cDNA Cloning, Chromosomal Mapping, and Functional Characterization of the Human Peroxisome Proliferator Activated Receptor‡

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ABSTRACT: The human peroxisome proliferator activated receptor (hPPAR) was cloned from a human liver cDNA library. The cDNA exhibited 85% and 91% DNA and deduced amino acid sequence identity with mouse PPAR (mPPAR), respectively. The hPPAR gene was mapped on human chromosome 22 slightly telomeric to a linkage group of six genes and genetic markers that are located in the general region 22q12–q13.1. Cotransfection assays of mouse Hepa 1 cells were used to roughly compare the ability of hPPAR- and mPPAR-expressed cDNAs to *trans*-activate the acyl CoA oxidase (ACO) PPAR response element located 5′ upstream to the minimal thymidine kinase promoter driving the expression of the chloramphenicol acetyl transferase (CAT) reporter gene. Both receptors elicited a response with the prototypical peroxisome proliferators nafenopin, clofibrate, and WY-14,643. Moreover, using cotransfection assays in which the CAT reporter plasmid contained the *CYP4A6* gene response element rather than the ACO element, it was shown that hPPAR is capable of very efficently *trans*-activating a second PPAR response element. These results indicate that the PPAR is present in humans in a form that is functional and can *trans*-activate response elements derived from two different genes, the rat ACO and the rabbit *CYP4A6*.

Peroxisome proliferators are a diverse group of chemicals which includes hypolipidemic drugs, herbicides, leukotriene antagonists, and plasticizers (for a review, see Reddy and Lalwai (1983), Moody et al. (1991), and Green (1992)). Two major categories of peroxisome proliferator chemicals play a significant role in current society. The first, the fibrate class of hypolipidemic drugs, has been found to be effective at reducing the levels of triglycerides and cholesterol in humans suffering from hyperlipidemia, a major risk factor for coronary heart disease (Thorp & Waring, 1962; Dujovne et al., 1970; Berioli et al., 1990). The second category relates to phthalate ester plasticizers used in the production of highly versatile flexible vinyl plastics (Reddy & Lalwai, 1983).

Peroxisome proliferators seem to affect most mammalian species that have been tested. They induce hepatomegaly resulting from liver hyperplasia and an increase in the size and number of peroxisomes (Reddy & Lalwai, 1983). Nevertheless, on the basis of hypolipidemic drug dose required to produce recognizable peroxisome proliferation, mice and rats are considered to be highly responsive to these agents, developing hepatocellular carcinoma following long-term drug administration, hamsters have intermediate responses, and guinea pigs, marmosets and other nonhuman primates are weakly responsive (Eacho et al., 1986; Lake et al., 1989; Reddy et al., 1984).

Examination of human liver biopsy samples revealed either no differences between fibrate-treated and control groups (Blumcke et al., 1983) or small changes in the density of mitochondria and peroxisomes but no toxic or carcinogenic effects (Hanefeld et al., 1983). Only one study indicated an increased morbidity of patients receiving clofibrate (Oliver et al., 1984). The carcinogenic effect in rodents together with the limited fibrate studies in humans (Green, 1992) raised the argument that humans treated with fibrate drugs could be at risk for cancer development.

Elucidation of the mechanism by which peroxisome proliferators induce carcinogenesis is a prerequisite for assessing the health risk to humans in the pharmaceutical use of fibrates. So far, the mechanism of action of clofibrate and similar drugs is unknown. Peroxisome proliferators are termed nongenotoxic carcinogens since they fail to directly cause DNA damage. The oxidative stress hypothesis suggests that enhanced production of hydrogen peroxide by increased peroxisomal fatty acid β-oxidation overcomes the ability of H_2O_2-degrading enzymes and results in a slow accumulation of oxidative damage to the genome (Reddy & Lalwai, 1983). Alternatively, it was suggested that peroxisome proliferators promote spontaneously initiated foci in liver (Schulte-Herman et al., 1983).

Studies on the long-term effects of two peroxisome proliferators inducing high and low carcinogenic activity, respectively, indicated that factors other than oxidative injury may be important in the carcinogenicity of such chemicals (Moody et al., 1991). Speculation on the existence of specific receptors for mediating the action of peroxisome proliferators (Reddy & Rao, 1986) has recently been supported by the isolation of new members of the steroid hormone receptor superfamily which are activated by peroxisome proliferators: the mouse peroxisome proliferator activated receptor (Issemann & Green, 1990), the *Xenopus laevis* PPAR[1] (Dreyer et al., 1992), and the rat PPAR (Gottlicher et al., 1992). Moreover, it has been shown that PPARs are capable of activating the promoter of the rat acyl coenzyme A oxidase gene, the key enzyme of peroxisomal fatty acid β-oxidation,

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[1] Abbreviations: ACO, acyl CoA oxidase; CAT, chloramphenicol acetyl transferase; hPPAR, human peroxisome proliferator activated receptor; mPPAR, mouse peroxisome proliferator activated receptor; PCR, polymerase chain reaction; PPAR, peroxisome proliferator activated receptor; RFLPs, restriction fragment length polymorphisms; tk, thymidine kinase.

and they mediate the induction of rabbit *CYP4A6*, a cytochrome P450 fatty acid ω-hydroxylase, by clofibric acid (Tugwood et al., 1992; Dreyer et al., 1992; Muerhoff et al., 1992). This activation is carried out through specific response elements that have recently been identified.

