can enhance binding of hPPARα to the PPRE. Similar results were also obtained with the mPPARα [31] and rPPARα [35, 36].

DISCUSSION

We have cloned a subtype of the human PPAR, hPPARα. Comparisons of the deduced amino acid sequence over the whole length of the cDNA confirms that this clone represents a *bonafide* PPAR. Inspection of the predicted amino acid sequence of this receptor in the DBD indicated that the two cysteine residues in the D-box of the second finger are separated by three amino acids (DRS, Fig. 1), as against five amino acids present in the same region of all other nuclear receptors. This sequence motif is a characteristic feature of all known PPARs [32]. The sequence of hPPARα closely resembles that of the mPPARα [16, 31] and rPPAR [17] demonstrating 93% overall amino acid sequence identity. Based on this sequence information, and its observed biological activity in a reconstituted transcription system in mammalian cells, we feel that hPPARα is the functional human homolog of mPPARα and rPPARα. Interestingly, Sher *et al.* [20] have recently reported the cloning of a hPPAR cDNA the predicted amino acid sequence of which differs by only two amino acids from the sequence of our hPPARα. These two amino acid differences are located in the hinge domain and LBD of hPPARα, respectively. We are presently unsure whether these differences are a result of a cloning artifact, splicing variations or random allelic variations that occur naturally in the population. We believe that the sequence of our PPAR reflects the natural receptor as these two amino acids occurring at positions 268 and 296 in the amino acid sequence of hPPARα are conserved in the rat and mouse PPARs. These amino acid differences may play an important role in the biology of hPPARs. Currently, we are investigating the role of these specific amino acids in PPAR function using reverse genetics.

It is possible that other forms of hPPARs exist, as clearly is the case in *Xenopus* where at least three receptor subtypes have been identified (xPPARα,β,γ). Work in our laboratory and that of others [33] suggests that multiple

