Identification and characterization of DNA elements implicated in the regulation of CYP4A1 transcription

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We have identified a peroxisome proliferator response element (PPRE) approx. 4300 nucleotides upstream of the rat cytochrome *P*-450 CYP4A1 gene. Two members of the steroid-hormone-receptor superfamily, the peroxisome proliferator-activated receptor- α (PPAR α) and the retinoid X receptor- α (RXR α), bind specifically to this element as a heterodimer, and this element confers responsiveness to the peroxisome proliferator Wyeth-14,643 when tested in co-transfection assays. A second element, located 35 nucleotides further upstream, fails to bind PPAR α /RXR α heterodimers and is unresponsive to Wy-14,643 in co-transfection assays. Both elements are, however, responsive to 9-*cis*-retinoic acid in the presence of RXR α , when tested in the

co-transfection assay. As RXR α fails to bind to either element as a homodimer, we suggest that RXR α interacts with PPAR α to regulate transcription via the proximal element, and interacts with some other cellular factor to regulate transcription via the more distal element. This is consistent with previous reports that a number of peroxisome proliferator-regulated genes contain PPRE-like elements as part of their regulatory sequences, which may be recognized by several receptor combinations. This provides further evidence that PPARs and their co-factors are important in mediating the pleiotropic action of peroxisome proliferators.

INTRODUCTION

The rat liver microsomal enzyme cytochrome *P*-450 (P450) IVA1 (CYP4A1) is involved in the hydroxylation of fatty acids and the metabolism of a range of xenobiotics [1,2]. Transcription of the CYP4A1 (4A1) gene is induced in the liver of rodents administered with a class of compounds termed peroxisome proliferators [3]. These non-genotoxic rodent carcinogens encompass a diverse range of compounds including plasticizers and fibrate hypolipidaemic drugs (for reviews, see [4–7]). The rapid induction of CYP4A1 [8] is followed by liver hypertrophy and hyperplasia, and an increase in the size and number of hepatic peroxisomes (peroxisome proliferation). Prolonged administration of these compounds results in pathological changes in the liver and eventually hepatocellular carcinoma. Induction of CYP4A1 is co-ordinated with the transcription of genes encoding enzymes of the peroxisomal β -oxidation pathway, namely the gene for the bifunctional enzyme acyl-CoA oxidase (ACO) and that for 3-oxoacyl-CoA thiolase [9–11]. As CYP4A1 induction precedes the peroxisomal response, it is perhaps the most important enzymic marker of peroxisome proliferation [3,12]. The pleiotropic effects of peroxisome proliferators are thought to be mediated by a class of nuclear receptors termed the ‘peroxisome-proliferator-activated receptors’ (PPARs) [13]. These closely related receptors are members of the steroid-hormone-receptor superfamily of ligand-activated transcription factors and have been isolated from a range of species [13–16]. As no natural ligands have yet been found for these PPARs, their physiological role is unclear, although it has been suggested that they may be involved in lipid homeostasis, since they can be activated by a variety of fatty acids [15,17].

We have shown previously that the transcriptional activation of the rat peroxisomal ACO gene is directly regulated by PPAR α , which binds to a regulatory sequence (PPRE) consisting of an

almost perfect direct repeat of the sequence TGACCT spaced by a single base-pair [18]. Other peroxisome proliferator-inducible genes, which include the rabbit CYP4A6 and rat bifunctional enzyme, have since been shown to possess PPREs closely resembling that of ACO [19–21]. It has been demonstrated that PPAR α and the retinoid X receptor (RXR) [22] bind as a heterodimer to these PPREs, thereby regulating gene expression [14,21 23–27]. Since peroxisome proliferators co-ordinately regulate the transcription of the CYP4A1 and peroxisomal β -oxidation-enzyme genes, it is possible that they are all regulated in a similar fashion. We therefore examined the upstream sequence of the rat CYP4A1 gene for the existence of a PPRE.

