Xenobiotic induction of cytochrome P450 2B1 (CYP2B1) is mediated by the orphan nuclear receptor constitutive androstane receptor (CAR) and requires steroid co-activator 1 (SRC-1) and the transcription factor Sp1

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The constitutive androstane receptor (CAR) activates the expression of a reporter gene attached to the phenobarbital-response element (PBRE) of the cytochrome P450 2B1 (*CYP2B1*) gene in response to the barbiturate phenobarbital and the plant product picrotoxin. The xenobiotic-mediated increase in transactivation occurs in transfected primary hepatocytes and in liver transfected by biolistic-particle-mediated DNA transfer, but not in the transformed cell lines HepG2, CV-1 and HeLa, which support only constitutive activation of gene expression by CAR. Steroid co-activator 1 (SRC-1) enhances both constitutive and xenobiotic-induced CAR-mediated transactivation via the *CYP2B1* PBRE in transfected primary hepatocytes. The nuclear receptor 1 (NR1) site of the PBRE is sufficient for CAR-

mediated transactivation, but additional sequences within the PBRE, and hence the proteins that bind to them, are required for the interaction of CAR with SRC-1. The NR2 site of the PBRE binds proteins other than CAR, including an unidentified nuclear receptor heterodimerized with retinoid X receptor α . By binding to the proximal promoter of *CYP2B1*, the transcription factor Sp1 increases both basal transcription and xenobiotic-induced expression via the PBRE. Thus induction of *CYP2B1* expression by xenobiotics is mediated by the nuclear receptor CAR and, for optimal expression, requires SRC-1 and Sp1.

Key words: gene regulation, hepatocytes, liver, phenobarbital, picrotoxin.

INTRODUCTION

A vast array of foreign chemicals, including dietary components, environmental pollutants and therapeutic drugs, require detoxification to ensure their safe elimination from the body. The initial stage in the detoxification of xenobiotics is usually carried out by cytochromes P450 (CYPs), in particular by members of the CYP1, CYP2, CYP3 and CYP4 families. The efficiency of this process is enhanced because many foreign chemicals selectively induce, via activation of gene transcription, the CYP required for their metabolism (reviewed in [1]).

Transcriptional activation of *CYP* genes by xenobiotics occurs predominantly in the liver and is mediated by the interaction of ligand–nuclear-receptor complexes with enhancer sequences that lie upstream of *CYP* gene promoters (reviewed in [2–4]). Polycyclic aromatic hydrocarbons increase the expression of *CYP1* genes via the aryl hydrocarbon (Ah) receptor (reviewed in [5,6]). The peroxisome-proliferator-activated receptor α [7] mediates regulation of *CYP4* genes by drugs, such as clofibrate, and non-genotoxic carcinogens [8]. The pregnane X receptor [9], also known as the steroid and xenobiotic receptor [10], activates *CYP3* genes by binding natural and synthetic steroids.

Less well understood, however, are the mechanisms by which *CYP2B* genes are regulated in response to numerous structurally diverse compounds, including the anti-epileptic and tumour promoter phenobarbital [11–13], and plant products, such as

picrotoxin [14] and the pinenes camphor and limonene [15]. A phenobarbital-response element (the PBRE) was first identified upstream (–2318 to –2155 bp) of the rat *CYP2B2* gene [16], and a similar sequence upstream of the mouse *Cyp2b10* gene was shown subsequently to confer inducibility, by 16 different compounds, on a reporter gene transfected into mouse primary hepatocytes [17]. Within the PBRE are two nuclear-receptor direct-repeat (DR) 4 (where the 4 denotes 4 bp spacing) -binding sites of the type AGGTCA [18], known as nuclear receptor 1 (NR1) and NR2 [19], separated by a nuclear factor 1 (NF1) site [20]. The identification, *in vivo*, of a phenobarbital-dependent DNA footprint in the region of the PBRE [21], and the increase *in vivo*, in response to phenobarbital, of the expression of a reporter gene attached to the PBRE [22], confirm a role for this sequence in the induction of *CYP2B* genes by phenobarbital. The orphan nuclear receptor constitutive androstane receptor (CAR) [23,24] has been shown to bind to the PBRE as a heterodimer with the retinoid X receptor α (RXR α) [19]. CAR, unlike other nuclear receptors, is able to bind to its response element and activate transcription in the absence of ligand [24]. In the absence of any exogenous chemical, CAR increases the transcription of a reporter gene, linked to the PBRE, in the hepatoma cell line HepG2 [19]. The constitutive activity of CAR is inhibited by androstanol (5 α -androstan-3 α -ol) and androstenol (5 α -androsten-16-en-3 α -ol), because these compounds promote the release of the co-factor steroid co-activator 1 (SRC-1) from the putative

Abbreviations used: Ah, aryl hydrocarbon; BTE, basal transcription element; CAR, constitutive androstane receptor; CYP, cytochrome P450; DR_n, direct repeat with *n* bp spacing; NF1, nuclear factor 1; NR1 or 2, nuclear receptor 1 or 2; PBRE, phenobarbital-response element; RXR α , retinoid X receptor α ; SRC-1, steroid co-activator 1.

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ligand-binding domain of CAR [25]. Interestingly, in HepG2 cells the ability of CAR to transactivate a *CYP2B6* PBRE reporter-gene construct is lost after treatment of cells with androsthenol, but is restored following subsequent treatment with phenobarbital [26]. It has been suggested that phenobarbital causes CAR to translocate from the cytoplasm to the nucleus, where the receptor is then able to activate the transcription of *CYP2B* genes [27].

