The effect of peroxisome-proliferator-activated receptor-α on the activity of the cholesterol 7α-hydroxylase gene

Dilip D. PATEL*, Brian L. KNIGHT*[1], Anne K. SOUTAR*, Geoffrey F. GIBBONS† and David P. WADE*[2]

*Lipoprotein Group, MRC Clinical Sciences Centre, Hammersmith Hospital, DuCane Road, London W12 ONN, U.K., and †Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Woodstock Road, Oxford OX2 6HE, U.K.

Cholesterol 7α-hydroxylase (Cyp7a1) plays a central role in the regulation of bile acid and cholesterol metabolism, and transcription of the gene is controlled by bile acids and hormones acting through a complex interaction with a number of potential steroid-hormone-binding sites. Transcriptional activity of the human *CYP7A1* gene promoter transfected into HepG2 cells was decreased in a concentration-dependent manner by co-transfection with an expression vector for peroxisome-proliferator-activated receptor-α (PPARα). This effect was augmented by 9-*cis*-retinoic acid receptor-α (RXRα) and activators of PPARα to give a maximum inhibition of approx. 80%. The region responsible for this inhibition contained a site known to bind hepatocyte nuclear factor 4 (HNF4), and mutation of this site greatly decreased the effect. Co-expression of HNF4 increased promoter activity and decreased the effect of PPARα. Gel-mobility-shift assays failed to detect any binding of PPARα/RXRα dimers to any regions of the promoter containing potential binding sites. Also the hepatic abundance of Cyp7a1 mRNA in mice in which the PPARα gene was disrupted was the same as in normal mice, both during the dark phase, when the animals were feeding, and during the light phase, when mRNA abundance was greatly increased. Cholesterol feeding produced the same increase in hepatic Cyp7a1 mRNA abundance in PPARα-null animals as in normals. It is concluded that, whereas PPARα can affect *CYP7A1* gene transcription *in vitro* through an indirect action, probably by competing for co-factors, this is unlikely to be a major influence on Cyp7a1 activity under normal physiological conditions.

Key words: diurnal rhythm, gene promoter, PPAR α-null mice, transcription.

INTRODUCTION

Cholesterol 7α-hydroxylase (Cyp7a1) is found exclusively in the liver, where it catalyses the first step in the major pathway responsible for the synthesis of bile acids [1,2]. Since secretion of cholesterol and bile acids in the bile is the major route for the removal of cholesterol from the body, regulation of Cyp7a1 activity plays an important role in cholesterol homoeostasis. Deletion of the cholesterol 7α-hydroxylase gene (*Cyp7a1*) in mice has dramatic effects on their development [3] and over-expression of the gene in hamsters greatly reduces plasma cholesterol concentrations [4]. In humans, polymorphisms in the *CYP7A1* gene have been linked to differences in the concentration of low-density lipoprotein [5]. It has been known for many years that the synthesis of bile acids can be regulated by a variety of hormonal and dietary factors, including cholesterol and bile acids themselves (reviewed in [2]). More recently, it was shown that much of this regulation involved modulation of *cyp7a1* gene transcription [6–12], which has prompted an intensive study of the promoter region of the gene.

Various sites in the proximal promoter of the *CYP7A1* gene bind the liver-specific factors hepatocyte nuclear factor 1 (HNF1) and HNF3 [8,13,14], although this is not apparently sufficient to drive maximal liver expression [15]. Feedback inhibition of gene activity by bile acids has been linked to two other regions in the promoter [16,17], each of which contains potential sites for the binding of nuclear hormone receptors. One, between about −70 bp and −50 bp from the translation start site, contains a possible DR4 sequence (two direct repeats of the nuclear hormone half-site separated by four nucleotides), and has been shown in the rat to bind the chicken ovalbumin upstream promoter transcription factor (COUP TFII) [18], as well as the liver nuclear oxysterol receptor-α (LXRα) [19]. It could also be the site of action of the bile-acid-activated farnesyl X receptor, which impedes the ability of LXRα to stimulate transcription [20]. However, this mechanism may not be universal, since the region is not completely conserved between species. The corresponding site in the human promoter does not bind COUPTFII [21] or LXRα [22], but prefers to bind HNF1 [22].

The second putative bile acid response element is about −130 bp to −150 bp upstream from the translation start site and contains a DR1 sequence and overlapping DR0 and DR5 sequences. The DR1 sequence is conserved between species and is known to bind HNF4 in both the rat [17] and the human [13] promoters. The overlapping DR0 sequence binds COUPTFII in the rat [18], but not in the human [21] promoter, in which it binds *CYP7A1* promoter binding factor (CPF), a homologue of the *Drosophila* nuclear receptor fushi tarazu F1 [23]. The DR5 sequence binds retinoic acid-activated factors in the rat [14], but is not conserved in humans. The presence of so many potential

Abbreviations used: ACO, acyl-CoA oxidase; COUP TFII, chicken ovalbumin upstream promoter transcription factor II; Cyp7a1, cholesterol 7α-hydroxylase; *CYP7A1*, human Cyp7a1 gene; *cyp7a1*, mouse Cyp7a1 gene; DR, direct repeat; ETYA, eicosa-5,8,11,14-tetraynoic acid; HNF, hepatocyte nuclear factor; LXR, liver nuclear oxysterol receptor; PPAR, peroxisome-proliferator-activated receptor; RXR, retinoid X receptor; CPF, *CYP7A1* promoter binding factor.