Our approach has been to examine whether differences between human and mouse PPAR could account for the marked differences in responsiveness to peroxisome proliferators displayed by both species. To achieve this goal, we isolated the human PPAR from a human liver cDNA library and compared its structure and ability to activate the ACO response element with those of mouse PPAR. The chromosomal location of the human PPAR gene was regionally mapped by use of somatic cell hybrids and linkage analysis in multigeneration families.

MATERIALS AND METHODS

Screening of cDNA Libraries and cDNA Sequence Analysis. The mPPAR cDNA was amplified by PCR (GeneAmp, Perkin- Elmer, Cetus, CT) from total mouse RNA following its conversion to cDNA with reverse transcriptase. As primers we used synthetic oligonucleotides comprising the 25 nucleotides of the 5′-end and the 25 nucleotides complementary to the 3′-end of the mPPAR cDNA coding region (Issemann & Green, 1990), with the addition of *Bam*HI restriction sites at each end.

5′ mPPAR primer: 5′- GGG ATC CAT GGT GGA CAC AGA GAG CCC CAT C -3′

3′ mPPAR primer: 5′- GGC ATC CGA TCA GTA CAT GTC TCT GTA GAT C -3′

The amplified mPPAR thus obtained was cloned in the M13mp18 vector and completely sequenced from both strands. It differs from the published cDNA sequence (Issemann & Green, 1990) in four amino acids: residue 75, Ala instead of Arg; residue 138, Glu instead of Lys; residue 308, Ser instead of Leu; and residue 341, Asn instead of Asp.

Three human cDNA libraries in λgt11 (Yamano et al., 1989) were screened with mPPAR cDNA, and the complete human PPAR cDNA coding sequence was obtained from one of them. cDNAs from positive plaques were subcloned in pUC18, and plasmid sequencing was performed from both strands using the dideoxy chain-termination method with Sequenase 2.0 (United States Biochemical Corp., Cleveland, OH) (Sanger et al., 1977). The complete cDNA coding sequence of the hPPAR was derived from three partial clones, as shown in Figure 1.

Plasmids. The human PPAR cDNA coding region was constructed in pUC18 from clones 135, 86, and 26 (Figure 1) as follows. Clone 135 was digested with *Mlu*I and *Sma*I and ligated to a double-stranded synthetic oligonucleotide comprising nucleotides 558–747 (*Mlu*I/blunt). To produce this oligo, two single-stranded oligonucleotides of 116 bases each were annealed; the 5′-strand contained nucleotides 558–674 and the complementary 3′-strand contained 631–747, to which a *Bam*HI sequence was added at its 3′-end. Klenow polymerase was then used to fill in the double-stranded fragment, which was subsequently digested with *Mlu*I to give the *Mlu*I/blunt fragment. The construct obtained following ligation of the double-stranded oligonucleotide was sequenced to exclude any undesirable errors introduced during synthesis. It was then digested with *Bsm*I and *Bam*HI and ligated to the *Bsm*I/*Bam*HI fragment derived from clone 26 to give the complete human PPAR cDNA. To improve expression of

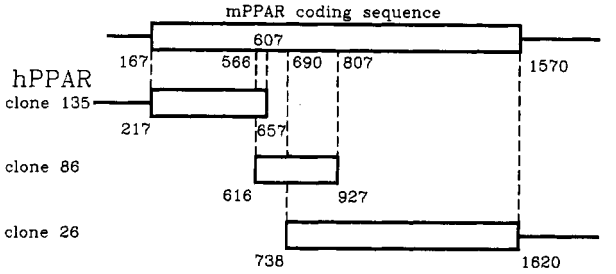

FIGURE 1: Schematic structure of hPPAR clones used for construction of hPPAR coding sequence. Three clones, 135, 86, and 26, were isolated from a human liver cDNA library using the mPPAR cDNA probe under low-stringency hybridization conditions. They were used for sequencing and construction of the complete cDNA coding sequence of hPPAR. The boxes represent coding sequences and the solid lines are noncoding sequences. The upper box relates to the mPPAR coding sequence and the nucleotide numbers were derived from Issemann & Green (1990). Below is a schematic description of the three hPPAR clones, the nucleotide numbers of which were derived from the cDNA sequence presented in Figure 3.

the receptor protein, the 3′ noncoding sequences were removed. The 1510–1623-bp fragment of hPPAR was amplified by PCR from clone 26 (*Bam*HI site was inserted in the 3′-end primer), treated with Klenow polymerase, digested with *Sph*I and *Bam*HI, and inserted in place of the original 1-kb fragment *Sph*I/*Bam*HI.

Expression of receptor by cotransfection assays was performed using the pSG5 vector (Stratagene, La Jolla, CA). The *Bam*HI/*Bam*HI fragment of the mPPAR cDNA coding region was excised from pUC18 and ligated to the *Bam*HI site of pSG5 (pSG5-mPPAR). hPPAR cDNA was excised from pUC18 as an *Nru*I/*Bam*HI fragment and inserted into pSG5 [(*Eco*RI + Klenow)/*Bam*HI] to give the pSG5-hPPAR plasmid. L15CAT was a kind gift of Mike Mitas at the National Institutes of Health. It is a pBLCAT2 vector (Luckow & Schutz, 1987) with additional restriction enzyme sites 5′ to the promoter.