In this report we show directly that, in primary hepatocytes and intact liver, CAR-mediated transactivation of *CYP2B1* gene expression via the PBRE is activated by phenobarbital and picrotoxin. This xenobiotic-induced enhancement of transcription by CAR does not require pretreatment with an inverse agonist of the receptor and is not observed in transformed cell lines. In addition, we demonstrate that the transactivation of *CYP2B1* gene expression by CAR is greatly stimulated by the transcription factor Sp1 [28] and by the co-activator SRC-1 [20].

MATERIALS AND METHODS

Plasmids

A plasmid containing nucleotides –3878 to +27 of the *CYP2B1* gene was provided by Dr P. Shaw (PanVera Corporation, Madison, WI, U.S.A.). The reporter plasmid (*CYP2B1* PBRE)-*SV40*-luc was constructed by inserting a *Sau3A* fragment, comprising nucleotides –2301 to –2142 of the *CYP2B1* 5'-flanking sequence, into the *Bgl*III site of the plasmid pGL3-SV40-Promoter (Promega). This placed the *CYP2B1* gene PBRE upstream of the firefly luciferase reporter gene. The reporter plasmid (*CYP2B1* NR1)₂-*SV40*-luc was constructed by inserting two copies of a double-stranded oligonucleotide (5'-TCTGTA-CTTTCCTGACCTT-3'), containing the NR1 site of the *CYP2B1* PBRE, into the *Sma*I site of the plasmid pGL3-SV40-Promoter. The reporter plasmid (*CYP2B1*PP)-luc was constructed by inserting a *Stu*I/*Hind*III fragment, corresponding to the region from –179 to –1 of the *CYP2B1* gene, which contains the minimal promoter, into the pGL3 Basic vector (Stratagene). The plasmid (*CYP2B1*PBRE)-PP-luc was constructed by inserting a *Sau3A*I-fragment (–2301 to –2142 of the *CYP2B1* gene) into the *Bam*HI site of the plasmid (*CYP2B1*PP)-luc. The plasmid pRL-TK (Promega), which contains the renilla luciferase gene under the control of the thymidine kinase promoter, was used as a control vector to normalize the results of transient-transfection assays. Expression plasmids for mouse CAR (cDM8-mCAR β), mouse RXR (pSG5-mRXR α), steroid receptor co-activator (pSG5-SRC-1) and Sp1 (CMV-SP1pBluescript) were provided, respectively, by Professor D. Moore (Baylor College of Medicine, Houston, TX, U.S.A.), Professor P. Chambon [CNRS/INSERM(ULP)/College de France, CU de Strasbourg, France], Professor M. Parker (Imperial Cancer Research Fund, London, U.K.) and Professor R. Tjian (Howard Hughes Medical Institute, University of California, Berkeley, CA, U.S.A.).

Cell culture and transfection

Primary rat hepatocytes were prepared by a two-step collagenase-perfusion method [29]. After attachment, cells were cultured in William's E medium, supplemented with 10% foetal calf serum, 1.7 μ M insulin, 0.1 μ M dexamethasone, gentamicin (200 μ g/ml) and 2 mM L-glutamine, on 60 mm plates coated with 200 μ l of Matrigel (1 mg/ml; Beckton Dickinson). HepG2 cells were grown in William's E medium containing 10% foetal calf serum. CV-1 cells and HeLa cells were cultured in Dulbecco's modified minimal essential medium containing 10% foetal calf serum,

supplemented with gentamicin (200 μ g/ml). Transfection mixtures contained 2.5 μ g of reporter plasmid and 0.25 μ g of control plasmid, and, when present, 0.25 μ g of an expression plasmid. Primary hepatocytes and cell lines were transfected using Tfx™-20 reagent (Promega) according to the supplier's recommendations. Briefly, cells were transfected with Tfx-20 reagent/DNA mixture (2:1) and serum-free William's E medium in a total volume of 2 ml. After 1 h, without removing the transfection mixture, 4 ml of serum-free William's E medium containing vehicle alone (0.1% DMSO), 0.1 mM sodium phenobarbital or 0.5 mM picrotoxin was added to the cells. Cells were harvested 24 h after transfection. Cell extracts were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) according to the supplier's recommendations. Mean $\pm$ S.D. values were obtained from a minimum of three independent experiments. Results were analysed using two-tailed paired Student's *t* tests. Where S.D. values are not shown, experiments were carried out in duplicate in two independent experiments.

In vivo transfection of liver by biolistic particle delivery

Gold particles (25 mg, 1 μ m; Bio-Rad), were coated with 100 μ g of DNA. The proportions of (*CYP2B1* PBRE)-*SV40*-luc, pRL-TK and the expression plasmid cDM8-mCAR β were 10:1:1. The amount of total DNA per cartridge was 2 μ g. Male Sprague-Dawley rats (200–250 g) were anaesthetized with ketamine (100 mg/kg of body weight) and xylazine (70 mg/kg). The abdominal area was shaved and opened to expose the liver. DNA-coated gold particles were delivered into the liver under a He pressure of 250 psi (1725 kPa). The liver was shot at two different positions in a single lobe. A single dose of phenobarbital (100 mg/kg) was given intraperitoneally, as soon as suturing was complete. Later (24 h) the liver was removed and the shot areas assayed for luciferase activity as above.

RNA analysis

Total RNA was isolated as described previously [30] from cultures of rat hepatocytes that were either untreated or had been treated with phenobarbital or picrotoxin. RNase protection assays were carried out as described previously [31].

Protein extracts for gel mobility-shift assays

Male Sprague-Dawley rats (200–250 g) were either untreated or treated with phenobarbital as described previously [30]. Liver nuclear protein extracts were prepared according to the method of Sierra [32], as described previously [33,34] and stored at –70 °C. Protein concentration was measured by the method of Lowry, and protein integrity was assessed by SDS/PAGE.