[1] To whom correspondence should be addressed (e-mail brian.knight@csc.mrc.ac.uk).
[2] Present address: CV Therapeutics Inc., 3172 Porter Drive, Palo Alto, CA 94304, U.S.A.

binding sites in the Cyp7a1 gene opens the way for modulation of gene activity by other nuclear-hormone receptors that could interfere directly with DNA binding or act indirectly by competing for common dimerization partners or co-activators.

Peroxisome-proliferator-activated receptors (PPARs) are members of the nuclear-hormone-receptor family that are activated by fatty acids and their derivatives to stimulate the transcription of many enzymes involved in lipid metabolism [24,25]. PPARα, the isoform found predominantly in liver, was initially identified as the factor that mediated the stimulation of peroxisomal fatty acid oxidation in response to peroxisomal activators such as the fibrate family of drugs [26]. Mice in which the PPARα gene has been disrupted do not respond to peroxisomal proliferators [27]. The fibrates are widely used clinically to lower plasma lipid concentrations and have been shown to significantly decrease rates of cholesterol 7α-hydroxylation *in vivo* [28]. This raises the possibility that PPARα could be involved in some way in the regulation of *CYP7A1* activity. PPARα binds as a dimer with retinoid X receptors (RXRs) to DR1 sequences, and so could directly interfere with the binding of HNF4 to the gene promoter. It can also interfere with thyroid-hormone-receptor binding to DR4 elements [29] and has been shown to bind directly to LXRα, as well as competing for its RXR binding partner [30]. In the present paper we report that PPARα reduces the activity of constructs driven by the human *CYP7A1* promoter by a mechanism that does not involve the binding of the factor to promoter DNA. However, the hepatic abundance of mRNA for Cyp7a1 was not increased at any point of the diurnal cycle in PPARα knockout mice, indicating that the effects observed *in vitro* play little part in the regulation of cyp7a1-gene transcription under physiological conditions.

EXPERIMENTAL

Materials

Plasmid pLUXF70 containing the human *CYP7A1* promoter region was kindly provided by Dr S. B. Kadin (Pfizer Central Research Division, Groton, CT, U.S.A.). Plasmids pSG5mPPARα, containing the full-length cDNA of the mouse PPARα gene, and pACO($-1273/+20$)CAT containing the PPARα-responsive acyl-CoA oxidase (ACO) promoter were kindly provided by Dr J. Tugwood (AstraZeneca, Macclesfield, Cheshire, U.K.), plasmid pSG5-hRXRα, containing the full-length human RXRα cDNA, by Dr A. Dejean (Institut Pasteur, Paris, France), expression vector containing the full-length HNF4 cDNA by Dr K. Schwartz (Falk Institute, Stanford University, Stanford, CA, U.S.A.) and pSG5LXRα, containing the full-length LXRα cDNA, from Dr J. Lehmann (Glaxo Wellcome Research and Development, Research Triangle Park, NC, U.S.A.). pGL3-basic, Luciferase Assay System, and the TnT Coupled Reticulocyte Lysate System were from Promega Co, Madison, WI, U.S.A. The Galacto Light reporter assay system was from Tropix Inc., Bedford, MA, U.S.A. Fenofibrate and ETYA (eicosa-5,8,11,14-tetraynoic acid) were from Sigma–Aldrich, Poole, Dorset, U.K., and were dissolved in ethanol before use.

Reporter and expression vectors

The immediate promoter region of the human *CYP7A1* gene, encompassing positions $+63$ to -588 relative to the transcription start site, was cut from the pLUX70 plasmid with *Nco*1and *Xba*1 and ligated upstream of the luciferase gene in plasmid pGL3-basic digested with *Nco*1 and *Nhe*1 to give pGL3-7αh588. To obtain constructs containing smaller fragments of the promoter, pGL3-7αh588 was used as template in PCR reactions with the oligonucleotide 5′-GGCCTTTCTTTATGTTTTTGGCGTCTTCCATGG-3′ (containing a 3′ *Nco*1 site) as the non-coding primer and the oligonucleotides 5′-GCTCTAGAGCTCAAGTATTGCAGGTCTCT-3′, 5′-GCTCTAGAGCAAGGCCAGTTACTACCACT-3′ or 5′-GCTCTAGAGCGGATCTGGATACTATGTAT-3′ (containing an *Xba*1 site) as coding primers. PCR products were digested with *Nco*1 and *Xba*1 and inserted into pGL3-basic digested with *Nco*1 and *Nhe*1 to give constructs pGL3-7αh194, pGL3-7αh134 and pGL3-7αh49 respectively. The sequence of the DR1 element at position -140 to -138 was changed from CTT to TCC using the Unique Site Elimination Mutagenesis kit (Amersham Pharmacia Biotech) with oligonucleotides 5′-CCACTTCTGATACATGTGGATCCAGTTCAAGGC-3′ to mutate the promoter and 5′-TCAAGGGCATCGACCGGTGGATCCTTATCG-3′ to abolish the single *Age*1 site in the vector. The mutant fragment was removed with *Nco*1 and *Mlu*1 and replaced in fresh pGL3-basic digested with the same enzymes.

The expression vector in which the PPARα cDNA was reversed was constructed by digesting pSG5mPPARα with *Bam*H1, phosphorylating the isolated PPARα cDNA and re-ligating it into pSG5 vector digested with the same enzyme. Clones containing the reversed PPARα cDNA were identified by digestion with *Eco*R1.

pACO($-1273/+20$)CAT was digested with *Eco*RV and *Kpn*1 and the isolated ACO promoter cDNA was ligated into pGL3-basic digested with *Sma*1 and *Kpn*1.