We report here the presence of two closely linked elements located approx. 4300 bp upstream of the transcriptional initiation site of the rat CYP4A1 gene, and demonstrate transcriptional activation by both these elements in the presence of ligand-activated PPAR α and/or RXR α . The co-operative binding of PPAR α and RXR α to one of these elements is also described.

MATERIALS AND METHODS

Isolation of the CYP4A1 genomic sequence

A CYP4A1 probe was produced using 25 PCR cycles [28] to amplify a region of upstream sequence (–1002/+20) from rat genomic DNA. Oligonucleotides (24-mers) used as the PCR primers were derived from the published rat CYP4A1 gene sequence [29]. A rat genomic library (Stratagene) was then screened using this probe, under stringent hybridization conditions [30]. One positive clone was isolated from the screening of 750 000 phages. This clone (λ 4A1) was characterized by restriction-enzyme digestion and partial DNA sequence analysis and compared with the published sequence of the CYP4A1 gene [29]. Clone λ 4A1 overlaps by 2200 bp with the published sequence

Abbreviations used: P450, cytochrome *P*-450; 4A1, P450 CYP4A1; ACO, acyl-CoA oxidase [bifunctional enzyme (enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase)]; PPAR, peroxisome-proliferator-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; CAT, chloramphenicol acetyltransferase; TREp, thyroid-hormone response element; DMSO, dimethyl sulphoxide.

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and extends approximately a further 14 kb upstream (−15000/+1200). Partial DNA sequence from the 3' end of clone λ 4A1 shows 100% sequence identity with that of the CYP4A1 gene (results not shown). DNA sequence was derived from both strands, using the chain-termination method of Sanger et al. [31].

Plasmid constructions

Subfragments of the upstream region of the 4A1 gene were generated either by restriction-endonuclease cleavage or by PCR amplification of the genomic clone λ 4A1 using oligonucleotides with restriction sites at their termini. These fragments were then inserted into the reporter plasmid pG.CAT [13], which contains the rabbit β -globin (G) promoter linked to the chloramphenicol acetyltransferase (CAT) gene, or pCAT in which the globin promoter had been deleted. p4A1(−1016/+20)CAT was generated by PCR amplification using primers with *Hind*III(5') and *Bam*HI(3') cleavage sites, enabling the fragment to be subcloned into pCAT. p4A1(*Bam*/+20)CAT was generated by inserting a 5 kb *Bam*HI genomic fragment into p4A1(−1016/+20) at the natural *Bam*HI site at position −1016. All other restriction fragments were inserted into pG.CAT. p4A1(*Bgl*/*Sma*)G.CAT and its deletion derivatives were generated by PCR amplification (10 cycles) with p4A1(*Bgl*/−1016)G.CAT as the template. Oligonucleotides used as primers possessed either *Hind*III(5') or *Bam*HI(3') sites, enabling directional cloning into *Hind*III/*Bam*HI-cleaved pG.CAT. All the above constructs were verified by DNA sequencing of the vector/insert junctions.

It should be noted that the nucleotide sequence of the *Bgl*II/*Sma*I fragment has been numbered with the *Bgl*II site being at position 1 and the *Sma*I site at position 1063. This format has been adopted because the DNA sequence has been determined for this fragment only.

Plasmid p4A1(171/218)G.CAT was constructed by cloning the following synthetic oligonucleotides into *Hind*III/*Bam*HI-cleaved pG.CAT:

5'-agctTCCACAGTCTGAGTCCTAGGTCAGAGGGGAA-
GAGTACAATGTAGATAG-3'

and its complement:

5'-gacTCTATCTACATTGTACTCTTCCCCTCTGACCTAG-
GACTCAGACTGTGGA-3'

[nucleotides shown in lower case were included to generate *Hind*III(5') and *Bam*HI(3') cohesive termini].

p4A1(226/255)G.CAT was constructed as described above using the following oligonucleotides:

5'-agcttAGGCATGAACTAGGGTAAAGTTTCAGTGAG-3'

and its complement:

5'-gacTCTACTGAACTTTACCCTAGTTTCATGCCTa-3'

The expression plasmid pSG5.PPAR α , which contains the complete coding sequence of the mouse PPAR α gene, and the ACO reporter plasmids pACO(−1273/−471)G.CAT and pACO(−605/−473)G.CAT have been described previously [18]. The expression vector pSG5.RXR α , which contains the mouse RXR α coding sequence, was a gift from Professor P. Chambon (LGME/CNRS, INSERM, Faculté de médecine, Strasbourg, France). The β -galactosidase expression plasmid pCH110 was obtained from Pharmacia, and the plasmid pBluescript SK- from Stratagene. All plasmid DNAs were purified using Qiagen columns (Hybaid).

Transient co-transfection assays

Hepa1c1c7 cells were grown and transfected with 10 μ g of DNA by the lipofectin method using *N*-[1-(2,3-dioleoyloxy)propyl]-*NNN*-trimethylammonium chloride as described previously [18]. Briefly, cells at 50–80% confluence in 9 cm-diameter Petri dishes were transfected with 1 μ g of 4A1 reporter plasmid, 1 μ g of PPAR expression vector, 3 μ g of the internal control pCH110 and 5 μ g pBluescript as carrier. After 5 and 24 h, either dimethyl sulphoxide (DMSO), Wyeth-14,643 (ChemSyn Science Laboratories, Lenexa, KS, U.S.A.) at 10^{-8} M final concentration and/or 9-*cis*-retinoic acid (Hoffmann-La Roche) at 10^{-6} M final concentration (both prepared at 1000 $\times$ in DMSO), was added to the cells in fresh medium. Cells were harvested after 48 h and normalized for β -galactosidase activity. Each transfection was performed in triplicate.

CAT assays

CAT enzyme activity was determined as described previously [18]. Data quantification was performed by scanning the chromatograms using an Autograph scanner (Saxon Micro). The percentage CAT acetylation was calculated and normalized to CAT induction observed with the control reporter plasmid pACO(−1273/−471)G.CAT, tested in the presence and absence of Wyeth-14,643. Results are mean $\pm$ S.D. ($n = 3$). Statistical significance was determined using the Student *t* test.

Gel-retardation analysis

Double-stranded (annealed) oligonucleotides representing the 4A1 sequences from the upstream regions 171/218, 226/255 and the thyroid-hormone response element (TREP) [32], were used in gel-retardation analysis along with the *Hind*III/*Bam*HI restriction fragment excised from reporter plasmids p4A1(171/273)G.CAT. The double-stranded oligonucleotides and restriction fragment were labelled at their 3' ends using [α - 32 P]dCTP and the Klenow fragment of DNA polymerase I. PPAR α and RXR α receptor proteins were synthesised from the pSG5 expression vectors using the TNT coupled rabbit reticulocyte *in vitro* transcription/translation system (Promega). DNA binding was performed using approx. 75 fmol of PPAR α and/or RXR α protein in 10 mM Hepes, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 2.5 mM MgCl $_2$, 1 μ g of poly(dI-dC) and 10% glycerol in a reaction volume of 20 μ l. After incubation for 30 min at 25 $^{\circ}$ C, with or without ligand (Wy-14,643 and/or 9-*cis*-retinoic acid at 10^{-8} M final concn.), radiolabelled oligonucleotides (50000 c.p.m.; 200 fmol) and fragments (50000 c.p.m.; 2 pmol) were added and the incubation continued for a further 20 min at 25 $^{\circ}$ C. Reaction mixtures were loaded directly on to a 5% native polyacrylamide gel, which had been pre-run for 30 min, buffered in either 6.7 mM Tris/HCl (pH 7.5)/3.3 mM sodium acetate/1 mM EDTA for heterodimer binding, or 0.5 $\times$ TBE for RXR α homodimer binding [1 $\times$ TBE is 0.089 M Tris/borate (pH 8.3)/0.089 M boric acid/2 mM EDTA]. Electrophoresis was performed at 4 $^{\circ}$ C and at 40 mA for 1–2 h without buffer recirculation. After soaking for 10 min in 5% (v/v) glycerol, gels were dried and autoradiographed.