Gel mobility-shift assays

CAR and RXR α were synthesized *in vitro* using a TNT® transcription/translation-coupled reticulocyte lysate system (Promega) according to the supplier's recommendations. Gel mobility-shift assay mixtures contained 10 mM Hepes (pH 7.6), 0.5 mM dithiothreitol, 15% (v/v) glycerol, 2.5 μ g of poly(dI·dC), 0.05% (v/v) Nonidet P-40, 50 mM NaCl, 1.5 μ l of *in vitro*-translated products or 5 μ g of nuclear protein extract, and 3 $\times$ 10⁴ c.p.m. of a ³²P-labelled double-stranded oligonucleotide. The binding reaction was performed at room temperature for 20 min. Competition assays were performed with a 100-fold molar excess of unlabelled double-stranded oligonucleotide. The following double-stranded oligonucleotides were

used as probes or competitors: NR1 (DR4), 5'-TCGATCTGT-
ACTTCTGACCTT-3'; NR2 short (DR4), 5'-TCGACATC-
AACTTGACTGACACCA-3'; NR2 long (DR4), 5'-TCGACC-
ACCATCAACTTGACTGACACCAGGA-3'; RXR α -binding
site (DR1), 5'-TCGAAGCTTCAGGTCAGAGGTCAGAGA-
GCT-3'; NF1, 5'-TCGATTTTGGATTGAAGCCAATATGA-
TA3'; CYP2B1 (-88/-66), 5'-TCGATAGCTAAAGCAGGA-
GGCGTGAAC-3'; CYP2B1 (-48/-26), 5'-TCGATGAGTG-
GAGGGGCGGATTGAGCA-3'; Sp1, 5'-TCGAATTCGATC-
GGGCGGGGCGAGC-3'. Only the sequences of the upper-
strand oligonucleotides are shown. The 4 bp, 5'-TCGA-3', present
at the 5' end of each oligonucleotide (upper and lower strands)
was included as non-specific flanking sequence. Double-
stranded oligonucleotides were formed by annealing overlapping
complementary single-stranded sequences (synthesized by
Amersham Pharmacia Biotech), and radiolabelled by filling-in
the 5' TCGA overhangs through the use of the Klenow fragment
of *Escherichia coli* DNA polymerase. For supershift assays,
rabbit polyclonal antibodies against RXR α (SC-553x; 2 μ g) or
NF1 (SC-870x; 2 μ g; Santa Cruz Biotechnology) were added
15 min after the start of the binding reaction. Incubation was
continued for another 20 min.

RESULTS

Xenobiotics increase the transactivation of *CYP2B1* by CAR in primary hepatocytes and liver but not in transformed cell lines

Primary rat hepatocytes were transiently transfected with a reporter plasmid, containing one copy of the *CYP2B1* PBRE upstream of the SV40 promoter and the firefly luciferase gene [(*CYP2B1* PBRE)-SV40-luc], alone or together with an expression vector for mouse CAR. Measurement of luciferase activity showed that CAR stimulated transcription of the reporter gene 5-fold (Figure 1a). Treatment of transfected cells with the barbiturate phenobarbital or the natural plant product picrotoxin increased reporter-gene expression 4- and 9-fold, respectively, above that obtained as a result of constitutive transactivation by CAR (Figure 1a). However, when either the pGL3 Basic vector or the pGL3-SV40-Promoter vector was used as a reporter construct, co-transfected CAR had no effect on reporter-gene expression, in either the presence or absence of phenobarbital or picrotoxin (results not shown).

Transcription of a reporter gene under the control of the PBRE upstream of a *CYP2B1* minimal promoter was stimulated about 3-fold by CAR and was increased a further 5-fold in response to phenobarbital (see Figure 6 below). Therefore, in primary hepatocytes, CAR supports both constitutive and xenobiotic-induced transactivation of reporter-gene expression via the *CYP2B1* PBRE.

Co-transfection of HepG2 cells with the CAR expression vector and the reporter plasmid (*CYP2B1* PBRE)-SV40-luc resulted in a 2-fold stimulation of reporter-gene expression above that obtained in cells transfected with the reporter plasmid alone (Figure 1b). However, in contrast to the situation in primary hepatocytes, treatment of this liver-derived cell line with phenobarbital resulted in no further activation of reporter-gene transcription. Similarly, two non-liver-derived cell lines, CV-1, a cell line much used for nuclear-receptor-mediated transactivation studies, and HeLa, were able to support constitutive CAR-mediated transactivation via the PBRE, 2.5- and 19-fold, respectively, but were unable to support a further increase in reporter-gene expression in response to phenobarbital (Figures 1c and 1d). In fact, treatment of HeLa cells with phenobarbital consistently decreased the constitutive activation of the luciferase reporter gene by CAR (Figure 1d).

Figure 1 CAR activation of reporter-gene expression

Primary rat hepatocytes (a), HepG2 cells (b), CV-1 cells (c), HeLa cells (d) and intact rat liver (e) were transfected with the reporter construct (*CYP2B1* PBRE)-SV40-luc together with (CAR) or without (no E.P.) an expression vector for mouse CAR. Transfected cells and animals were treated with vehicle alone (DMSO), or with phenobarbital (PB) or picrotoxin (PCX). Results are shown as fold inductions of luciferase activity over that obtained in vehicle-treated cells or animals transfected with the reporter construct alone. * $P < 0.05$, ** $P < 0.01$, compared with the corresponding control value (two tailed paired Student's t test).