Transfection

HepG2 cells in 24 mm wells were grown and transfected in 1 ml of medium using the lipofectin reagent essentially as described previously [31]. Up to 6 μg of total plasmid DNA was added to each well, together with 0.5 μg of cytomegalovirus β-galactosidase vector to correct for transfection efficiency. Within each experiment the total amount of DNA added was kept constant by the addition of pSG5 vector. Cells were lysed 48 h later and assayed for luciferase and β-galactosidase activity. When required, 20 μM ETYA and 250 μM fenofibrate were added for 24 h before the cells were harvested.

Gel-mobility-shift assays

PPARα, RXRα and LXRα mRNAs were prepared and translated from pSG5mPPARα, pSG5-hRXRα and pSG5LXRα respectively using the reticulocyte lysate system as directed by Promega. An oligonucleotide probe was synthesized corresponding to positions -160 to -125 in the human *CYP7A1* promoter [8]. A further oligonucleotide probe was synthesized containing a known binding site for PPARα [32]. Oligonucleotides complementary to these forward strands were designed and synthesized to create four-base overhangs after annealing, and the resulting double-stranded probes were labelled by filling in with the appropriate radioactive dNTPs. Gel-mobility-shift assays were performed as described by Kliewer et al. [33].

PPARα-defective mice

PPARα-null mice bred on to an Sv/129 genetic background were kindly provided by Dr J. Peters and Dr F. J. Gonzalez (National Institutes of Health, Bethesda, MD, U.S.A.). Wild-type Sv/129 mice were used as controls. All animals were kept on a 12 h-light/12 h-dark cycle and were provided with food and water *ad*

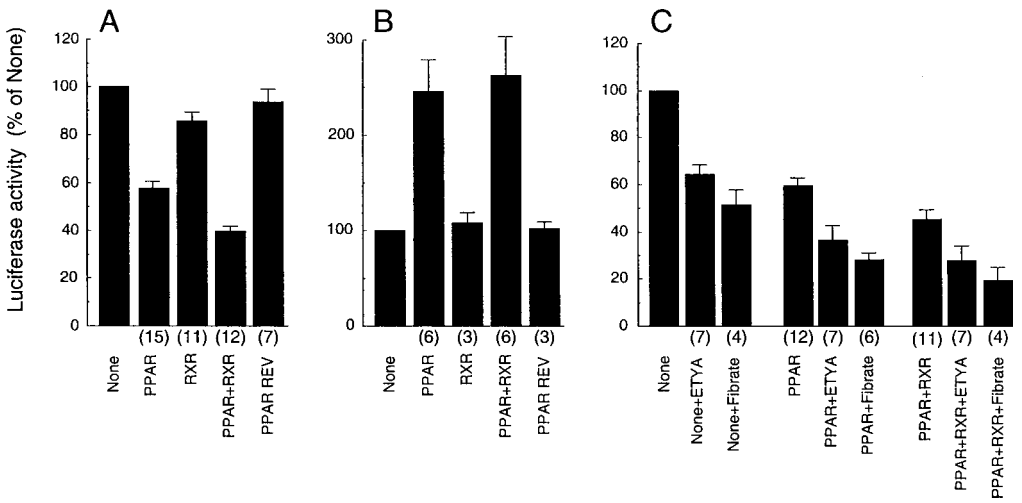

Figure 1 *CYP7A1* promoter activity *in vitro*

HepG2 cells were transfected with reporter constructs containing the luciferase gene driven by the human *CYP7A1* promoter (**A** and **C**) or the ACO promoter (**B**). They were co-transfected, as indicated, with expression vectors for PPARα (4 μg/ml), RXRα (2 μg/ml) or vector in which the PPARα gene was reversed (PPAR REV; 4 μg/ml). When required, cells were incubated for 24 h before harvesting with ETYA (20 μM) or fenofibrate (250 μM) as shown. Results are presented as a percentage of the promoter activity observed with no additions and are means ± S.E.M. with the number of independent observations in parentheses beneath. The effects of PPARα and RXRα on the *CYP7A1* promoter activity in (**A**) were all statistically significant ($P < 0.0001$, Student's *t*-test). The effects of PPARα on the ACO promoter activity in (**B**) were also significant ($P < 0.01$). In (**C**) the effects of PPARα and of PPARα + RXRα were all statistically significant ($P < 0.02$), as were the effects of ETYA ($P < 0.02$) and fenofibrate ($P < 0.05$).

libitum. Mice were killed at the midpoint of the light or dark cycle and their livers removed and frozen immediately in liquid N_2. The livers were ground to a powder under liquid N_2 and portions were used to prepare total RNA by the acid guanidine thiocyanate method [34]. Cyp7a1 mRNA was assayed by reverse transcription [35] followed by real-time PCR using an ABI PRISM Sequence Detection System (PE Applied Biosystems, Foster City, CA, U.S.A.) with the oligonucleotides 5′-TCTCTGAAGCCATGATGCAAA-3′ and 5′-TGACCCAGACAGCGCTCTT-3′ as primers and 5′-FAM-CAATCTGTCATGAGACCTCCGGGCCT-TAMRA-3′ as the fluorescent probe (FAM is carboxyfluorescein and TAMRA is *N′*,*N′*,*N′*,*N′*-tetramethyl-6-rhodamine). Reactions took place in 30 μl of TaqMan Universal PCR Master Mix (PE Applied Biosystems) containing 300 nM of each primer and 200 nM probe under the standard conditions recommended by the manufacturer. Primers (5′-GAGCTATGAGCTGCCTGACG-3′, 5′-AGTTTCATGGATGCCACAGGA-3′ 67 nM each) and probe (5′-VIC-CATCACTATTGGCAACGAGCGGTTCC-TAMRA-3′ 85 nM) for β-actin were included to provide an internal standard, and all values were related to a standard liver preparation.