RESULTS

Transfection analysis

The reporter construct p4A1(−1016/+20)CAT, containing the CYP4A1 upstream fragment −1016 to +20, was co-transfected into the mouse hepatoma cell line, Hepa1c1c7, in the presence or

e 1 Localization of the CYP4A1 PPRE

infection analysis of 4A1 reporter plasmids containing large deletions. A restriction map CYP4A1 upstream sequence is shown at the top of the Figure. p4A1CAT and p4A1G.CAT constructs (see the Materials and methods) were tested for peroxisome proliferator-induced activation by co-transfection assays in Hepa1 cells. Percentage acetylated chloramphenicol was determined (right hand column) in the absence (-R/-Wy) or the presence (+Wy) of pSG5.PPAR α (1 μ g) and Wyeth 14,643 (10^{-5} M) and represents the mean $\pm$ S.D. Student *t* tests were performed on all data pairs, significant differences being marked by asterisk (**P* < 0.05; ***P* < 0.01). The 4A1 upstream region in each construct is shown on the left. (b) Transfection analysis of reporter plasmids containing small deletions. The *Bgl*II/*Sma*I fragment was tested in co-transfection assays, along smaller fragments generated using the PCR. Percentage acetylated chloramphenicol is shown on the right for each construct tested and represents the mean of three determinations $\pm$ S.D. Student *t* tests were performed on all data pairs, significant differences being marked by asterisk (**P* < 0.05; ***P* < 0.01). 4A1 regions tested are shown on the left. Nucleotide positions 1-1063 refer to the *Bgl*II/*Sma*I region. Data for -R/+Wy and -Wy variables have been omitted as no significant induction was observed with any of the reporter constructs tested. Abbreviation: G, rabbit β -globin promoter.

nance of the PPAR α expression vector, pSG5.PPAR α , and the rat peroxisome proliferator Wy-14,643. Activated PPAR α was unable to stimulate transcription from this plasmid (Figure 1a). Assuming the PPRE may be present further upstream, we constructed the plasmid p4A1(Bam/+20)CAT (see the Materials and methods section). This construct also failed to stimulate CAT activity (Figure 1a). As previous studies with the ACO gene have only shown CAT induction when the reporter plasmid possessed the rabbit β -globin promoter [18], we constructed two reporter plasmids, p4A1(-1016/-50)G.CAT and p4A1(Bam/

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1  AGATCTAGGAGGAAGCTGGGAGCAGATGGATATATCAAAACATGTTGTAT 50
   BglII

51  AAAATCTCAAAAAAAGCTCCAAAGAACAGTAAGTACAAAGCAGATGTAAAT 100

101 TCCAAAAGCTCTTTTACAATGTGTTCCCTCATTTTAGGGGTGAAAAATGGGG 150
    I
151 TTTTGGGTAAAGAGGGGAAATCCACAGTCTGAGTCTTAGGTCAGAGGGGA 200
    TCCAGT TCCcct
201 AGAGTACAATGTAGATAGAAAAGGCAGGCATGAACACTAGGGTAAAGTTCA 250
    TCCcct TCaAGT
251 GTGAGATGGAGTGTAAATTTTC 273

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Figure 2 Nucleotide sequence of the region containing the CYP4A1 PPRE

The sequence shown corresponds to the first 273 bp of the *Bgl*II/*Sma*I region. Nucleotide position 1 corresponds to the *Bgl*II-restriction-enzyme site. Two sequence motifs resembling the consensus PPRE-binding site are indicated (elements I and II). Nucleotides shown in lower case represent divergence from the consensus TGACCT half-site.