To confirm that activation of CAR by phenobarbital occurs *in vivo*, rat liver was transfected, via biolistic-particle-mediated DNA transfer, with (*CYP2B1* PBRE)-SV40-luc alone or together with a CAR expression vector. Luciferase activity was measured 24 h post-operation in phenobarbital-treated and untreated animals. Reporter-gene activity was stimulated by CAR and was increased a further 2.5-fold in response to phenobarbital (Figure 1e).

The co-activator SRC-1 stimulates both constitutive and xenobiotic-induced CAR activation of transcription via the *CYP2B1* PBRE

CAR enhancement of transcription is mediated by the co-activator SRC-1 in a ligand-independent manner in CV-1 cells [25]. It was of interest, therefore, to determine whether SRC-1 stimulated CAR-mediated transactivation of reporter-gene

Figure 2 Effect of SRC-1 on CAR transactivation of gene expression

Primary rat hepatocytes were transfected with the reporter constructs (*CYP2B1* PBRE)-SV40-luc (**a**) or *CYP2B1* (NR1)₂-SV40-luc (**b**) either alone (no E.P.) or together with expression vectors for SRC-1, mouse CAR or both. Transfected cells were treated with vehicle alone (DMSO) or with phenobarbital (PB) or picrotoxin (PCX). Results are shown as fold induction of reporter-gene activity over that obtained in vehicle-treated cells transfected with a reporter plasmid alone. **P* < 0.005, compared with the value obtained when untreated hepatocytes are transfected with the CAR expression plasmid (two tailed paired Student's *t* test).

expression, via the *CYP2B1* PBRE, in primary hepatocytes. Cells were co-transfected with the (*CYP2B1* PBRE)-SV40-luc reporter plasmid and expression plasmids for SRC-1 and/or CAR (Figure 2a). Reporter activity was increased 3-fold by SRC-1, 5-fold by CAR and 16-fold when both SRC-1 and CAR were present. Phenobarbital and picrotoxin caused further 2- and 3.6-fold increases, respectively, in reporter-gene expression when SRC-1 was co-expressed with CAR. Thus both constitutive and xenobiotic-induced CAR activation of transcription via the *CYP2B1* PBRE are stimulated by SRC-1. When the reporter plasmid used was (*CYP2B1* NR1)₂-SV40-luc, which contains only the NR1 site of the *CYP2B1* PBRE, CAR-mediated activation of reporter-gene expression was maintained, but could not be stimulated by SRC-1 (Figure 2b). Thus the NR1 site is sufficient to support CAR-mediated transactivation, but sequences other than the NR1 element within the PBRE are required for the stimulation of this activation by SRC-1.

The *CYP2B1* PBRE comprises two nuclear-receptor-binding sites, NR1 and NR2, separated by an NF1-binding site [19]. Each nuclear-receptor site has two direct-repeat half-sites separated by four nucleotides. Rat liver nuclear proteins formed a complex in a gel mobility-shift assay with the NR1 sequence and with either of two oligonucleotides containing the NR2 sequence of the *CYP2B1* PBRE (NR2 short, 5'-TCGACATCAACTTGACT-

Figure 3 NR1 and NR2 sites of the *CYP2B1* PBRE bind different proteins

Gel mobility-shift assays using as probe a ³²P-labelled double-stranded oligonucleotide corresponding to either the NR1 or NR2 site of the *CYP2B1* PBRE (as indicated). (**a**) Liver nuclear proteins (5 μg) isolated from phenobarbital-treated rats were assayed in the presence (+) or absence (−) of a 100-fold molar excess of an unlabelled double-stranded oligonucleotide competitor corresponding to the NR1 or NR2 site of the PBRE, or to a consensus RXRα-binding site. (**b**) Liver nuclear-protein extracts (NE, 5 μg) isolated from either untreated (U) or phenobarbital-treated (PB) rats were assayed in the presence (+) or absence (−) of antibodies to RXRα or NF1. (**c**) Mouse CAR (mCAR-β) and RXRα, produced by *in vitro* transcription/translation, were assayed in the presence (+) or absence (−) of an antibody against RXRα or of a 100-fold molar excess of an unlabelled double-stranded oligonucleotide competitor corresponding to the NR1, NR2 or NF1 site of the PBRE.

GACACCATCGA-3', and NR2 long, 5'-TCGACCACCATCAACTTGACTGACACCAGGATCGA-3'; Figure 3a and results not shown). An excess of an oligonucleotide for NR1 did not compete for proteins bound to the NR2 and vice versa. However, an oligonucleotide containing a consensus binding site for RXRα competed for proteins bound to either NR1 or NR2 (Figure 3a). An antibody against RXRα was able to supershift DNA-protein complexes formed between both NR1 and NR2 and liver nuclear proteins isolated from untreated or phenobarbital-treated rats, whereas an antibody against NF1 (used as a control) had no effect (Figure 3b). Gel-shift assays of proteins produced by *in vitro* transcription/translation (Figure 3c and results not shown) showed that when present alone, neither CAR nor RXRα could bind to the NR1 or the NR2 site. However, when both proteins were present a complex was formed on the NR1, but not the NR2 site (irrespective of whether the short or long NR2 oligonucleotide was used as a probe; Figure 3c and results not shown). This complex was competed by an excess of NR1, but not by NR2 (Figure 3c) or by oligonucleotides containing either the NF1 site of the *CYP2B1*

Figure 4 Phenobarbital and picrotoxin induction of CYP2B1 mRNA

Time course of CYP2B mRNA induction in primary hepatocytes. Total RNA was isolated at various times from untreated cells (U), or from cells treated with 0.1 mM phenobarbital (PB) or 0.5 mM picrotoxin (PCX). CYP2B1 mRNA was assayed by RNase protection.