RESULTS

The immediate promoter of human *CYP7A1* was cloned upstream of the luciferase gene. This reporter gene was transfected into HepG2 cells together with expression vectors for PPARα and RXRα. Promoter activity was decreased by approx. 40 % on co-transfection with PPARα (Figure 1A). Co-transfection with RXRα had little effect alone, but augmented the effect of PPARα. A vector in which the PPARα gene was reversed had no effect. To check that this could not be explained by an inhibition of luciferase expression or activity, transfections were performed at the same time with a construct containing the ACO promoter (Figure 1B). Transfection with PPARα increased ACO promoter activity, as expected. RXRα and the reversed vector had little effect.

Activators of PPARα, such as ETYA and fenofibrate, decreased activity in HepG2 cells transfected with the *CYP7A1* reporter gene alone and also enhanced the effects of co-transfected PPARα (Figure 1C). The inhibitory effect of transfected PPARα was dose-dependent (Figure 2A). A 50 % inhibition was obtained with approx. 3 μg/ml expression vector in the absence of activators and with about 1 μg/ml of vector in their presence. A

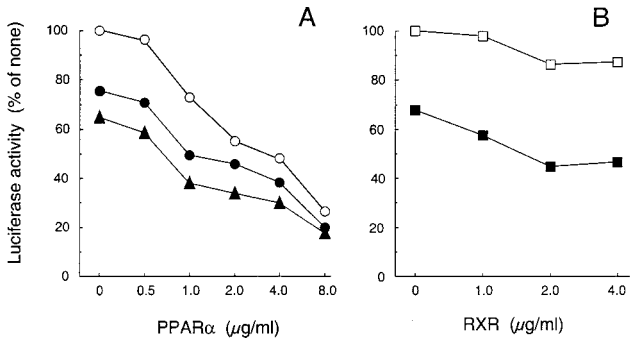

Figure 2 Concentration-dependence of PPARα and RXRα on inhibition of *CYP7A1* transcriptional activity

HepG2 cells were transfected with the *CYP7A1* reporter gene construct. (**A**) The effect of co-transfecting with increasing concentrations of PPARα expression vector. Cells were incubated with 20 μM ETYA (●), 250 μM fenofibrate (◆) or with no activator (○) for 24 h before harvesting. (**B**) The effect of co-transfecting with increasing concentrations of RXRα vector with (■) and without (□) the PPARα expression vector (4 μg/ml). Results are presented as a percentage of the promoter activity observed with no additions and are the means of values from triplicate wells. Similar, though less detailed, results were obtained in other independent experiments.

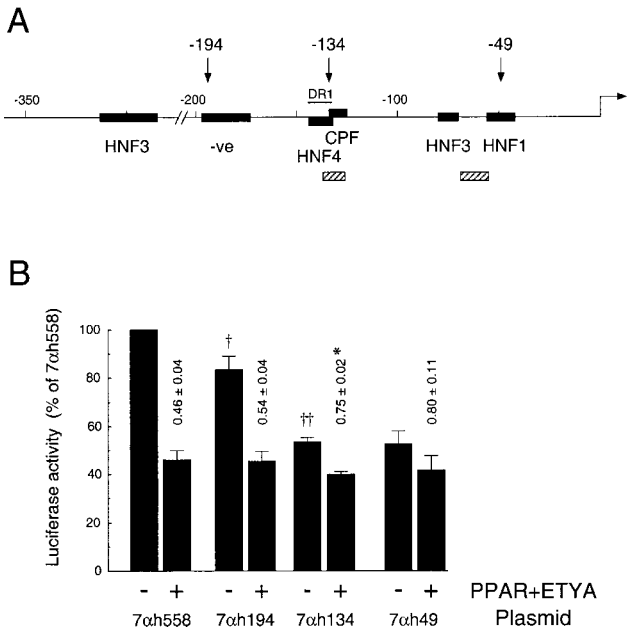

Figure 3 Localization of the inhibitory effect of PPARα on *CYP7A1* transcriptional activity

(**A**) Diagram of the human *CYP7A1* promoter region showing the positions of the enzyme cuts used to make the deleted promoter constructs, and their relationship to the DR1 site, to the sites known to bind the transcription factors HNF1, HNF3, HNF4 and CPF on the human promoter and to the sites that bind COUP TF in the rat promoter (hatched bars). (**B**) Transcriptional activity of equivalent amounts of constructs containing progressively shorter regions of the promoter transfected into HepG2 cells. Where indicated (+) cells were co-transfected with expression vectors containing PPARα (2 μg/ml) and RXRα (1 μg/ml) and were incubated for 24 h with 20 μM ETYA before harvesting. Constructs used contained the region from position +63 to the (−) number shown. Results are means ± S.E.M. of results from three independent experiments. Activity values for each experiment were expressed as a percentage of that given with the pGL3-7αh588 construct alone: †$P < 0.05$, pGL3-7αh194 versus pGL3-7αh588; ††$P < 0.01$, pGL3-7αh134 versus pGL3-7αh194. The proportion present after treatment with PPARα, RXR and ETYA is shown vertically above the appropriate column: *$P < 0.01$, pGL3-7αh134 versus pGL3-7αh194.

