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A Conserved Nuclear Receptor Consensus Sequence (DR-4) Mediates Transcriptional Activation of the Chicken *CYP2H1* Gene by Phenobarbital in a Hepatoma Cell Line*

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Phenobarbital-responsive DNA elements were identified in the 5′-flanking region of the chicken *CYP2H1* gene by in reporter gene assays in a chicken hepatoma cell line (leghorn male hepatoma (LMH)). A 264-base pair (bp) enhancer sequence (phenobarbital-responsive unit (PBRU)) responded to phenobarbital and a variety of phenobarbital-type inducers. Analysis of putative transcription factor binding sites within the 264-bp element revealed a nuclear receptor half-site repeat (DR-4) neighboring a putative nuclear factor-1 site. This motif resembles phenobarbital response elements in the flanking regions of three phenobarbital-inducible genes, rat *CYP2B2*, mouse *Cyp2b10*, and human *CYP2B6*. Activation of the 264-bp element was eliminated after site-directed mutagenesis of the DR-4 hexamer half-sites. Evidence for evolutionary conservation of this recognition site was indicated by activation in LMH cells of a mouse *Cyp2b10* phenobarbital-responsive enhancer by the same spectrum of inducers that activate the *CYP2H1* 264-bp PBRU. Inhibition of this activation by okadaic acid may explain the reported inhibitory effects on induction of *CYP2B1/2* and *Cyp2b10* by this phosphatase inhibitor. We show that this inhibition occurs directly on the 264-bp PBRU, whereas the proximal promoter of *CYP2H1* is induced by okadaic acid in reporter gene assays. These experiments exploit the unique phenobarbital inducibility of the hepatoma-derived cell line LMH.

The cytochrome P-450 (CYP)[1] gene superfamily encodes proteins responsible for the metabolism of numerous xenobiotic and endobiotic substrates (1). In addition, the transcription of drug-metabolizing enzymes from *CYP1, CYP2,* and *CYP3* families can be induced a variety of compounds, including substrates of these CYPs (2–7). Five different classes of prototypical inducer-drugs that activate distinct but overlapping classes of CYPs have been defined. For example, phenobarbital (PB) induces predominantly enzymes from the *CYP2B, -2C,* and *-3A* subfamilies (2, 5, 7). These CYPs, as well as more than 50 other genes, are affected by PB and a number of structurally unrelated compounds classified as PB-like inducers (8). PB induction is observed in a wide variety of species ranging from *Bacillus megaterium* (9) to human. In vertebrates, drug induction occurs predominantly in the liver and the intestine and to a lesser extent in other extrahepatic tissues, such as skin, kidney, lung, and brain (2, 5).

Phosphorylation and dephosphorylation events, cytokines, and hormones affect enzyme induction by as yet unknown mechanisms (for a review, see Ref. 10). Until recently, little was known about the molecular mechanisms of PB-type induction and a receptor for these drugs remains elusive. A major breakthrough has been the recent observation in several laboratories that orphan nuclear receptors are involved in CYP regulation (for reviews, see Refs. 6, 11, and 12). These findings emerged from the discovery and analysis of well defined PB-responsive elements in the flanking region of rat *CYP2B2* and *CYP3A1* (13–16), mouse *Cyp2b10* (17, 18), and human *CYP2B6* and *CYP3A4* (19, 20). In particular, the pregnane X receptor is involved in the induction of *CYP3A*s (21, 22) and the constitutive androstane receptor (CAR) mediates induction of *CYP2B*s (19, 23). There is considerable overlap between the pregnane X receptor and the CAR systems in regard to inducers and genes affected.

In chicken liver, *CYP2H1* and *CYP2H2* are major PB-inducible enzymes (24). The 5′-flanking region of *CYP2H1* has been analyzed by Hahn *et al.* (25), and a 4.8-kb PB-responsive enhancer fragment (–5898 to –1057) was identified. Within this large DNA region, a small enhancer element of 240 bp was isolated (26). *DNase*I footprinting, gel shift assays, and mutational analysis of the 240-bp responsive element suggest that proteins binding to this element are different from the rodent *Cyp2b10*-responsive element and consequently that induction mechanisms may be different in chicken and mammals (26). A limiting factor in these studies was that the 240-bp fragment mediated only a small response to PB in primary hepatocyte cultures when compared with activation of transcription *in vivo* (26).