PBRE (Figure 3c) or a consensus Sp1 site (results not shown), and was supershifted by an antibody against RXR α (Figure 3c). Taken together, the results demonstrate that CAR binds, as a heterodimer with RXR α , to the NR1 site of the CYP2B1 PBRE, whereas the NR2 site interacts with other proteins, including RXR α heterodimerized with an unknown partner.

Both phenobarbital and picrotoxin are potent inducers of CYP2B1 in the liver, each compound increasing CYP2B1 mRNA by more than 50-fold [14,30]. A time course of CYP2B mRNA induction in primary hepatocyte cultures shows that the kinetics of induction by the two compounds are very similar (Figure 4). This suggests that phenobarbital and picrotoxin induce CYP2B gene expression via the same activation pathway. However, the level of expression of a reporter gene under the control of the CYP2B1 PBRE is greater in response to picrotoxin than to phenobarbital (Figures 1a and 2a). The NR1 site of the PBRE is sufficient to support both constitutive and phenobarbital-induced CAR-mediated transactivation of gene expression, but not the increased induction in response to picrotoxin (Figures 2a and 2b), which requires additional sequences of the PBRE, i.e. the NF1 or NR2 sites.

The transcription factor Sp1 stimulates both basal and xenobiotic-induced transcription of CYP2B1

The proximal promoters of several CYP genes contain a sequence, the basal transcription element (BTE), which has been shown to confer transcriptional activity on the CYP1A1 gene [35]. Two proteins bind to the CYP1A1 BTE, Sp1 and BTE-binding protein [35]. Mutation of the CYP2B1 promoter BTE (located between -82 and -67) reduced transcriptional activity 70–80% in HepG2 cells [22]. Putative Sp1-binding sites are present at -45 to -36 and -79 to -70 of the CYP2B1 promoter, the latter within the BTE. Double-stranded oligonucleotides specifying either the -88/-65 or -48/-26 regions of the CYP2B1 promoter formed complexes with proteins present in rat liver nuclear extracts (Figure 5). Proteins bound to these oligonucleotides were competed by a 100-fold molar excess of

Figure 5 Binding of Sp1 to two sites in the CYP2B1 proximal promoter

Rat liver nuclear protein extracts (NE, 14 μ g) were incubated with 32 P-labelled double-stranded oligonucleotides, corresponding to sequences from -88 to -66 or -48 to -26 of the CYP2B1 proximal promoter, in the presence (+) or absence (-) of a 100-fold molar excess of an unlabelled double-stranded oligonucleotide competitor containing a consensus Sp1-binding site.

Figure 6 Stimulation of reporter-gene expression by Sp1

Primary rat hepatocytes were transfected with pGL3 Basic vector (a), (CYP2B1PP)-luc (b) or (CYP2B1PBRE)-PP-luc (c), either with (+) or without (-) expression vectors for Sp1 or mouse CAR. Transfected cells were treated with vehicle alone (DMSO; open bars) or with phenobarbital (filled bars). Results are given as fold induction of reporter-gene activity compared with that obtained in vehicle-treated cells transfected with pGL3 alone.

an oligonucleotide containing an Sp1-binding-site consensus sequence (Figure 5).

Does Sp1 play a functional role in CYP2B1 expression or its induction by xenobiotics? To address this, hepatocytes were co-transfected with an expression vector for Sp1 and a construct containing the luciferase gene under the control of the CYP2B1 proximal promoter either alone [(CYP2B1PP)-luc] or attached to

the *CYP2B1* PBRE [(*CYP2B1*PBRE)-PP-luc] (Figure 6). No stimulation of reporter-gene expression was observed in hepatocytes co-transfected with expression vectors for CAR or Sp1 and the reporter plasmid pGL3, which constitutes the backbone of (*CYP2B1*PP)-luc and (*CYP2B1*PBRE)-PP-luc (Figure 6a). In cells transfected with (*CYP2B1*PP)-luc, reporter-gene expression was increased 27-fold by Sp1, but was unaffected by phenobarbital (Figure 6b). Co-expression of CAR decreased the effect of Sp1 (Figure 6b). Sp1 had a similar effect in cells transfected with (*CYP2B1*PBRE)-PP-luc. But, in this case, reporter-gene expression was increased a further 3-fold by phenobarbital (Figure 6c). These results show that Sp1, by binding to sites within the proximal promoter of the *CYP2B1* gene, increases basal transcription of the gene and, presumably via interaction with protein(s) bound to the *CYP2B1* PBRE, stimulates the induction of gene expression in response to phenobarbital. Co-expression of CAR and Sp1 in hepatocytes transfected with (*CYP2B1*PBRE)-PP-luc resulted in no further enhancement of reporter-gene expression, in either the presence or absence of phenobarbital (Figure 6c).

DISCUSSION

A previous study [26] used an indirect method to implicate CAR in the activation by phenobarbital of a *CYP2B6* reporter-gene construct. Transfected HepG2 cells were first treated with androsthenol, an inverse agonist of CAR, which represses constitutive activity of the receptor [25]. Subsequent treatment of the cells with phenobarbital increased reporter-gene expression, but only to the level observed in cells before androsthenol treatment. Here we show directly that, in transfected primary hepatocytes and intact liver, constitutive CAR-mediated transactivation of a reporter gene linked to the *CYP2B1* PBRE is increased in response to phenobarbital. An even larger increase was obtained in response to picrotoxin. CAR thus mediates the induction of *CYP2B* gene expression not only by phenobarbital, but also by other, structurally unrelated, compounds.