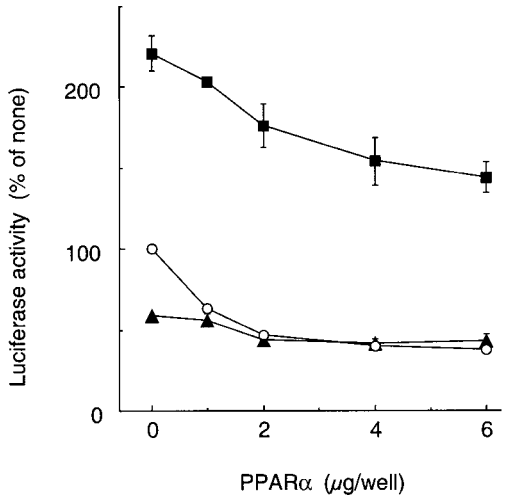

Figure 4 Effect of PPARα and HNF4 on the transcriptional activity of reporter gene constructs

HepG2 cells were transfected with the pGL3-7αh588 construct (○) or the same construct mutated at positions −140 to −138 within the DRI region (◆), and were co-transfected with RXRα expression vector (2 μg/ml) and increasing amounts of PPARα expression vector, as shown. In some wells the pGL3-7αh588 construct was co-transfected with an expression vector for HNF4 (■). Results in each experiment were expressed as a percentage of that given by the pGL3-7αh588 construct without PPARα and are shown as means ± S.E.M. for four independent experiments. Values without PPARα were significantly different from each other ($P < 0.0005$).

concentration of 2 μg/ml RXRα was enough to elicit the maximal effect of PPARα (Figure 2B). An inhibition of reporter-gene activity of approx. 80% could be achieved with the highest concentrations of PPARα in the presence of RXRα and activators.

To discover which areas of the *CYP7A1* promoter were responsible for the inhibitory effect of PPARα, reporter-gene constructs were made with progressively smaller fragments of the region (Figure 3). In these experiments, activity driven by the first 588 bp upstream of the transcription start site was decreased by 54% by PPARα and ETYA. Deletion of the bases upstream of position −194 slightly decreased activity, but did not significantly affect the inhibition by PPARα. However, further deletion to position −134, which removed the DR1 site (Figure 3A), significantly decreased both the promoter activity and its inhibition by PPARα (Figure 3B). Deletion to position −49 had surprisingly little effect on promoter activity in view of the loss of potential HNF3- and HNF1-binding sites, and had no further effect on inhibition by PPARα.

The results from these experiments indicated that part, at least, of the effect of PPARα on *CYP7A1* promoter activity was mediated by the DR1 site around position −138. Further evidence that PPARα was acting at this site came from experiments in which the DR1 sequence was mutated. The construct containing the mutated sequence showed greatly decreased promoter activity and a much smaller effect of PPARα (Figure 4). It is known from previous work that this DR1 sequence binds the liver-specific transcription factor HNF4 [13,14]. Co-transfection of the reporter construct with an expression vector for HNF4 increased promoter activity over 2-fold (Figure 4). In the presence of the over-expressed HNF4, the ability of PPARα to decrease promoter activity was greatly diminished. Thus, in the absence of added HNF4, transfection with 2 μg/ml PPARα vector produced an almost maximal 60% inhibition of activity, whereas in the presence of extra HNF4 it produced an inhibition of only 20% (Figure 4).

To discover if PPARα bound directly to the DR1 element in the human *CYP7A1* promoter, gel-mobility-shift assays were performed with PPARα and RXRα synthesized *in vitro* using a reticulocyte-lysate system. A mixture of PPARα and RXRα produced a single shifted complex when incubated with a labelled oligonucleotide containing a known PPARα response element (Figure 5, lane 1). The appearance of the radioactive band was abolished in the presence of the same non-radioactive oligonucleotide (Figure 5, lane 2), but not in the presence of a non-radioactive oligonucleotide encompassing the DR1 region of the human *CYP7A1* promoter (Figure 5, lane 3). When labelled, the promoter probe containing the DR1 sequence did not produce a shifted complex of the same size when incubated with the PPARα and RXRα mixture (Figure 5, lane 5). The only bands observed were smaller and were produced by the reticulocyte-lysate system alone (Figure 5, lane 8).

The availability of mice in which the PPARα gene has been disrupted has made it possible to assess whether an effect observed *in vitro* has any impact under physiological conditions. The expression of the *cyp7a1* gene in mice follows a diurnal variation

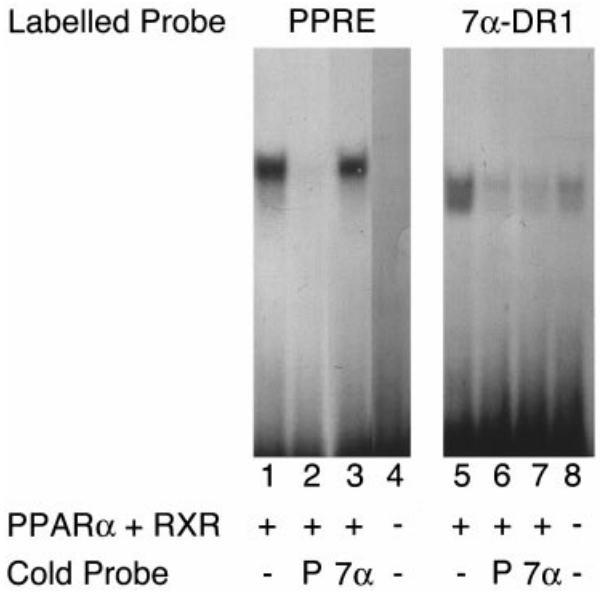

Figure 5 Gel-mobility-shift assays with the DR1 region of the human *CYP7A1* promoter

In-vitro-synthesized PPARα and RXRα were incubated with a radioactive oligonucleotide containing a known PPARα response element (PPRE, lanes 1–4) or with a radioactive oligonucleotide corresponding to positions −160 to −125 of the human *CYP7A1* promoter (7α-DR1, lanes 5–8), with or without an excess of non-radioactive ('Cold Probe') PPRE (P) or 7α-DR1 (7α) and were separated by PAGE. Lanes 4 and 8 contained labelled oligonucleotide incubated with the translation system alone.