In the present study, we have analyzed the 5′-flanking region of *CYP2H1* by reporter gene assays in the hepatoma cell line LMH that uniquely maintains a pleiotropic response to PB. We identified a 264-bp enhancer element that overlaps with the 240-bp element found by Dogra *et al.* (26) and discovered a conserved nuclear receptor consensus sequence that mediates PB induction. This motif is also present in PB-inducible genes in mammals. Evolutionary conservation of this element was further supported by trans-species activation of a mouse *Cyp2b10* PB-responsive enhancer module in LMH cells. Our

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF236668.

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[1] The abbreviations used are: CYP, cytochrome(s) P-450; bp, base pair(s); CAR, constitutive androstane receptor; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer-binding protein; ELISA, enzyme-linked immunosorbent assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; kb, kilobase(s); LMH, leghorn male hepatoma; NF-1, nuclear factor-1; OA, okadaic acid; PB, phenobarbital; PBREM, phenobarbital-responsive enhancer module; PBRU, phenobarbital-responsive unit; PCN, 5-pregnen-3β-ol-20-one-16α-carbonitrile; PCR, polymerase chain reaction; tk, thymidine kinase.

results thus indicate that the proteins involved in the activation of the *CYP2H1* 264-bp PB-responsive element are identical or similar to proteins binding to the mouse and rat PB-responsive enhancer sequences. These findings document the experimental potential of the LMH cell line for PB induction studies.

MATERIALS AND METHODS

Reagents—Okadaic acid sodium salt and 1-nor-okadaone were purchased from Alexis Biochemicals (Läufelfingen, Switzerland). Dexamethasone, 2-methyl-1,2-di-3-pyridyl-1-propanone (metyrapone), 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN), and rifampicin were obtained from Sigma. Propylisopropylacetamide was generously provided by Peter Sinclair, Department of Veterans Affairs Medical Center (White River Junction, VT). Glutethimide and β-naphthoflavone were purchased from Aldrich. 5-Ethyl-5-phenylbarbituric acid sodium salt (phenobarbital sodium salt) was obtained from Fluka (Buchs, Switzerland). All other reagents and supplies were obtained from standard sources.

Culture and Transfection of LMH Cells—LMH cells were obtained from the ATCC (catalog no. 2117-CRL, batch no. F12213) and thawed immediately after arrival. They were cultivated in William's E medium supplemented with 10% fetal calf serum, 1% glutamine (2 mM), and 1% penicillin/streptomycin (50 IU/ml) on gelatin-coated dishes. Cells were seeded onto gelatin-coated plates at 70–80% surface density 24 h prior to transfection. The cells were removed by trypsinization, and transfections were performed using the FuGENE 6 transfection reagent (Roche Molecular Biochemicals) in a serum-free suspension according to the supplier's manual. This procedure ensured equal transfection efficiencies for control and treated cells. The cells were then plated on six-well dishes, and medium was replaced 4 h after transfection by either induction or control medium.

Construction of Vectors—5.9 kb of the *CYP2H1* flanking region were analyzed as follows. An initial 4.8-kb fragment was kindly provided by Dr. Brian May, Dept. of Biochemistry, University of Adelaide (Adelaide, Australia). The fragment was cut with *Bam*HI and inserted into *Bam*HI-linearized pBLCAT5 vector generously provided by Dr. Günther Schütz, Institute of Cell and Tumor Biology, German Cancer Research Center (Heidelberg, Germany). The proximal 1.1 kb of the flanking region were amplified by PCR using the primers 5′ ACT GAG TTG TGT TTT GGG TCC-3′ and 5′-GGT GGG CAG AGG AAG AGA TGT-3′. The amplified product was then cut at the 5′ end with *Bam*HI and ligated into a pBLCAT6 vector lacking the thymidine kinase-promoter. This vector was prepared by digestion with *Xho*I followed by Klenow fill-in and further digestion with *Bam*HI to generate a 5′ *Bam*HI-3′ blunt-ended product. Both the 4.8-kb and the 1.1-kb fragment were sequenced by standard methods with an ABI Prism 310 Genetic Analyser (PE Applied Biosystems, Rotkreuz, Switzerland). The nucleotide sequence of the complete 5.9-kb 5′-flanking region of *CYP2H1* has been deposited in the GenBank™ nucleotide sequence data base.