All three of the transformed cell lines used in our study, HepG2, HeLa and CV-1, were found to support constitutive activation of reporter-gene expression by co-transfected CAR. But, in contrast to primary hepatocytes and the intact liver, none was able to support an increase in CAR-mediated transactivation in response to phenobarbital. The ability of the transformed cell lines to regulate CAR-mediated activation of gene expression thus differs from that of liver *in vivo*. In transformed cell lines, a signalling pathway that interacts with CAR may be permanently activated, thus preventing further activation in response to xenobiotics. Alternatively, such cells may lack a key component involved in modulating CAR activity. It is clear, therefore, that even liver-derived transformed cells, such as HepG2, are not good models for the xenobiotic induction of CAR-mediated transactivation of gene expression.

Nuclear receptors are thought to shuttle continually between the cytoplasm and the nucleus to maintain an equilibrium, which is perturbed in the presence of ligand [36]. It has been suggested that CAR is maintained in a repressed state in the cytoplasm and is translocated to the nucleus in response to phenobarbital [27]. However, the results of our co-transfection experiments show that, in primary hepatocytes and intact liver, CAR can enter the nucleus and transactivate gene expression via the PBRE in the absence of xenobiotics. Thus mechanisms other than, or in addition to, nuclear translocation of CAR may be responsible for the xenobiotic induction of gene expression via CAR.

We have found that CAR-mediated transactivation of gene expression via the PBRE is stimulated by the co-activator

SRC-1. Furthermore, we show that, although the NR1 site of the PBRE is sufficient to support transactivation by CAR, additional sequences within the PBRE are required for the stimulation of this activity by SRC-1, and thus for the maximal induction of *CYP2B1* in response to xenobiotics. That sequences other than NR1 may be important for the function of the PBRE has been suggested by the results of *in vivo* DNA footprinting experiments [21]. These showed that, in rat liver, a footprint centred on the NF1 site of the PBRE expanded, in response to treatment of animals with phenobarbital, to encompass the whole of the adjacent NR1 and NR2 sites. Mutations of the NF1 site of the PBRE reduced responsiveness to phenobarbital of a reporter gene in primary hepatocytes [37,38] but not in transgenic mice [39]. The contribution of the NF1 site to the function of the PBRE is thus unclear.

The results of our gel mobility-shift assays show that CAR binds, as a heterodimer with RXR α , to the NR1, but not to the NR2, site of the *CYP2B1* PBRE, thus providing an explanation for why only the NR1 site of the PBRE is apparently necessary for CAR-mediated transactivation of the *CYP2B1* gene. These results are in contrast to those of Tzamelli et al. [40], who showed that CAR can heterodimerize with human RXR α on the NR2 site of the mouse *Cyp2b10* gene. This discrepancy may be due to conformational differences adopted by CAR when heterodimerized to mouse RXR α (used by us) or to human RXR α (used by Tzamelli et al. [40]), or to the difference in sequence between the half-site spacer region of the rat and mouse NR2 sites. The human orthologue of CAR can bind to both the NR1 and NR2 sites of the human *CYP2B6* PBRE [26]. This is probably due to differences between the nucleotide sequences of the rat and human NR2 sites: the human *CYP2B6* NR2 is very similar in sequence to the *CYP2B6* NR1, whereas the NR1 and NR2 of the rat *CYP2B1* gene differ by two nucleotides in each of their half-sites and within the spacer region. This was confirmed by the finding that human CAR, produced by *in vitro* transcription/translation, is able to bind to the NR1, but not the NR2, site of the *CYP2B1* gene (results not shown). The apparent requirement of the NR2 site for stimulation of CAR-mediated transactivation by SRC-1 suggests that proteins that bind to this site may mediate the interaction between CAR, bound to the NR1 site, and SRC-1.

The mechanisms by which the barbiturate phenobarbital and the plant product picrotoxin induce *CYP2B1* both involve the binding of CAR to the NR1 site of the PBRE. It seems likely that the induction mechanism utilized by these compounds evolved to enable animals to protect themselves against potentially harmful chemicals, such as picrotoxin, present in plants they ingest. It is fortuitous that a synthetic compound, such as phenobarbital, is able to use the same basic induction pathway.

Our results demonstrate that, by binding to sites within the proximal promoter of the *CYP2B1* gene, the ubiquitous transcription factor Sp1 [28] increases both basal transcription and xenobiotic-induced expression via the PBRE. Sp1 may thus form a molecular bridge, allowing proteins bound to the PBRE to interact optimally with the transcriptional pre-initiation complex. For instance, Sp1 has been shown to interact with the Ah receptor and the Ah receptor nuclear translocator to increase the expression of *CYP1A1* [41]. The identity of the protein(s) bound to the *CYP2B1* PBRE that interact with Sp1 is not known. However, the lack of the effect of CAR on the activity of Sp1 implies that other proteins, possibly those that bind to the NR2 site, are involved. The changes that occur in the chromatin structure of the proximal promoter and PBRE of the *CYP2B1* gene in response to phenobarbital [42] may aid recruitment of co-activators, such as SRC-1, and the interaction of PBRE-bound

proteins with transcription factors, such as Sp1, bound to the proximal promoter.