The ACO-L15CAT vector was constructed as follows. The region 5′ upstream to the rat ACO promoter between -640 and -472 containing the ACO PPAR response element (Osumi et al., 1991) was amplified from total rat cellular DNA (Osumi et al., 1987). *Hin*dIII and *Sal*I sites were added to the 5′- and 3′-ends of the primers, respectively.

5′ ACO primer: 5′- GGA AGC TTA CAG AGG GTT GTG AGC CCC CAT G -3′

3′ ACO primer: 5′- GAG TCG ACT CGG GCG GAG TGA AGA GGC TAA T -3′

This *Hin*dIII/*Sal*I fragment was ligated to the *Hin*dIII and *Sal*I sites within the L15CAT polylinker, 5′ to the minimal thymidine kinase promoter driving the CAT gene.

The 3xZ-L15CAT reporter plasmid was constructed as follows. Two oligonucleotides comprising both complementary strands and each containing three copies of the Z-element of *CYP4A6* (Muerhoff et al., 1992) flanked by a *Hin*dIII site at the 5′- end and a *Sal*I site at the 3′-end were synthesized and annealed to create a double-stranded DNA fragment. This was inserted into the *Hin*dIII/*Sal*I sites of L15CAT, respectively. Bluescript KS II⁻ (Stratagene, La Jolla, CA) was used as a carrier in the CAT assay to bring the total DNA concentration up to 5 μg per sample (see below). pSV232LΔ5′ (de Wet et al., 1987), generously supplied by Suresh Subraman (University of California at San Diego), was used as a control for transfection efficiencies.

Transfection. Mouse Hepa 1 cells were cultured in Dulbecco's modified Eagle's Medium supplemented with 10%

fetal calf serum (Gibco Life Technologies, Inc., Grand Island, NY), glutamine, and antibiotics. The day before transfection, cells were transferred to 25-cm^2 flasks with the same medium but with 1.5% dextran-coated, charcoal-treated fetal calf serum. This medium was used throughout the experiment. The next morning, when the cells were 70–80% confluent, the medium was changed and 3 h later transfection was carried out using the calcium phosphate precipitation technique (Graham & Van der Eb, 1973). The cotransfection mixture for results shown in Figure 4 contained 1 μg of ACO-L15CAT, 1 μg of either pSG5-mPPAR, pSG5-hPPAR, or pSG5 as control, 1 μg of luciferase vector pSV232Δ5′, and Bluescript KS II$^-$ as carrier to bring the total DNA content to 5 μg/25-cm^2 flask. The cotransfection mixture for results depicted in Figure 5 contained 1 μg of 3xZ-L15CAT, 1 μg of either pSG5-hPPAR or pSG5 as control, 1 μg of luciferase vector pSV232Δ5′, and Bluescript KS II$^-$ to bring the total DNA content to 5 μg/flask. The next morning the medium was changed and fresh medium with the appropriate inducer was added. The inducers were [4-chloro-6-(2,3-xylidino)-2-pyrimidinyl]thioacetic acid (WY-14,643) (U1025 Chemsyn Science Laboratories, Lenexa, KS), nafenopin (a kind gift of Janardan K. Reddy, Northwestern University Medical School, Chicago, IL), 2-(*p*-chlorophenoxy)-2-methylpropionic acid (clofibrate), and dimethyl sulfoxide (DMSO). The latter two reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Wy-14,643 and nafenopin were dissolved in DMSO and diluted 1:1000 into the medium. Clofibrate was disolved in H_2O and neutralized with NaOH. After 24 h, cells were harvested, and cell lysates were prepared by four cycles of consecutive freeze–thawing and were then assayed for both CAT activity (Gorman et al., 1982) and luciferase activity (de Wet et al., 1987), which was used to normalize the CAT activity. The latter measurements were carried out using a Monolight 2010 luminometer (Analytical Luminescent Laboratories, San Diego, CA).

Chromosomal Localization of the hPPAR Gene. The hPPAR gene was localized by Southern analysis of DNAs from a panel of human–rodent somatic cell hybrids retaining subsets of human chromosomes in a rodent chromosome background as previously described (McBride et al., 1982). The probe was a 0.9-kb *Bsm*I/*Bam*HI cDNA fragment comprising the 3′-end of the hPPAR coding sequence. The hybrids were characterized for the presence of each human chromosome except Y by isoenzyme analysis, Southern analysis with previously mapped probes, and frequently karyotypic analysis. The human–hamster hybrids consisted of 29 primary clones and 14 subclones (18 positive of 43 total), and human–mouse hybrids represented 19 primary clones and 30 subclones (10 positive of 49 total).

Detection of DNA Restriction Fragment Length Polymorphisms (RFLPs). DNA was isolated from the peripheral leukocytes of 10 unrelated normal individuals, separately digested with 12 different restriction endonucleases (*Eco* RI, *Bam*HI, *Hin*dIII, *Xba*I, *Sac*I, *Taq*I, *Msp*I, *Pvu*II, *Pst*I, *Eco* RV, *Bgl*II, and *Kpn*I), size-fractionated by gel electrophoresis, transferred to nylon membranes, and hybridized with the hPPAR cDNA probe. When RFLPs were detected, DNAs from an additional 80 individuals were examined to determine allele frequencies.