-50)G.CAT, and tested these in the same assay (Figure 1a). Again no significant CAT induction was observed. The deletion of sequences close to the transcriptional start site of the bifunctional enzyme gene stimulated greater induction of CAT activity in those constructs, perhaps due to the removal of inhibitory sequences [21]. As negative regulatory elements have been shown to be present in the promoter region of certain P450 genes [33], we constructed and analysed the reporter plasmid p4A1(Bgl/-1016)G.CAT. PPAR α in the presence of Wy-14,643 stimulated sufficient CAT activity to acetylate 4.5% of the labelled chloramphenicol, compared with the background level of 0.9%. This induction is statistically significant, with a *P* value of 0.014 determined by the Student *t* test (Figure 1a). In order to localize the PPRE, large deletions of both the 5' and 3' regions of p4A1(Bam/-1016)G.CAT were generated using convenient restriction-enzyme sites, and these reporter plasmids tested in the co-transfection assay with activated PPAR α . No significant induction of CAT activity was observed in the reporter plasmids with deletions of the *Bam*HI/*Sma*I region, p4A1(*Sma*/-1016)G.CAT, or the *Bgl*II/*Bam*HI region, p4A1(Bam/*Bgl*)G.CAT. Therefore the PPRE is located between the *Bgl*II and *Sma*I sites (nucleotide positions 1-1063).

To map more accurately the position of the response element, reporter plasmids containing smaller deletions of the *Bgl*II/*Sma*I region were made and tested in the co-transfection assay. The first of these, p4A1(1/1063)G.CAT, corresponding to the *Bgl*II/*Sma*I region, gave a 2-3-fold induction (Figure 1b). This level of activation was maintained with the sequence between 1 and 273 (Figure 1b). The larger regions 1-523 and 1-773 gave a slightly greater stimulation, 6- and 5-fold respectively, although this was due entirely to a lower level of CAT activity in the absence of PPAR and Wy-14,643 (Figure 1b). Regions 500-773 and 250-773 were inactive, localizing the PPRE within the region 1-250 (Figure 1b).

The DNA sequence of the *Bgl*II/*Sma*I region was examined for potential PPRE consensus sequences. Two possible PPRES were found within the 1-273 region at positions 188-200 and 238-250, designated elements I and II respectively (Figure 2).

To determine whether either or both of these elements were necessary to obtain the observed induction, three further reporter plasmids were tested in co-transfection assays with PPAR α in the presence of Wy-14,643. Region 171-273, containing both ele-

Figure 3 Localization of the CYP4A1 PPRE

Transfection analysis of reporter plasmids containing small 4A1 fragments. Reporter constructs with 4A1 regions corresponding to elements I and II, and their flanking sequences, were tested for CAT activity in the presence of pSG5, PPAR α and Wy-14,643. 4A1 regions tested are shown on the left, and percentage of chloramphenicol acetylated on the right. All values represent the mean of three determinations $\pm$ S.D. Student *t* tests were performed on all data pairs, significant differences being marked by asterisk (**P* < 0.05; ***P* < 0.01). Shaded areas indicate the positions of elements I and II. The sequence of both elements and their nucleotide positions are shown at the bottom. Lower-case nucleotides represent divergence from the consensus TGACCT half-site. Data for -R/+Wy and +R/-Wy variables have been omitted as no significant induction was observed with any of the reporter constructs tested. Abbreviation: G, rabbit β -globin promoter.