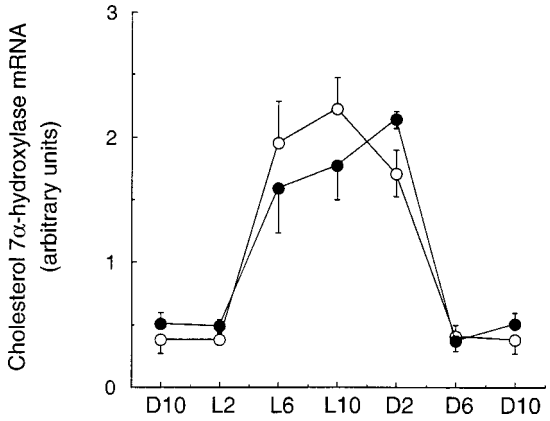

Figure 6 Diurnal rhythm of Cyp7a1 mRNA content of livers from normal (○) and PPARα-null (●) mice

Mice were killed at various times during the diurnal cycle, expressed as the number of hours into the dark (D) or light (L) phase. Total RNA was extracted from their livers and assayed for Cyp7a1 mRNA as described in the Experimental section. Results are shown as means ± S.E.M. of the values from 12 animals at D6 or L6 and from four animals at all other times.

with a peak during the light phase (Figure 6). The abundance of Cyp7a1 mRNA in the livers of PPARα-null mice during this period were essentially identical at all points of the diurnal cycle. To assess whether PPARα is involved in the response of the *cyp7a1* gene to cholesterol, animals were placed on a diet supplemented with 2% cholesterol for 7 days. Hepatic Cyp7a1 mRNA content at the D6 time point (6 h into the dark period) increased from 0.345 ± 0.068 to 0.804 ± 0.198 in control animals and from 0.475 ± 0.122 to 1.030 ± 0.176 in PPARα-null animals [means (arbitrary units) ± S.E.M. for six mice]. The increases were statistically significant ($P < 0.05$; Student's *t*-test) and were of the same order (2.33-fold and 2.18-fold respectively) in the control and PPARα-null mice.

DISCUSSION

The results presented here show that the transcription of the human *CYP7A1* gene can be decreased by PPARα in HepG2 cells *in vitro*. This inhibition appeared to be specific, since a construct containing the reversed PPARα sequence was without effect. Some inhibition was obtained with PPARα activators alone, indicating that the cells contained appreciable amounts of PPARα. However, this was not enough to elicit a maximum response, which required co-transfection with a PPARα expression vector. The over-expressed PPARα was inhibitory by itself, suggesting that the cells also contained endogenous activators. An inhibition of 75–80% was achieved in these experiments, but, since it is not possible to be sure of the true basal value, because of the presence of endogenous PPARα and its activators, the potential effect of PPARα may be significantly greater than this.

The region of the promoter responsible for the inhibition of human Cyp7α1 activity by PPARα was located between −134 and −194 bp upstream from the transcription start site. This contains a previously identified DR1 sequence as well as other possible sites for the binding of nuclear hormone receptors. The region further downstream (−70 to −50 bp from the transcription start site), containing a possible DR4 binding site, was not implicated in these effects. The involvement of the DR1 site in the response to PPARα was confirmed by mutagenesis. Mutating part of the DR1 sequence greatly decreased the effect of PPARα. It also lowered the basal transcriptional activity, suggesting that it had decreased the effect of some activating transcription factor. This was unlikely to be COUP TFII or CPF, since the mutation was outside the region to which these factors would be predicted to bind. However, the site was known to bind HNF4 [13,14], so the clear inference was that PPARα somehow affected the action of HNF4. If this were so, it would be expected that HNF4 would compete with PPARα, and this was found to be the case. The inhibitory effect of PPARα was greatly decreased at each concentration tested when the cells were co-transfected with an expression vector for HNF4. However the PPARα could not have competed with HNF4 for binding to the promoter itself. Despite extensive investigation using *in vitro* synthesized PPARα and RXRα, as well as nuclear extracts from cells expressing these factors (results not shown), we could detect no binding of the PPARα/RXRα dimers to DNA sequences contained in the region between −160 and −125 bp from the transcription start site. We could also confirm that the downstream −70 to −50 bp region of the human *CYP7A1* promoter did not bind PPARα/RXRα dimers and did not bind LXRα or LXRβ (results not shown). In each case the transcription factors bound to oligonucleotides containing known PPAR- or LXR-binding sequences.