Linkage Analysis. DNAs from 40 large three-generation CEPH families (Dausset et al., 1990) were used for these analyses. RFLP typing was performed by standard Southern blot analysis under high-stringency conditions. Both *Msp*I and *Taq*I digests of all parental DNAs were examined with the hPPAR cDNA probe, and all family members from informative matings (i.e., one or both parents heterozygotes) were then analyzed. Two-point linkage analyses versus other loci typed in CEPH database version 5 were performed using the LINKAGE computer program (Lathrop et al., 1984), version 5.1. All recombination events between closely linked markers were rechecked against the original autoradiograms.

RESULTS

Isolation and Sequence of Human PPAR cDNA. Nine positive clones were isolated from a human liver cDNA library by low-stringency hybridization with an mPPAR cDNA probe. Out of these clones, three were used for construction of the full length of the human PPAR cDNA coding region, as shown in Figure 1. The human PPAR cDNA sequence followed by a comparison between the human and the mouse PPAR amino acid sequences is presented in Figure 2. The hPPAR cDNA sequence exhibits 85% identity with that of mPPAR, while 91% similarity was found between the two amino acid sequences. The amino acid sequence of the putative hPPAR DNA binding domain is identical to that of the mPPAR, as reported by Issemann and Green (1990), but differs at residue 138 of our PCR product mPPAR in which Glu is substituted for Lys. There are two main regions enriched with amino acid variations between hPPAR and mPPAR. The first one, comprising the 19 amino acids preceding the putative DNA binding domain, shows only 63% similarity. The second, consisting of 18 amino acids in front of the putative ligand binding domain, displays 66% identity.

Chromosome Mapping of the hPPAR Gene. The chromosome location of the hPPAR gene was determined by analyzing a panel of somatic cell hybrids segregating human chromosomes. The hPPAR locus could be assigned to human chromosome 22, and it segregated discordantly ($\geq$18%) with all other human chromosomes (Table I). It was possible to regionally localize the hPPAR gene to 22q11.2-qter by examination of two human–hamster hybrids containing spontaneous breaks involving chromosome 22. One hybrid retained both the immunoglobulin λ gene and G_{Za} gene, but none of the more distal genes including MYH9, IL2RB, PDGFB, CYP2D, and G22P1; hPPAR was absent in this hybrid. The other hybrid also contained a break between IGL and MYH9 with retention of all of the distal markers, as well as hPPAR (data not shown).

RFLPs. Restriction fragment length polymorphisms were detected in *Taq*I and *Msp*I digests with the hPPAR cDNA probe (Figure 3). The allele sizes and frequencies in the *Taq*I digest were A1:A2(4.0 kp:3.6 kb) = 0.84:0.16 in 79 CEPH parents and a 3.8-kb allele was found in a single individual. In *Msp*I digests, B1:B2 (4.2 kb:3.7 kb) = 0.03:0.97 in 79 CEPH parents.

Mapping the hPPAR Locus by Genetic Linkage Analysis. *Taq*I and *Msp*I digests of DNAs from all CEPH parents were typed with the hPPAR probe, and all offsprings and grandparents were also typed in informative families (i.e., 17/40 with *Taq*I and 5/40 with *Msp*I). Using the *Taq*I polymorphism, hPPAR could be linked to a series of genes and anonymous DNA markers on chromosome 22 by two-point linkage analysis (Table II). Highly significant Lod scores were obtained, with all of these markers providing strong evidence for linkage. These loci previously have been assigned to the general region 22q12–q13.1 (Emanuel et al., 1991). Preliminary results of multipoint linkage analysis (not shown) suggest that the hPPAR gene is located slightly telomeric to this entire cluster of linked genes.