ments, gave 4–5-fold elevated induction (Figure 3). Significant induction (3–4-fold) was also observed with region 200–273 containing element II alone, but region 1–218, containing element I, showed no induction (Figure 3). To confirm this result, synthetic oligonucleotides corresponding to region 171–218 (element I) and 226–255 (element II) were placed upstream of the globin promoter of pG.CAT and these reporter plasmids tested in co-transfection assays with activated PPAR α . Significant induction was only observed with the reporter plasmid containing element II (Figure 3). These data suggest element II contains a functional PPRE, whereas element I does not. In order to determine whether RXR α in the presence of 9-*cis*-retinoic acid could stimulate transcription from either or both of the elements, further co-transfection assays were performed using the reporter plasmids p4A1(171/273)G.CAT which contains both elements I and II, p4A1(171/218)G.CAT (element I) and p4A1(226/255)G.CAT (element II). Co-transfection of p4A1(171/218)G.CAT (element I) with PPAR α either in the presence or absence of Wy-14,643 and/or 9-*cis*-retinoic acid showed no stimulation of activity (Figure 4a, lanes 5–8). However, when RXR α was co-transfected with the reporter 2.3-fold induction was observed in the presence of 9-*cis*-retinoic acid (lane 11), which increased slightly with the addition of Wy-14,643 (lane 12). No induction with Wy-14,643 alone was observed (lane 10). When the reporter was co-transfected with both PPAR α and RXR α the response to Wy-14,643 was slightly increased to 1.6-fold (lane 14). However, the response to both ligands was effectively the same as that observed with RXR α and 9-*cis*-retinoic acid alone (compare lanes 16 and 11). This result confirms that element I is non-responsive to activated PPAR α but can respond to RXR α alone in the presence of 9-*cis*-retinoic acid. This suggests that either RXR α homodimers or RXR α /co-factor heterodimers are capable of causing activation from this element.

Figure 4 CYP4A1 induction is receptor/ligand-dependent

Co-transfection of the 4A1 reporter constructs (a) p4A1(171/218)G.CAT, corresponding to element I, (b) p4A1(226/255)G.CAT (element II) and (c) p4A1(171/273)G.CAT (elements I + II) with pSG5, PPAR α , RXR α or PPAR α and RXR α together. Each combination was tested either with DMSO ('-Ligand'), Wy-14,643 (10^{-5} M final concn.), 9-*cis*-retinoic acid (10^{-6} M final concn.) or both ligands together. The y-axes show the percentage of chloramphenicol acetylated. All values represent the mean of three determinations, error bars showing the S.D. The values above each bar represents fold induction above background (pSG5/without ligand). Student *t* tests were performed on all data pairs, significant induction above background ('-Ligand') for each co-transfection being marked by asterisk (**P* < 0.05; ***P* < 0.01).

Co-transfection of the reporter p4A1(226/255)G.CAT (element II) with PPAR α alone produced a 1.5-fold increase in CAT activity, probably as a consequence of the presence of endogenous PPAR activators in the Hepa1c17 cells (Figure 4b, lane 5). The addition of Wy-14,643 produced a 3.2-fold increase (lane 6). Interestingly, a similar level of induction (2.9-fold) was observed with 9-*cis*-retinoic acid alone (lane 7). The addition of both ligands produced an enhanced effect, increasing the induction to 4.6-fold (lane 8). As expected Wy-14,643 had no effect

Figure 5 Gel-retardation analysis

(a) PPAR α and RXR α bind to element II, but not element I. Gel-retardation analysis was performed using *in vitro*-translated PPAR α and RXR α as described in the Materials and methods section. A 4A1 fragment corresponding to the region 171–273 (elements I and II) was tested with lysate alone (lane 1), PPAR α alone (lane 2), RXR α alone (lane 3) and PPAR α /RXR α in combination (lane 4). Double-stranded (annealed) oligonucleotides representing regions 171–218 (element I) and 226–255 (element II) were tested with lysate alone (lanes 5 and 9 respectively), PPAR α alone (lanes 6 and 10 respectively), RXR α alone (lanes 7 and 11 respectively) or with PPAR α /RXR α in combination (lanes 8 and 12 respectively). (b) RXR α homodimers do not bind to either element I or II. Gel-retardation analysis was performed using *in vitro*-translated RXR α as described in the Materials and methods section. Double-stranded (annealed) oligonucleotides representing the TREp (positive control) was tested in the absence of ligand with either lysate (lane 1) or with RXR α (lane 2) and in the presence of 9-*cis*-retinoic acid (10^{-5} M final concn.) with either lysate (lane 3) or with RXR α (lane 4), the TREp/RXR α complex indicated by the arrow. Double-stranded (annealed) oligonucleotides representing 4A1 regions 171–218 (element I) and 226–255 (element II) were also tested in the absence of ligand with either lysate (lanes 5 and 9 respectively) or with RXR α (lanes 6 and 10 respectively) and with 9-*cis*-retinoic acid with either lysate (lanes 7 and 11 respectively) or RXR α (lanes 8 and 12 respectively).