HNF4 binds to DNA as a homodimer, so there is no potential for competition between HNF4 and PPARα for a common binding partner. It is possible, however, that they could compete for cofactors required to transduce their response, such as the p160 family of co-activators and the DRIP–TRAP complexes [36]. Investigation of these cofactor complexes is still in its infancy, and it is not possible at this stage to pinpoint any likely mechanism. To some extent this is immaterial, since the effects observed *in vitro* were not apparently reproduced *in vivo*. The

abundance of Cyp7a1 mRNA in the livers of mice in which the PPARα gene had been disrupted was the same as that in the livers of control mice. This was so both during the dark phase, when the animals were feeding, and during the light phase, when mRNA abundance was greatly increased. In these experiments the animals were fed a standard carbohydrate-rich diet, and it remains possible that normal animals on a regimen higher in polyunsaturated fat could show a greater response, revealing some difference from the PPARα-null mice. However, there is no indication from the current data that PPARα competes with HNF4 or with any other transcription factor *in vivo* to affect the transcription of the *cyp7a1* gene or its response to the normal pattern of food intake. There was also no evidence for the involvement of PPARα in the stimulation of Cyp7a1 activity by cholesterol. The increase in the hepatic abundance of Cyp7a1 mRNA on cholesterol feeding was the same in the PPARα-null animals as in the controls. These results were obtained with mice, but it is likely that they also apply to humans. The human *CYP7A1* promoter contains no extra potential PPARα-binding sites and the concentration of cofactors would not be expected to be greatly different. Indeed, any effect of PPARα activators in humans would be predicted to be less than in rodents, since human liver contains a much lower concentration of PPARα [37].

The discrepancy between the results obtained *in vitro* and the effects observed in whole animals is probably a reflection of a suboptimal concentration of the stimulating factor, presumed to be HNF4, in the HepG2 cells used for these experiments. Although the cells contained some of the factor, as evidenced by the decrease in activity on mutating the DR1 site, activity was greatly increased on transfection with an expression vector for HNF4. Thus the results can be explained by an inability of the limited amount of HNF4 in HepG2 cells to compete with PPARα for various cofactors, allowing PPARα to exert an effect which is not normally apparent in untransformed hepatoctes. Overall these experiments indicate that transcription of the Cyp7a1 gene can be influenced by factors that interfere with the action of a positive factor, probably HNF4, at a DR1 site in the promoter, but that PPARα is unlikely to be responsible for regulating the activity of the gene under normal physiological circumstances.