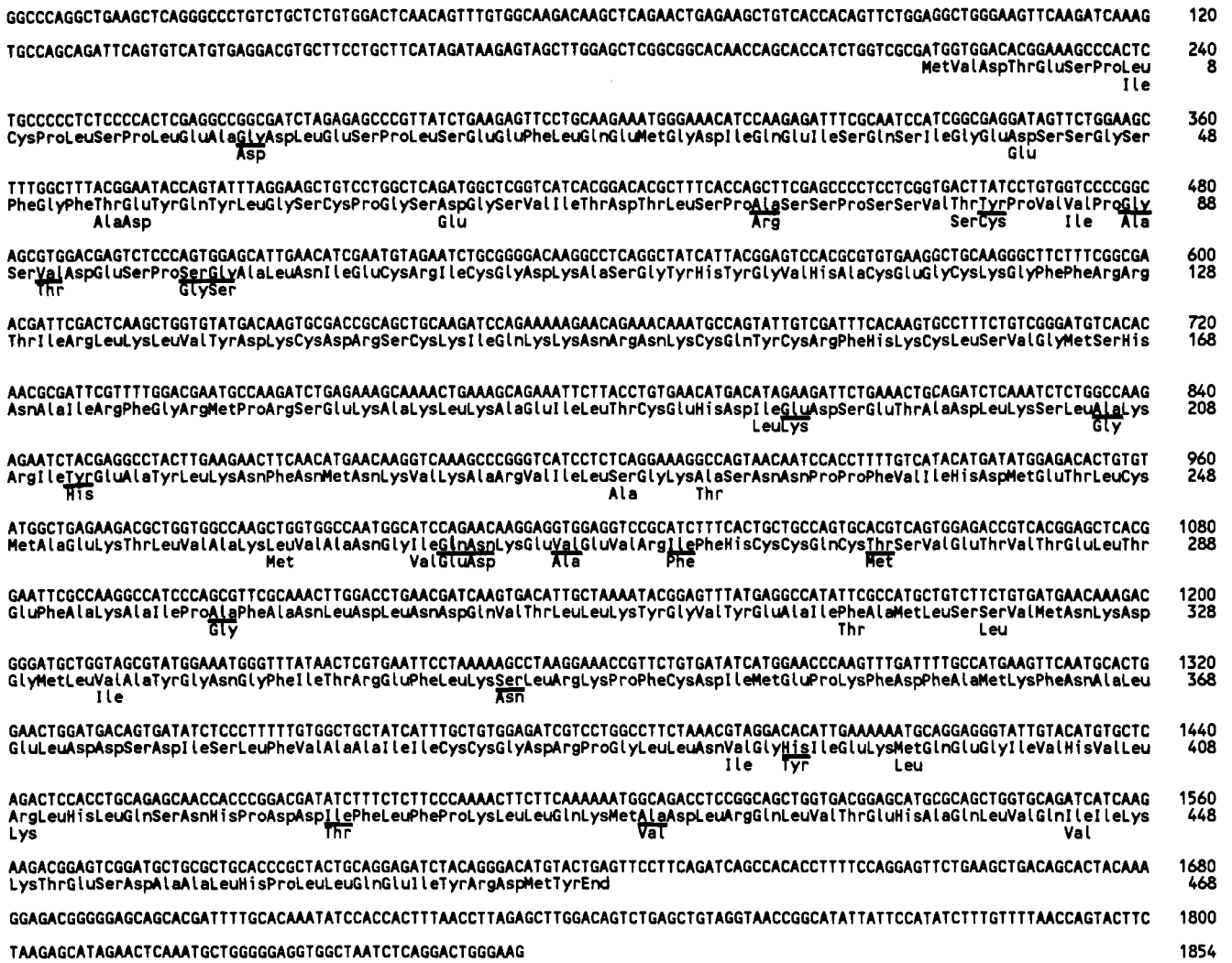

FIGURE 2: cDNA and cDNA-deduced amino acid sequences of hPPAR. The complete cDNA nucleotide sequence of hPPAR as derived from clones 135, 86, and 26 is presented, and the nucleotide positions are denoted on the right. The deduced amino acid sequence and numbering is presented below, respectively. Differences in the predicted amino acids between hPPAR and mPPAR (Issemann & Green, 1990) are indicated and significant differences are underlined.

Trans-Activation of CAT Activity via the Acyl CoA Oxidase Response Element by hPPAR and mPPAR. The ACO-L15CAT reporter plasmid contains the rat acyl CoA oxidase response element corresponding to positions -680 to -470 (Osumi et al., 1991) in the correct orientation upstream of the minimal thymidine kinase promoter driving expression of the CAT gene. This reporter plasmid was cotransfected into the mouse Hepa 1 cell line with either pSG5-mPPAR, pSG5-hPPAR, or pSG5 alone as control. The cotransfected cells were exposed for 24 h to various concentrations of either Wy-14,643, nafenopin (dissolved in DMSO), clofibrate, or 0.1% DMSO as control. CAT activity was normalized for luciferase activity, and the results are shown in Figure 4. Both pSG5-hPPAR and pSG5-mPPAR induced high levels of CAT activity following induction by similar concentrations of Wy-14,643, nafenopin, and clofibrate. Differences in the extent of induction were also noted between mPPAR and hPPAR, the significance of which remains to be determined.

Trans-Activation of CAT Activity by hPPAR via the Acyl CoA Oxidase and a Triplicate of the Z-Oligo of the CYP4A6 Response Element. We further examined whether hPPAR is capable of *trans*-activating another response element, the Z-oligo of the rabbit *CYP4A6* gene (Muerhoff et al., 1992). To this end, we used the 3xZ-L15CAT reporter plasmid, which contains three copies of the Z-oligo of the rabbit *CYP4A6* response element upstream of the tk promoter driving expression of the CAT gene, respectively. Each of the reporter plasmids 3xZ-L15CAT and ACO-L15CAT was cotransfected into the mouse Hepa I cell line with either pSG5-hPPAR or pSG5 alone as control group. The cotransfected cells were exposed for 24 h to various concentrations of either Wy-14,643, nafenopin (dissolved in DMSO), clofibrate, or 0.1% DMSO as control. CAT activity was normalized for luciferase activity, and the results are shown in Figure 5. *Trans*-activation of ACO-L15CAT by hPPAR in the presence of 10^{-4} M nafenopin, which displayed the highest values of CAT activity for the ACO-L15CAT reporter plasmid, was defined as 100% of CAT activity. pSG5-hPPAR *trans*-activated both the ACO and the *CYP4A6* response elements in the presence of three peroxisome proliferators: Wy-14,643, nafenopin, and clofibrate, as depicted in Figure 5. However, CAT activity elicited following *trans*-activation of 3xZ-L15CAT was >5-fold higher compared to that displayed by *trans*-activated ACO-L15CAT (Figure 5). It should be noted though that the constitutive background activity of the 3xZ-L15CAT plasmid was about 6-fold higher than that of ACO-L15CAT in 0.1% DMSO. Another control group of pSG5 alone cotransfected with each of the reporter plasmids gave values of CAT activity similar to those produced by ACO-L15CAT in the presence of 0.1% DMSO (Figure 5), and this activity was either unchanged or