in combination with RXR α alone (lane 10), whereas 9-*cis*-retinoic acid produced a 2.4-fold stimulation (lane 11), which effectively remained unchanged by the further addition of Wy-14,643 (lane 12). When both PPAR α and RXR α were co-transfected with reporter in the presence of both Wy-14,643 and 9-*cis*-retinoic acid a 5.8-fold induction was observed (lane 16), which we interpret as an additive response of each receptor with its appropriate ligand (compare lanes 6 and 11 with lane 16). Element II therefore appears responsive to both PPAR α and RXR α .

When the reporter p4A1(171/273)G.CAT (elements I+II) was co-transfected with PPAR α , the overall pattern of induction was effectively the same as with element II, although greater levels of stimulation were observed (Figure 4c). When the reporter was co-transfected with PPAR α in the presence of Wy-14,643, a 6.1-fold stimulation was observed (lane 6), which increased very slightly to 6.6-fold with the further addition of 9-*cis*-retinoic acid (lane 8). With 9-*cis*-retinoic acid alone, stimulation was reduced to 4.4-fold (lane 7). When RXR α was co-transfected with the reporter, Wy-14,643 again had no effect on transcription (lane 10). 9-*cis*-Retinoic acid alone, however, stimulated transcription 6.4-fold (lane 11), which increased only slightly to 6.6-fold in the

presence of both ligands (lane 12). When both PPAR α and RXR α were co-transfected with reporter in the presence of Wy-14,643, a 12.0-fold induction was observed (lane 14), but this only represents a 2.0-fold increase above background. 9-*cis*-Retinoic acid increased stimulation to 13.2-fold (lane 15), while in the presence of Wy-14,643 and 9-*cis*-retinoic acid, induction increased to 18.6-fold (lane 16). The two elements together, therefore, have a synergistic effect on transcription levels.

The high background level of induction in the absence of ligand (6-fold, lane 13) accounts for the unusually high levels of induction observed.

These data confirm element II as a functional PPRE and show that both elements I and II can act as a response element for 9-*cis*-retinoic acid-activated RXR α .

DNA-binding studies

The ability of PPAR α and RXR α to bind to elements I and II was determined by performing gel-retardation analysis using *in vitro*-synthesized PPAR α and RXR α . Neither PPAR α nor RXR α alone were able to bind to a DNA fragment (region 171–273)

containing both elements I and II (Figure 5a, lanes 2 and 3). However, in the presence of both receptors, significant binding was observed (Figure 5a, lane 4). No binding to element I was observed in the presence of both PPAR α and RXR α (lane 8), whereas strong binding was seen with element II alone (lane 12), indicating that the second element alone is able to bind PPAR α /RXR α heterodimers. The binding of PPAR α /RXR α to the PPRE in the gel-retardation analysis was independent of ligand, the addition of Wy-14,643 and 9-*cis*-retinoic acid having no effect on the amount of complex formed or its mobility (results not shown).

The ability of RXR α homodimers to bind to elements I and II was then examined (Figure 5b). As RXR α requires the presence of 9-*cis*-retinoic acid to form homodimers in solution [32], gel-retardation analysis was performed using *in vitro*-synthesized RXR α which had been preincubated with 9-*cis*-retinoic acid. The palindromic TREp was used as a positive control in these experiments. In the absence of ligand RXR α did not bind effectively to the TREp (lane 2), but, with the addition of 9-*cis*-retinoic acid, strong binding was observed (lane 4). No binding to either element I or II was observed with or without the addition of 9-*cis*-retinoic acid (lanes 6, 8, 10 and 12).