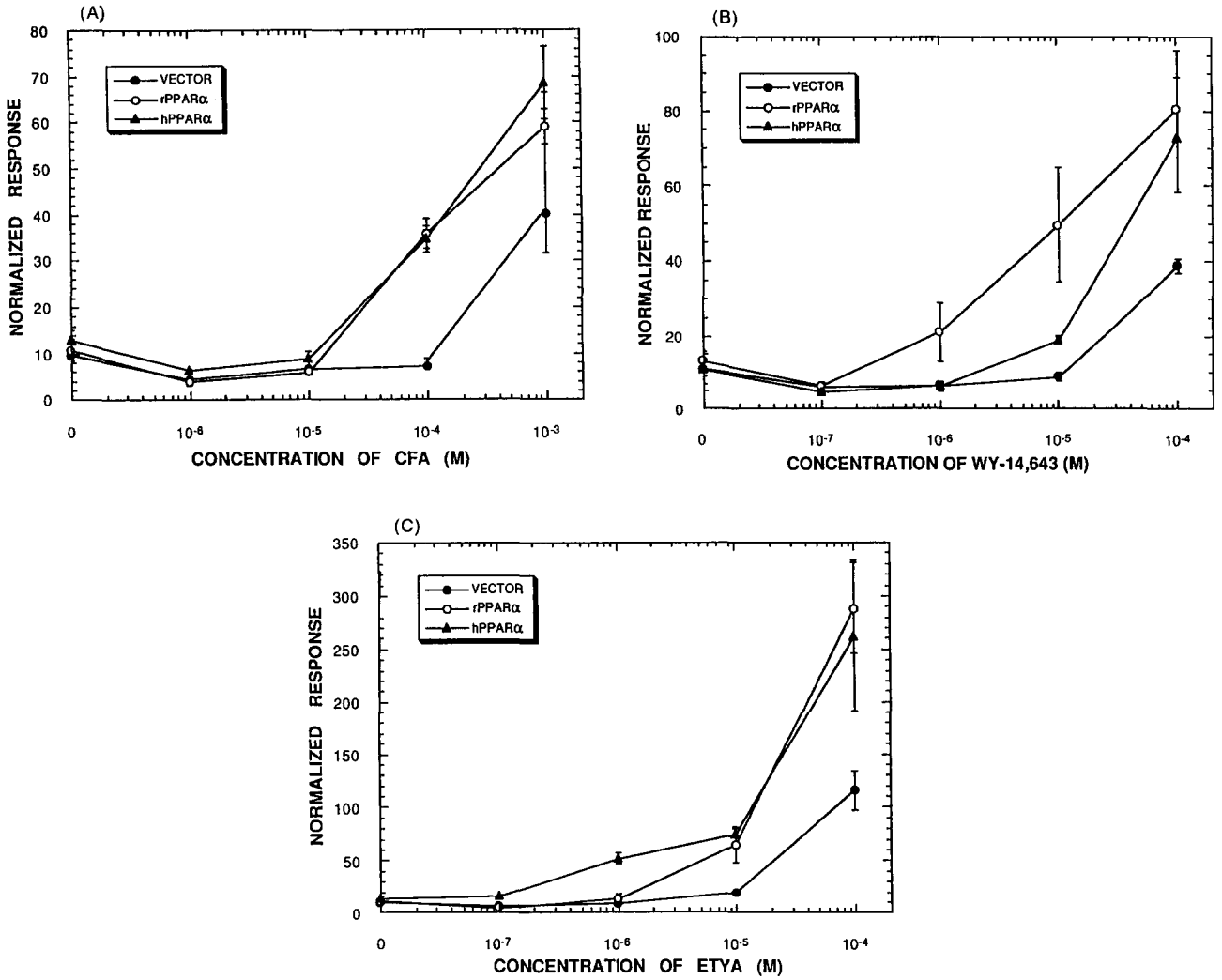

Fig. 5. Activation of hPPARα and rPPARα in CV-1 cells. Cells were transfected with pBKCMV (vector) or with pCMVhPPARα (hPPARα) or pCMVrPPARα (rPPARα) and treated with (A) CFA, (B) WY-14,643 and (C) ETYA at the concentrations indicated. The reporter construct is pPPREA3-tk-luc and the luciferase activity is shown normalized to the β-galactosidase activity of the same extract.