The identification of androstane metabolites as inverse agonists of the constitutive activity of CAR [25] raises the possibility that, in the liver, *CYP2B1* gene expression is normally repressed by the presence of a compound that binds to and inactivates CAR, but can be displaced by certain xenobiotics. Although *CYP2B1* can hydroxylate various steroids, including oestrogen and testosterone [43,44], the endogenous substrate(s) for this CYP are not known. Perhaps a hydroxylated steroid, a product of a reaction catalysed by *CYP2B1* (or by another CYP), regulates *CYP2B1* gene transcription via a feedback mechanism in which the metabolite, as its concentration rises, binds to CAR, thus preventing transactivation. A decrease in the concentration of the metabolite, or its displacement by a xenobiotic, would restore the ability of CAR to transactivate *CYP* gene expression through its interaction with the co-activator SRC-1. It will be of interest to determine whether compounds such as phenobarbital and picrotoxin bind directly to CAR, and whether this interaction displaces a CAR inhibitory compound similar to the steroids androstanol and androstenol. If this proves to be the case then the rationale for the dramatic increase in *CYP* gene expression in response to foreign chemicals may be to ensure the rapid removal of xenobiotics, thus allowing maintenance of steroid homeostasis within organs such as the liver.

Phenobarbital has multiple effects in the liver: it causes proliferation of the endoplasmic reticulum, promotes tumour formation, dramatically increases liver size and regulates the expression of more than 30 genes [45–47]. It remains to be established whether all of these effects are mediated by CAR.

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REFERENCES

- Gonzalez, F. J. (1989) The molecular biology of cytochrome P450s. *Pharmacol. Rev.* **40**, 243–288
- Waxman, D. J. (1999) P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. *Arch. Biochem. Biophys.* **369**, 11–23
- Kliwer, S. A., Lehmann, J. M. and Willson, T. M. (1999) Orphan nuclear receptors: shifting endocrinology into reverse. *Science* **284**, 757–760
- Honkakoski, P. and Negishi, M. (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. *Biochem. J.* **347**, 321–337
- Swanson, H. I. and Bradfield, C. A. (1993) The Ah-receptor: genetics, structure and function. *Pharmacogenetics* **3**, 213–230
- Whitlock Jr, J. P., Okino, S. T., Dong, L., Ko, H. P., Clarke-Katzenberg, R., Ma, Q. and Li, H. (1996) Cytochromes P450. 5. Induction of cytochrome P4501A1: a model for analyzing mammalian gene transcription. *FASEB J.* **10**, 809–818
- Issemann, I. and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature (London)* **347**, 645–650
- Johnson, E. F., Palmer, C. N., Griffin, K. J. and Hsu, M. H. (1996) Role of the peroxisome proliferator-activated receptor in cytochrome P450 4A gene regulation. *FASEB J.* **10**, 1241–1248
- Kliwer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H. et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* **92**, 73–82
- Blumberg, B., Sabbagh Jr, W., Juguilon, H., Bolado Jr, J., van Meter, C. M., Ong, E. S. and Evans, R. M. (1998) SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12**, 3195–3205
- Hardwick, J. P., Gonzalez, F. J. and Kasper, C. B. (1983) Cloning of DNA complementary to cytochrome P-450 induced by pregnenolone-16 α -carbonitrile. Characterization of its mRNA, gene, and induction response. *J. Biol. Chem.* **258**, 10182–10186
- Atchison, M. and Adesnik, M. (1983) A cytochrome P-450 multigene family. Characterization of a gene activated by phenobarbital administration. *J. Biol. Chem.* **258**, 11285–11295
- Pike, S. F., Shephard, E. A., Rabin, B. R. and Phillips, I. R. (1985) Induction of cytochrome P-450 by phenobarbital is mediated at the level of transcription. *Biochem. Pharmacol.* **34**, 2489–2494
- Yamada, H., Fujisaki, H., Kaneko, H., Ishii, Y., Hamaguchi, T. and Oguri, K. (1993) Picrotoxin as a potent inducer of rat hepatic cytochrome P450, CYP2B1 and CYP2B2. *Biochem. Pharmacol.* **45**, 1783–1789
- Austin, C. A., Shephard, E. A., Pike, S. F., Rabin, B. R. and Phillips, I. R. (1988) The effect of terpenoid compounds on cytochrome P-450 levels in rat liver. *Biochem. Pharmacol.* **37**, 2223–2229
- Trottier, E., Belzil, A., Stoltz, C. and Anderson, A. (1995) Localization of a phenobarbital-responsive element (PBRE) in the 5'-flanking region of the rat CYP2B2 gene. *Gene* **158**, 263–268
- Honkakoski, P., Moore, R., Washburn, K. A. and Negishi, M. (1998) Activation by diverse xenochemicals of the 51-base pair phenobarbital-responsive enhancer module in the CYP2B10 gene. *Mol. Pharmacol.* **53**, 597–601
- Glass, C. K. (1994) Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocrine Rev.* **15**, 391–407
- Honkakoski, P., Zelko, I., Sueyoshi, T. and Negishi, M. (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the CYP2B gene. *Mol. Cell. Biol.* **18**, 5652–5658
- Oate, S. A., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W. (1995) Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270**, 1354–1357
- Kim, J. and Kemper, B. (1997) Phenobarbital alters protein binding to the CYP2B1/2 phenobarbital-responsive unit in native chromatin. *J. Biol. Chem.* **272**, 29423–29425
- Park, Y., Li, H. and Kemper, B. (1996) Phenobarbital induction mediated by a distal CYP2B2 sequence in rat liver transiently transfected *in situ*. *J. Biol. Chem.* **271**, 23725–23728
- Baes, M., Gulick, T., Choi, H. S., Martinoli, M. G., Simha, D. and Moore, D. D. (1994) A new orphan member of the nuclear hormone receptor superfamily that interacts with a subset of retinoic acid response elements. *Mol. Cell. Biol.* **14**, 1544–1551
- Choi, H. S., Chung, M., Tzameli, I., Simha, D., Lee, Y. K., Seol, W. and Moore, D. D. (1997) Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. *J. Biol. Chem.* **272**, 23565–23571
- Forman, B. M., Tzameli, I., Choi, H. S., Chen, J., Simha, D., Seol, W., Evans, R. M. and Moore, D. D. (1998) Androstane metabolites bind to and deactivate the nuclear receptor CAR- β . *Nature (London)* **395**, 612–615
- Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P. and Negishi, M. (1999) The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J. Biol. Chem.* **274**, 6043–6046
- Kawamoto, T., Sueyoshi, T., Zelko, I., Moore, R., Washburn, K. and Negishi, M. (1999) Phenobarbital-responsive nuclear translocation of the receptor CAR in induction of the CYP2B gene. *Mol. Cell. Biol.* **19**, 6318–6322
- Dynan, W. S. and Tjian, R. (1983) Isolation of transcription factors that discriminate between different promoters recognized by RNA polymerase II. *Cell* **32**, 669–680
- Seglen, P. O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29–83
- Phillips, I. R., Shephard, E. A., Ashworth, A. and Rabin, B. R. (1983) Cloning and sequence analysis of a rat liver cDNA coding for a phenobarbital-inducible microheterogeneous cytochrome P-450 variant: regulation of its messenger level by xenobiotics. *Gene* **26**, 41–52
- Akrawi, M., Rogiers, V., Vandenberghe, Y., Palmer, C. N., Vercruysse, A., Shephard, E. A. and Phillips, I. R. (1993) Maintenance and induction in co-cultured rat hepatocytes of components of the cytochrome P450-mediated mono-oxygenase. *Biochem. Pharmacol.* **45**, 1583–1591
- Sierra, F. (1990) Laboratory Guide to In Vitro Transcription, *Biomethods*, vol. 2, Birkhauser Verlag, Basel
- Dignam, J. D., Lebovitz, R. M. and Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489
- Dell, H., Wong, S. C., Phillips, I. R. and Shephard, E. A. (1998) Identification of regulatory protein-binding sites in cytochrome P450 genes by means of gel-retardation assay. In *Cytochrome P450 Protocols*, vol. 107 (Phillips, I. R. and Shephard, E. A., eds.), pp. 381–394, Humana Press, Totowa
- Imataka, H., Sogawa, K., Yasumoto, K., Kikuchi, Y., Sasano, K., Kobayashi, A., Hayami, M. and Fujii-Kuriyama, Y. (1992) Two regulatory proteins that bind to the basic transcription element (BTE), a GC box sequence in the promoter region of the rat P-4501A1 gene. *EMBO J.* **11**, 3663–3671
- Defranco, D. B., Madan, A. P., Tang, Y., Chandran, U. R., Xiao, N. and Yang, J. (1995) Nucleocytoplasmic shuttling of steroid receptors. *Vitam. Horm.* **51**, 315–338