Table I: Segregation of Human PPAR Gene with Human Chromosome 22[a]

human chromosome	gene/chromosome +/+	+/−	−/+	−/−	% discordancy
1	17	11	15	49	28
2	14	14	14	50	30
3	19	9	12	52	23
4	22	6	33	31	42
5	18	10	7	57	18
6	23	5	27	37	35
7	14	14	28	36	46
8	18	10	19	45	32
9	20	8	13	51	23
10	13	15	7	57	24
11	19	9	12	52	23
12	11	17	18	46	38
13	11	17	25	39	46
14	12	16	32	32	52
15	16	12	29	35	45
16	13	15	26	38	45
17	19	9	40	24	53
18	19	9	27	37	39
19	17	11	13	51	26
20	21	7	20	44	29
21	22	6	37	27	47
22	28	0	0	64	0
X	18	10	28	36	41

[a] The human PPAR gene was detected as 12.5-, 8.5-, and 4-kb bands in *Eco*RI digests of somatic cell hybrid DNAs after Southern hybridization with a 0.9-kb hPPAR cDNA probe. These bands co-segregated, and they were all resolved from 11.4-, 9.3-, and 6-kb or 10- and 17-kb cross-hybridizing bands in Chinese hamster and mouse DNAs, respectively. Detection of the human gene is correlated with the presence or absence of each human chromosome in the group of somatic cell hybrids. Discordancy indicates the presence of the gene in the absence of the chromosome (+/−) or absence of the gene despite the presence of the chromosome (−/+), and the sum of these numbers divided by total hybrids examined multiplied by 100 represents the percent discordancy.

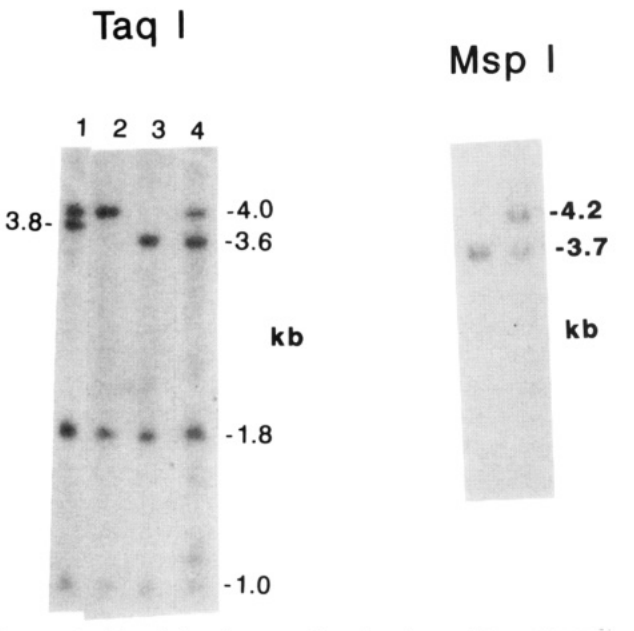

FIGURE 3: Restriction fragment length polymorphisms (RFLPs). RFLPs detected in representative DNAs from CEPH parents after Southern hybridization with a human hPPAR cDNA probe. Allelic bands of 3.6, 3.8, and 4.0 kb were observed in *Taq*I digests (left); invariant bands of 1.0 and 1.8 kb were also present. Individuals homozygous for a 3.7-kb allele (lane 1) or heterozygous for 3.7- and 4.2-kb alleles (lane 2) were found in *Msp*I digests (right).

increased less than 2-fold following induction by each of the peroxisome proliferators depicted in Figure 5 (data not shown). In addition, no significant differences were detected in the

Table II: Two-Point Lod Scores for hPPAR versus Other Chromosome 22 Loci

locus[a]	Z at Θ[b] 0	0.05	0.1	0.2	0.3	0.4	Θ_{max}	Z_{max}[d]	confidence interval[c]
MYH9	−∞	2.6	5.3	6.6	5.0	2.7	0.179 (0.099, 0.269	6.23 6.97)*	0.10–2.28
IL2RB	−∞	8.0	10.3	10.2	7.7	4.0	0.141 (0.144, 0.140	10.74 10.74)*	0.09–0.22
PDGFB	−∞	2.7	3.3	3.3	2.6	1.5	0.142 (0.248, 0.125	3.44 3.52)*	0.04–0.32
CYP2D	−∞	8.4	9.6	8.8	6.5	3.3	0.116 (0.053, 0.194	9.68 10.34)*	0.06–0.21
G22P1	−∞	6.9	8.0	7.4	5.5	3.0	0.123 (0.094, 0.134	7.52 7.55)*	0.04–0.25
D22S29	−∞	0.58	2.79	3.73	3.05	1.59	0.193 (0.128, 0.328	3.74 4.13)*	0.10–0.32
D22S18							0.034	6.53	