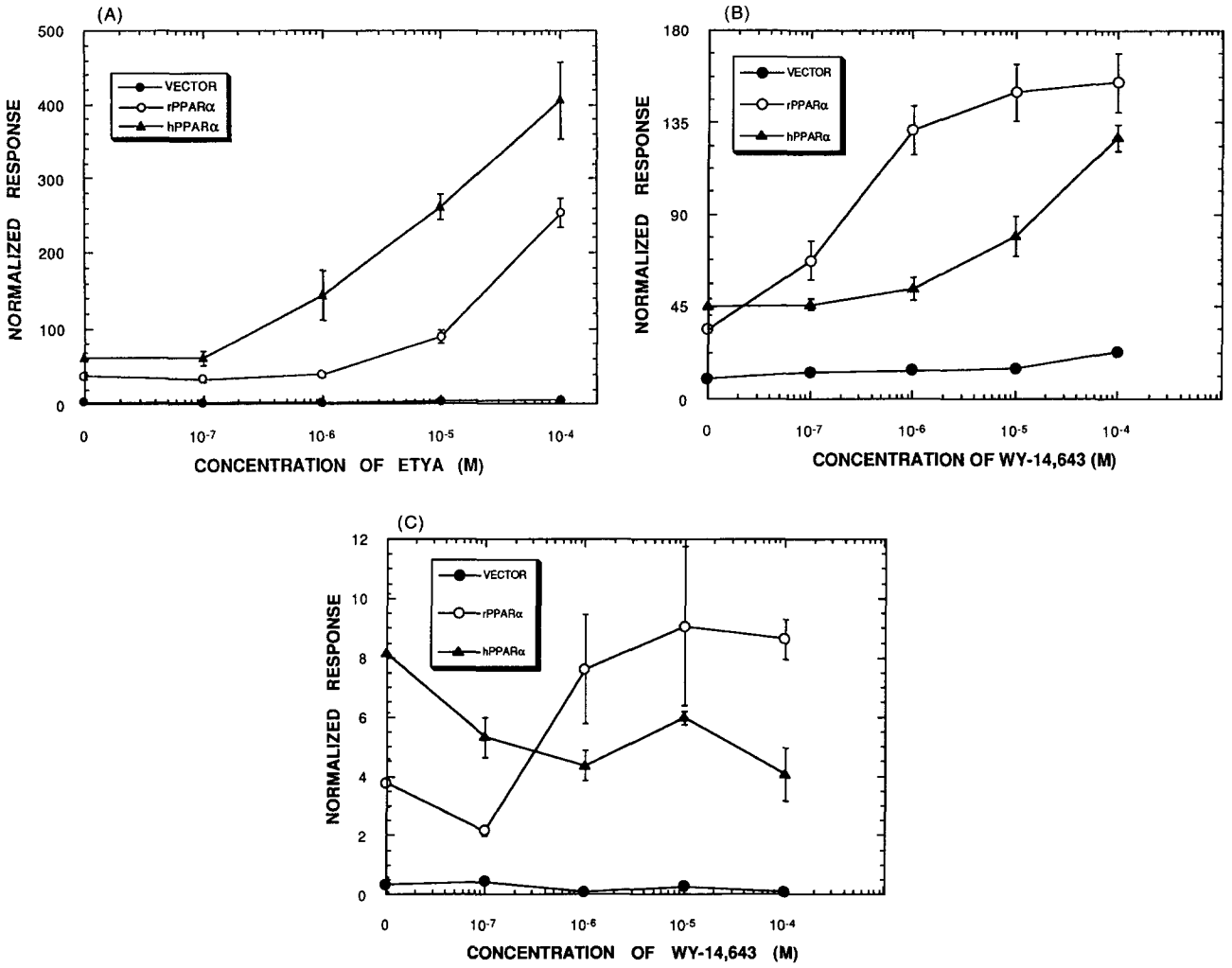

Fig. 6. Activation of hPPARα and rPPARα in hepatoma cells. HepG2 cells were transfected with pBKCMV (vector) or with pCMVhPPARα (hPPARα) or pCMVrPPARα (rPPARα) and treated with (A) ETYA and (B) WY-14,643, at the concentrations indicated. H4IIEC3 cells were similarly transfected and treated with WY-14,643 (C). The reporter construct is pPPREA3-tk-luc and the luciferase activity is shown normalized to the β-galactosidase activity of the same extract.

PPARs exist in both humans and mice. Furthermore, hPPARα is related to NUC1, another PPAR related cDNA that was cloned from a human osteosarcoma library [34]. However, there is only 62% amino acid sequence identity between these two receptor types. It has been postulated that NUC1 is a new member of the steroid hormone receptor superfamily [34].

The tissue distribution pattern of hPPARα mRNA is similar to that of rPPARα. They are expressed at roughly equivalent levels in the heart, kidney and liver. However, without knowing how mRNA expression relates to the expression of receptor protein, or understanding how these expression levels equate to biological activity, it is difficult to come to any firm conclusions as to the significance of the absolute levels of hPPARα mRNA that is expressed in a given tissue. However, the Northern analysis presented here gives a qualitative description of hPPARα expression in human tissues. We are in the process of generating antibodies to hPPARα to specifically examine expression of hPPARα in target tissues.

We have experimentally demonstrated that activation of the rPPARα and hPPARα by some known peroxisome proliferators and hypolipidemic agents are not identical. CFA clearly activates both rPPARα and hPPARα with the same potency and efficacy in CV-1 cells and in HepG2 cells (data not shown). However, the biological activity of WY-14,643 and ETYA is somewhat dependent on the receptor analyzed and the cell context where activity is measured. This demonstrated cell (and by inference tissue) specific differences in the biological activity of PPAR activators, and additional evidence demonstrating multiple PPAR subtypes, suggest that one might be able to reconcile the clinical profiles of different fibrates with cell specific expression of different PPAR subtypes.

One possible explanation for the cell context and species specific differences in activity of the various PPAR activators is that PPARs may require an auxillary protein in order to bind their DNA response elements. The retinoic acid related receptors (RXRs) can function in this regard. We have demonstrated that hRXRα can