REFERENCES

1 Myant, N. B. and Mitropoulos, K. A. (1977) Cholesterol 7α-hydroxylase. J. Lipid Res. **18**, 135–153
2 Russell, D. W. and Setchell, K. D. (1992) Bile acid biosynthesis. Biochemistry **31**, 4737–4749
3 Ishibashi, S., Schwarz, M., Frykman, P. K., Herz, J. and Russell, D. W. (1996) Disruption of cholesterol 7α-hydroxylase gene in mice. I. Postnatal lethality reversed by bile acid and vitamin supplementation. J. Biol. Chem. **271**, 18017–18023
4 Spady, D. K., Cuthbert, J. A., Willard, M. N. and Meidell, R. S. (1995) Adenovirus-mediated transfer of a gene encoding cholesterol 7α-hydroxylase into hamsters increases hepatic enzyme activity and reduces plasma total and low density lipoprotein cholesterol. J. Clin. Invest. **96**, 700–709
5 Wang, J., Freeman, D. J., Grundy, S. M., Levine, D. M., Guerra, R. and Cohen, J. C. (1998) Linkage between cholesterol 7α-hydroxylase and high plasma low-density lipoprotein cholesterol concentrations. J. Clin. Invest. **101**, 1283–1291
6 Li, Y. C., Wang, D. P. and Chiang, J. Y. (1990) Regulation of cholesterol 7α-hydroxylase in the liver. Cloning, sequencing, and regulation of cholesterol 7α-hydroxylase mRNA. J. Biol. Chem. **265**, 12012–12019
7 Pandak, W. M., Li, Y. C., Chiang, J. Y., Studer, E. J., Gurley, E. C., Heuman, D. M., Vlahcevic, Z. R. and Hylemon, P. B. (1991) Regulation of cholesterol 7α-hydroxylase mRNA and transcriptional activity by taurocholate and cholesterol in the chronic biliary diverted rat. J. Biol. Chem. **266**, 3416–3421
8 Molowa, D. T., Chen, W. S., Cimis, G. M. and Tan, C. P. (1992) Transcriptional regulation of the human cholesterol 7α-hydroxylase gene. Biochemistry **31**, 2539–2544
9 Hylemon, P. B., Gurley, E. C., Stravitz, R. T., Litz, J. S., Pandak, W. M., Chiang, J. Y. and Vlahcevic, Z. R. (1992) Hormonal regulation of cholesterol 7α-hydroxylase mRNA levels and transcriptional activity in primary rat hepatocyte cultures. J. Biol. Chem. **267**, 16866–16871
10 Stravitz, R. T., Hylemon, P. B., Heuman, D. M., Hagey, L. R., Schteingart, C. D., Ton, N. H., Hofmann, A. F. and Vlahcevic, Z. R. (1993) Transcriptional regulation of cholesterol 7α-hydroxylase mRNA by conjugated bile acids in primary cultures of rat hepatocytes. J. Biol. Chem. **268**, 13987–13993
11 Crestani, M., Stroup, D. and Chiang, J. Y. (1995) Hormonal regulation of the cholesterol 7α-hydroxylase gene (CYP7). J. Lipid Res. **36**, 2419–2432
12 Spady, D. K., Cuthbert, J. A., Willard, M. N. and Meidell, R. S. (1996) Feedback regulation of hepatic 7α-hydroxylase expression by bile salts in the hamster. J. Biol. Chem. **271**, 18623–18631
13 Cooper, A. D., Chen, J., Botelho-Yetkinler, M. J., Cao, Y., Taniguchi, T. and Levy-Wilson, B. (1997) Characterization of hepatic-specific regulatory elements in the promoter region of the human cholesterol 7α-hydroxylase gene. J. Biol. Chem. **272**, 3444–3452
14 Crestani, M., Sadeghpour, A., Stroup, D., Galli, G. and Chiang, J. Y. (1998) Transcriptional activation of the cholesterol 7α-hydroxylase gene (CYP7A) by nuclear hormone receptors. J. Lipid Res. **39**, 2192–2200
15 Goodart, S. A., Huynh, C., Chen, W., Cooper, A. D. and Levy-Wilson, B. (1999) Expression of the human cholesterol 7α-hydroxylase gene in transgenic mice. Biochem. Biophys. Res. Commun. **266**, 454–459
16 Chiang, J. Y. and Stroup, D. (1994) Identification and characterization of a putative bile acid-responsive element in cholesterol 7α-hydroxylase gene promoter. J. Biol. Chem. **269**, 17502–17507
17 Stroup, D., Crestani, M. and Chiang, J. Y. (1997) Identification of a bile acid response element in the cholesterol 7α-hydroxylase gene CYP7A. Am. J. Physiol. **273**, G508–G517
18 Stroup, D., Crestani, M. and Chiang, J. Y. (1997) Orphan receptors chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) and retinoid X receptor (RXR) activate and bind the rat cholesterol 7α-hydroxylase gene (CYP7A). J. Biol. Chem. **272**, 9833–9839
19 Lehmann, J. M., Kliewer, S. A., Moore, L. B., Smith, O. T., Oliver, B. B., Su, J. L., Sundseth, S. S., Winegar, D. A., Blanchard, D. E., Spencer, T. A. and Willson, T. M. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J. Biol. Chem. **272**, 3137–3140
20 Wang, H., Chen, J., Hollister, K., Sowers, L. C. and Forman, B. M. (1999) Endogenous bile acids are ligands for the nuclear receptor FXR/BAR. Mol. Cell **3**, 543–553
21 Stroup, D. and Chiang, J. (2000) HNF4 and COUP-TFII interact to modulate transcription of the cholesterol 7α-hydroxylase gene (CYP7A1). J. Lipid Res. **41**, 1–11
22 Chen, J., Cooper, A. D. and Levy-Wilson, B. (1999) Hepatocyte nuclear factor 1 binds to and transactivates the human but not the rat CYP7A1 promoter. Biochem. Biophys. Res. Commun. **260**, 829–834
23 Nitta, M., Ku, S., Brown, C., Okamoto, A. Y. and Shan, B. (1999) CPF: an orphan nuclear receptor that regulates liver-specific expression of the human cholesterol 7α-hydroxylase gene. Proc. Natl. Acad. Sci. U.S.A. **96**, 6660–6665
24 Lemberger, T., Desvergne, B. and Wahli, W. (1996) Peroxisome proliferator-activated receptors: a nuclear receptor signaling pathway in lipid physiology. Annu. Rev. Cell Dev. Biol. **12**, 335–363
25 Schoonjans, K., Staels, B. and Auwerx, J. (1996) Role of the peroxisome proliferator-activated receptor (PPAR) in mediating the effects of fibrates and fatty acids on gene expression. J. Lipid Res. **37**, 907–925
26 Issemann, I. and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature (London) **347**, 645–650
27 Lee, S. S., Pineau, T., Drago, J., Lee, E. J., Owens, J. W., Kroetz, D. L., Fernandez, S. P., Westphal, H. and Gonzalez, F. J. (1995) Targeted disruption of the α isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. Mol. Cell. Biol. **15**, 3012–3022
28 Bertolotti, M., Concari, M., Loria, P., Abate, N., Pinetti, A., Guicciardi, M. E. and Carulli, N. (1995) Effects of different phenotypes of hyperlipoproteinemia and of treatment with fibric acid derivatives on the rates of cholesterol 7α-hydroxylation in humans. Arterioscler. Thromb. Vasc. Biol. **15**, 1064–1069
29 Ren, B., Thelen, A. and Jump, D. B. (1996) Peroxisome proliferator-activated receptor alpha inhibits hepatic S14 gene transcription. Evidence against the peroxisome proliferator-activated receptor alpha as the mediator of polyunsaturated fatty acid regulation of S14 gene transcription. J. Biol. Chem. **271**, 17167–17173
30 Miyata, K. S., McCaw, S. E., Patel, H. V., Rachubinski, R. A. and Capone, J. P. (1996) The orphan nuclear hormone receptor LXRα interacts with the peroxisome proliferator-activated receptor and inhibits peroxisome proliferator signalling. J. Biol. Chem. **271**, 9189–9192

31 Wade, D. P., Puckey, L. H., Knight, B. L., Acquati, F., Mihalich, A. and Taramelli, R. (1997) Characterization of multiple enhancer regions upstream of the apolipoprotein(a) gene. J. Biol. Chem. **272**, 30387–30399
32 Tugwood, J. D., Issemann, I., Anderson, R. G., Bundell, K. R., McPheat, W. L. and Green, S. (1992) The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl CoA oxidase gene. EMBO J. **11**, 433–439
33 Kliewer, S. A., Umesono, K., Noonan, D. J., Heyman, R. A. and Evans, R. M. (1992) Convergence of 9-*cis*-retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature (London) **358**, 771–774
34 Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. **162**, 156–159
35 Sambrook, J., Frisch, E. F. and Maniatis, T. (1989) Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
36 Freedman, L. P. (1999) Increasing the complexity of coactivation in nuclear receptor signaling. Cell **97**, 5–8
37 Tugwood, J. D., Aldridge, T. C., Lambe, K. G., Macdonald, N. and Woodyatt, N. J. (1996) Peroxisome proliferator-activated receptors: structures and function. Ann. N.Y. Acad. Sci. **804**, 252–265

Received 5 June 2000/24 July 2000; accepted 22 August 2000