- 37 Liu, S., Park, Y., Rivera-Rivera, I., Li, H. and Kemper, B. (1998) Nuclear factor-1 motif and redundant regulatory elements comprise phenobarbital-responsive enhancer in CYP2B1/2. *DNA Cell Biol.* **17**, 461–470
- 38 Stoltz, C., Vachon, M. H., Trottier, E., Dubois, S., Paquet, Y. and Anderson, A. (1998) The CYP2B2 phenobarbital response unit contains an accessory factor element and a putative glucocorticoid response element essential for conferring maximal phenobarbital responsiveness. *J. Biol. Chem.* **273**, 8528–8536
- 39 Ramsden, R., Beck, N. B., Sommer, K. M. and Omiecinski, C. J. (1999) Phenobarbital responsiveness conferred by the 5'-flanking region of the rat CYP2B2 gene in transgenic mice. *Gene* **228**, 169–179
- 40 Tzamelis, I., Pissios, P., Schuetz, E. G. and Moore, D. D. (2000) The xenobiotic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is an agonist ligand for the nuclear receptor CAR. *Mol. Cell. Biol.* **20**, 2951–2958
- 41 Kobayashi, A., Sogawa, K. and Fujii-Kuriyama, Y. (1996) Cooperative interaction between AhR, Arnt and Sp1 for the drug-inducible expression of CYP1A1 gene. *J. Biol. Chem.* **271**, 12310–12316
- 42 Kim, J., Rivera-Rivera, I. and Kemper, B. (2000) Tissue-specific chromatin structure of the phenobarbital-responsive unit and proximal promoter of CYP2B1/2 and modulation by phenobarbital. *Nucleic Acids Res.* **28**, 1126–1132
- 43 Ryan, D. E., Wood, A. W., Thomas, P. E., Walz Jr, F. G., Yuan, P. M., Shively, J. E. and Levin, W. (1982) Comparisons of highly purified hepatic microsomal cytochromes P-450 from Holtzman and Long-Evans rats. *Biochim. Biophys. Acta* **709**, 273–283
- 44 Waxman, D. J. (1988) Interactions of hepatic cytochromes P-450 with steroid hormones. Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem. Pharmacol.* **37**, 71–84
- 45 Waxman, D. J. and Azaroff, L. (1992) Phenobarbital induction of cytochrome P-450 gene expression. *Biochem. J.* **281**, 577–592
- 46 Frueh, F. W., Zanger, U. M. and Meyer, U. A. (1997) Extent and character of phenobarbital-mediated changes in gene expression in the liver. *Mol. Pharmacol.* **51**, 363–369
- 47 Meyer, U. A. and Hoffmann, K. (1999) Phenobarbital-mediated changes in gene expression in the liver. *Drug Metab. Rev.* **31**, 365–373

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