DISCUSSION

We have identified a PPRE located approx. 4300 bp upstream of the rat CYP4A1 gene. The element consists of a pair of TGACCT-like motifs separated by a single base-pair, which represents the consensus binding site for a PPAR α /RXR α heterodimer [18,24]. As shown by gel-retardation analysis, PPAR α and RXR α bind to the CYP4A1 PPRE (element II) as a heterodimer. The transfection data have shown that peroxisome proliferator-induced transcription of 4A1 reporter plasmids is dependent on the presence of PPAR α and occurs via binding of PPAR α /RXR α heterodimers to the CYP4A1 PPRE (element II). RXR α activated by 9-*cis*-retinoic acid could also induce transcription from this element, with the effect of both PPAR α and RXR α in the presence of both ligands being additive. This result is similar to that observed with the ACO gene [14,17,23], but is in contrast with that of the bifunctional-enzyme gene [21], where no synergistic or additive effect was observed.

As no direct binding of RXR α to element II was observed in gel-retardation assays, one must assume that RXR α activates transcription from this element by binding as a heterodimer with co-factor(s) present in the Hepa1c1c7 cells. Interestingly, element I, located 35 bp upstream from the CYP4A1 PPRE also resembles the preferred PPRE sequence, having one perfect and one divergent half-site separated by a single base-pair. This element, however, did not bind PPAR α /RXR α heterodimers in the gel-retardation analysis and was incapable of eliciting any transcriptional activation in the co-transfection assays with PPAR α and Wy-14,643. The element was, however, responsive to RXR α in the presence of 9-*cis*-retinoic acid, giving a 2.3-fold stimulation of transcription, which remained unchanged by the addition of PPAR α and Wy-14,643. No direct binding of RXR α to this element was observed in gel-retardation assays. Both elements, I and II, therefore represent RXR α /co-factor response elements. The situation found here is very similar to that observed in the rabbit equivalent of rat p450 IVA1, CYP4A6 [19]. In the rabbit, the two PPRE-like sequences were located 677 bp upstream of the gene and were separated by approx. 70 nucleotides. Both elements showed good agreement with the consensus PPRE sequence, with the distal element sharing 10 out of 12 base-pairs with the consensus PPRE (TGACCT N TGACCT), and the proximal element sharing nine out of 12 base-pairs. As with rat,

the distal rabbit 4A6 element was unable to activate transcription when tested in co-transfection assays in the presence of both PPAR and the peroxisome proliferator clofibrate. The proximal element, however, mediated a 4.2-fold activation of transcription in the same assay. Therefore, there is an appreciable degree of structural and functional similarity in the regulatory sequences of the rat and rabbit CYP4A genes.

As discussed in [25], the diversity in both PPRE sequence and the factors able to bind to them may provide the basis for positive or negative regulation of their target genes. The co-ordinated induction of peroxisomal and microsomal enzymes could therefore be explained by the affinity of PPRES for similar if not identical ligand-activated receptors. Therefore the structure of a PPRE, its location and the presence of other positive and negative regulatory elements may together determine the extent and timing of the response to peroxisome proliferators via PPARs. Our findings support the existence of such a signal-transduction pathway and emphasize the role played by PPARs in mediating hepatic enzyme induction by peroxisome proliferators. One interesting aspect of the peroxisome proliferator phenomenon is the marked species differences in response [5,6,34]. It is conceivable that inter-species and inter-strain differences are a consequence of variations in PPAR and/or PPRES structure, and it may be significant that thus far no PPRES has been identified in a 'non-responsive' species. Further data are required, however, to clarify this, and to assess the hazard to man of such compounds.

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