[a] Probe–enzyme combinations for these loci are as follows: MYH9 = 1.36 kb nonmuscle myosin heavy chain A cDNA with *Bgl*II (Simons et al., 1991); IL2RB = interleukin 2 receptor β gene using PCR amplification of an $(ATT)_{17}$ repeat in intron 1 with analysis of amplified fragments by denaturing PAGE; PDGFB = protooncogene SIS using a 1.2-kb insert from v-sis as a probe with *Hin*dIII; CYP2D = cDNA probe of CYP2D1 using haplotype generated with enzymes *Bam*HI, *Xba*I, and *Eco*RI; G22P1 = 0.65-kb *Hin*dIII/*Eco*RI 3′ cDNA insert of a gene for an autoantigen in Grave's disease and systematic lupus erythematosis with enzyme *Sac*I; D22S29 = 1W22D with *Taq*I (Dumanski et al., 1991); D22S18 = CRI-L1272 probe with enzyme *Taq*I (Donis-Keller, 1987). [b] The LOD scores (Z) at selected recombination factors (Θ) are shown. The most likely recombination fraction (Θ_{max}) and LOD scores (Z_{max}) are also shown. All values are computed assuming no sex difference in recombination frequencies, except those values in parentheses giving sex-specific (male–female order) recombination fractions and Z_{max}. [c] The confidence intervals for recombinant fractions over a 10-fold range of likelihood. [d] * indicates sex-specific recombination fractions and LOD scores.

abilities of pSG5-hPPAR and pSG5-mPPAR to activate 3xZ-L15CAT following clofibrate administration (data not shown).

DISCUSSION

We cloned the human cDNA homologous to the mouse PPAR cDNA which had been identified as a member of the steroid hormone receptor superfamily (Issemann & Green, 1990). The hPPAR gene was mapped on chromosome 22 slightly telomeric to a linkage group of six genes and genetic markers that are located in the general region 22q12–q13.1. It is noteworthy that only a simple pattern of fragments was obtained on Southern blots, suggesting the presence of only a single hPPAR gene with no closely related family members in contrast to the results in frogs where three forms were identified (Dreyer et al., 1992). These data do not exclude multiple PPAR receptors in humans that exhibit lower sequence relatedness, such as the NUCI receptor cDNA recently cloned from a human osteosarcoma cell library (Schmidt et al., 1992).

As a member of the nuclear hormone receptor superfamily, the PPAR is believed to be a ligand-dependent transcriptional modulator (Green, 1992). Indeed, a specific "hormone response element" has been identified in the 5′ upstream region of the acyl CoA oxidase promoter which is activated by PPAR following exposure to its cognate ligand (Tugwood et al., 1992; Dreyer et al., 1992). We used these findings in the construction of an assay system to compare and evaluate the ability of pSG5-hPPAR and pSG5-mPPAR to activate a CAT reporter plasmid via the ACO response element located 5′ upstream

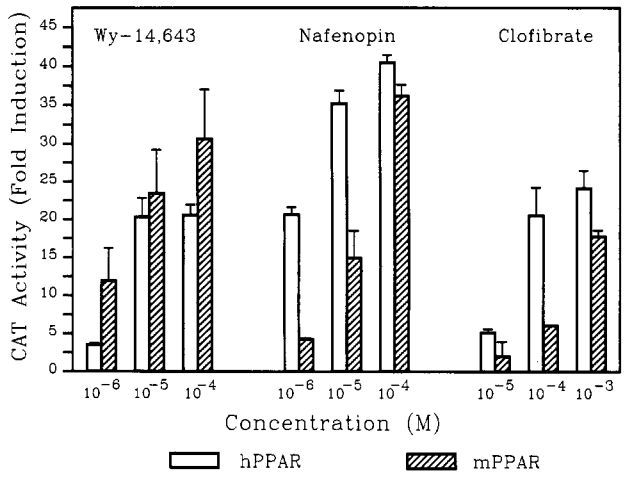

FIGURE 4: CAT activity induction via ACO response element by pSG5-hPPAR compared to pSG5-mPPAR. Hepa I cells were cotransfected with the ACO-L15CAT reporter plasmid and with either pSG5-mPPAR, pSG5-hPPAR, or pSG5 as control. The transfected cells were treated for 24 h with various concentrations of Wy-14,643, nafenopin, clofibrate, or 0.1% DMSO as control. All of these chemicals were toxic for Hepa I cells at concentrations higher than those shown. CAT activity was determined by calculating the dpm fraction of acetylated forms of chloramphenicol per total dpm of acetylated and nonacetylated forms. This value was divided by luciferase activity to normalize differences in transfection efficiency. Fold induction of CAT activity above control (induction of pSG5 cotransfected with ACO-L15CAT by 0.1% DMSO) is presented. The open bars represent activity induced by pSG5-hPPAR, and the hatched bars represent that of pSG5-mPPAR. The standard deviation is depicted as well. At least two independent experiments, each one in duplicate, were carried out for each point.