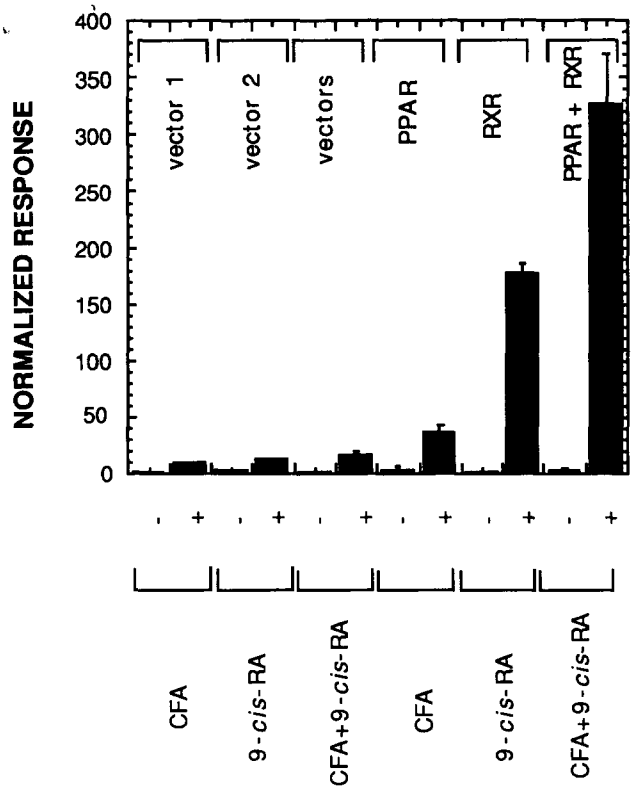

Fig. 7. hPPARα and hRXRα co-operatively activate transcription through a PPRE. CV-1 cells were transfected with 0.5 μg of reporter construct along with 0.05 μg of pCMVhPPARα (PPAR) and/or pRShRXRα (RXR) [35]. As controls pBKCMV (vector 1) and/or pRS-CAT (vector 2) [35] were used. Cells were treated with vehicle (–) or compounds (+). Clofibric acid (CFA) and 9-*cis* retinoic acid (9-*cis* RA) were added to a final concentration of 1 mM and 1 μM, respectively. Luciferase and β-galactosidase assays were performed as described in Materials and Methods.

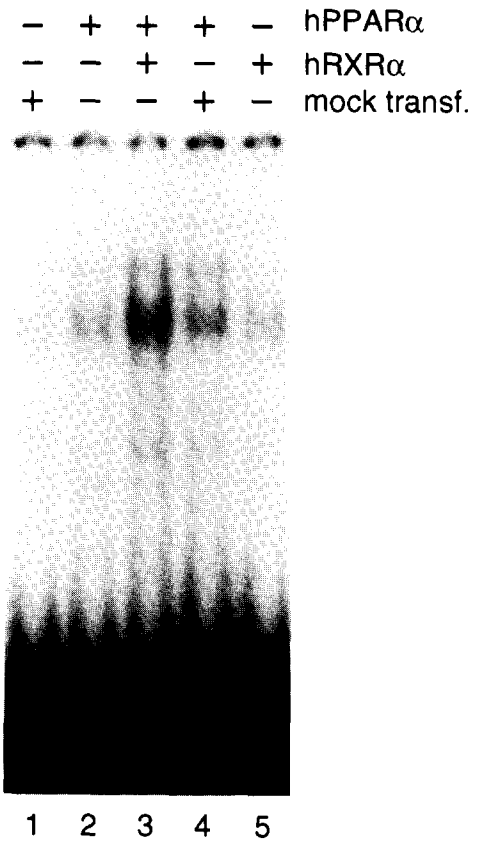

Fig. 8. Co-operative binding of hPPARα and hRXRα to a PPRE. DNA binding assays were performed with extracts from COS cells transfected with pCMVhPPARα or pRShRXRα as described in Materials and Methods. As controls, extracts from mock transfected (mock transf.) cells were used.

enhance binding of hPPARα to the PPRE from the AOX gene and also synergistically activate transcription through the PPRE. Three distinct forms of RXR exist in humans and so it is possible that cell specific activation of human PPARs by peroxisome proliferators could be due to differences in the RXR complement in the two cell types. An additional level of complexity was revealed when it was shown that PPARs and RXRs form heterodimers that can activate the AOX gene in the presence of either PPAR or RXR specific ligands (our data and Refs [35, 36]). This convergence of the retinoid and PPAR signal transduction pathways is clinically relevant as it suggests that the biological profiles of fibrates can be modified by compounds effecting the retinoic acid signal transduction pathway.

The mechanism of activation of PPARs by small molecules is unknown. It is interesting that the cell must be treated with high concentrations of these compounds in order to transcriptionally activate the PPARs. This is in contrast to the related steroid, thyroid and vitamin D receptors which respond to their ligands at subnanomolar concentrations. Furthermore, a wide variety of structurally diverse compounds seem to activate the PPARs. There is no structural feature common to these compounds, except for the presence of a carboxylic acid group. This is different from the often rigid structural requirements for agonists or antagonists of the steroid receptors. One possibility is that these compounds induce a cellular process that secondarily activates the receptor or yields the active ligand. The possibility therefore exists that these compounds could be metabolized differently in different cell types giving rise to tissue specific effects. This coupled with different expression levels of the PPAR subtypes and the required RXRs in tissues could give rise to a wide range of effects by peroxisome proliferators.

We have isolated a cDNA that codes for hPPARα. The tissue distribution of hPPARα is similar to rPPARα. However, the activation profiles of the two receptors to certain PPAR activators are different and depend on the cell line used for the assay. The identification of hPPARα and the subsequent demonstration that it mediates the transcriptional responses to fibrate derived drugs will enable a detailed dissection of the molecular mechanism of action of this group of drugs. In addition, the reconstituted PPAR responsive transcription system in mammalian cells provides a robust *in vitro* assay allowing the development of compounds with improved therapeutic profile.

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