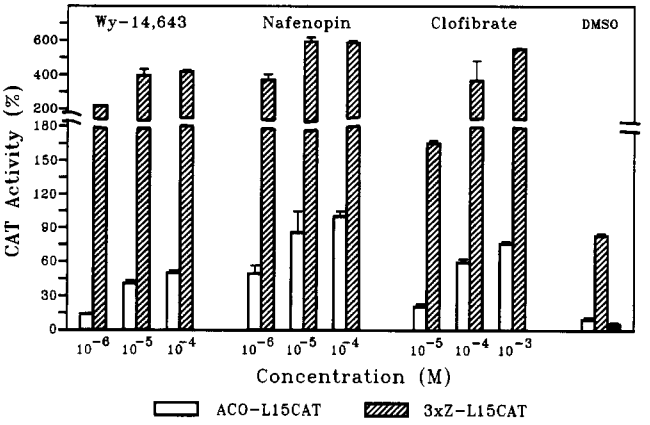

FIGURE 5: CAT activity induction by hPPAR via *trans*-activation of the response element of either ACO or *CYP4A6* genes. Hepa I cells were cotransfected with the ACO-L15CAT reporter plasmid and either pSG5-hPPAR or pSG5 as control or with the 3xZ-L15CAT reporter plasmid together with either pSG5-hPPAR or pSG5 as control. The transfected cells were treated for 24 h with various concentrations of Wy-14,643, nafenopin, clofibrate, or 0.1% DMSO as control. CAT activity determination and normalization were done as described for Figure 4. Results are expressed as percent of CAT activity. The value 100% of CAT activity refers to CAT activity exhibited by *trans*-activation of ACO-L15CAT by pSG5-hPPAR in the presence of 10^{-4} M nafenopin. The open bars represent CAT activity (%) displayed following *trans*-activation of ACO-L15CAT by pSG5-hPPAR, the hatched bars represent such activity after *trans*-activation of 3xZ-L15CAT by pSG5-hPPAR, and the black bar represents activity after *trans*-activation by pSG5 alone in the presence of 0.1% DMSO. The DMSO concentration in Figure 5 is 0.1% as was its concentration in the final dilution of each peroxisome proliferator used in the assay. The standard deviation is depicted as well. At least two independent experiments, each one in duplicate, were carried out for each point.

to the minimal thymidine kinase promoter driving CAT gene expression. The purposes of such a study were to elucidate whether hPPAR is functional and whether variations in the potency of hPPAR versus mPPAR to activate the ACO response element could account for the marked differences in responsiveness to peroxisome proliferators exhibited by mice as compared to humans (Reddy & Lalwai, 1983; Moody et al., 1991; Green, 1992). We found that the human PPAR was a very active receptor, exhibiting an ability to *trans*-activate the acyl CoA oxidase promoter comparable to that of the mouse receptor. Marked inductions of 20–40-fold over control were observed with three known peroxisome proliferator chemicals.

We noted some differences in our ligand dose–response curves between hPPAR and mPPAR. In the absence of direct measurements of PPAR contents in our experiments, however, we cannot draw definitive conclusions regarding the apparent differences in ligand responsiveness between the human and mouse receptors.

hPPAR was shown to *trans*-activate another response element of the *CYP4A6* gene, as was previously shown for mPPAR (Muerhoff et al., 1992; our unpublished data). Interestingly, a higher overall response including the constitutive response (no addition of ligand) was noted. This may be due to the presence of endogenous ligands (Osmundsen et al., 1991) that are capable of eliciting a response with the triplicate Z-element and the higher activity of the *CYP4A6* Z-element as compared to the single ACO element.

Peroxisome proliferation has not been clearly demonstrated in humans (Blümcke et al., 1983; Hanefeld et al., 1983). A study done in monkeys also indicated that nafenopin did not induce peroxisomal β-oxidation in marmoset primary hepatocyte cultures, in contrast to its induction effect on rat primary hepatocyte cultures (Bieri et al., 1988). Our results suggest that malfunctioning of the hPPAR protein per se cannot account for the fact that humans are weakly responsive while rodents are highly responsive to hypolipidemic drugs (Reddy & Lalwai, 1983). Actually it has been demonstrated that the peroxisomal enzymes and β-oxidation system do exist in human cells (Watkins et al., 1991).

In our experiments we used the rat ACO and the rabbit *CYP4A6* response elements, both of which are derived from species highly responsive to peroxisome proliferators. It may well be that the mechanism of regulation of the response element controlling the human ACO gene, and perhaps other human genes induced by PPAR and its ligands, fundamentally differs from that of rats or mice, resulting in low responsiveness to peroxisome proliferators. One possibility is that the human genes have lost the *cis*-acting regulatory elements found in rodent genes. Therefore, a possible approach is to characterize the human ACO response element and evaluate its ability to be *trans*-activated by hPPAR. Unfortunately, the human ACO gene and its corresponding response element have yet to be isolated. It will be interesting to determine whether there is an inducible human ACO response element, which can be activated by hPPAR in the presence of a suitable ligand, or whether some inhibition of the induction process can account for the low responsiveness of humans to peroxisome proliferators. For example, it has been reported for another member of the nuclear hormone receptors, the thyroid hormone (T3) receptor, that although it bound to the estrogen response element with high affinity *in vitro* it failed to activate transcription from the same response element *in vivo* and inhibited estrogen-dependent transcription. It was proposed that the T3 receptor was bound to the estrogen response element in a transcriptionally inactive form (Glass et al., 1988). Another factor which may affect the activity of the hPPAR

in the human system is a possible requirement for the coupling of another receptor to augment *trans*-activation of the peroxisome proliferator response element. Such a phenomenon has been shown for mPPAR coupled to the 9-*cis* retinoid acid receptor (Kllewer et al., 1992). Distinction among these possibilities awaits further experimentation.

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