Construction of Subfragments—The subfragments of the 5.9-kb flanking region were obtained either by restriction digest or PCR amplification (see Fig. 1*A*) and cloned into the pBLCAT5 vector containing a tk promoter. The 4.8-kb enhancer was cut with *Xba*I and *Bam*HI to obtain fragments of 1376 and 3465 bp. Digestion of the 4.8-kb enhancer with *Acc*I, *Xba*I, and *Bam*HI resulted in fragments of 1865 and 1599 bp. The 1599-bp fragment was further digested with *Acc*I and *Stu*I or with *Bam*HI and *Stu*I to obtain 1016- or 583-bp fragments, respectively. The 331-bp fragment was produced by digesting the 583-bp fragment with *Bsp*MI and subsequent religation. The PB-responsive element from the 5′-flanking region of *CYP2H1* was amplified with the primers 5′-CCC AAG CTT ACA CAA CAG GTG ATA AGG C-3′ and 5′-CGG GAT CCA TGG AAG GAC TCG AGA TAA G-3′, which contain *Hin*dIII and *Bam*HI sites, respectively, at their 5′ ends. The amplified fragment was 264 bp in length and was subsequently digested with *Bam*HI and *Hinc*II, resulting in the generation of a 142-bp fragment. The vector was previously cut with *Acc*I, blunt-ended with Klenow enzyme, and further digested with *Bam*HI. A 122-bp element was obtained by digesting the 264-bp fragment with *Hin*dIII and *Hinc*II. The vector was prepared by digestion with *Bam*HI, and ends were filled in with Klenow enzyme followed by digestion with *Hin*dIII.

The published 205-bp promoter fragment from *CYP2H1* (27) was produced by PCR amplification using the primers 5′-CCC AAG CTT GGG ATG ATT ACC CAA AGT CA-3′ and 5′-CGC GGA TCC GCG CTG AAC TGG GAC CTG C-3′ containing *Hin*dIII and *Bam*HI sites at their respective 5′ ends. The amplified PCR product was subsequently cleaved with *Hin*dIII and *Bam*HI and subcloned into pBLCAT6. All constructs were verified by sequencing.

Site-specific Mutagenesis—Mutations in the putative nuclear receptor binding half-sites (see Fig. 5*A*) were introduced by PCR using standard overlap techniques. Briefly, subfragments were amplified with overlapping, mutated primers and vector primers. These subfragments were chosen as template in a second round of PCR using the vector primers alone for amplification of the full-length, mutated fragment. The vector primers in the first and the second round of PCR were 5′-TCA TGT CTG GAT CTC GAA GC-3′ and 5′-TTC GCC AAT GAC AAG ACG C-3′. The complete, mutated 264-bp fragments were then digested with *Bam*HI and *Hin*dIII and cloned into the pBLCAT5 vector. The 5′ half-site (AGTTCA) of the DR-4 element was mutated with 5′-GGC C**CC GCG G**TC CTT GCC CTT CAG AGA CC-3′ and 5′-GGA **CCG CGG** GGC CTT ATC ACC TGT TGT GTA-3′, exchanging the 6 bp of the half-site with a *Sac*II site. This mutation was designated HSc, and the mutation is depicted in boldface letters in both of the primers here and in Fig. 5*A*. The 3′ half-site mutation (AGGGCA) was obtained using the primers 5′-TCC T**GA TAT C**TT CAG AGA CCG AGC CAA TAC-3′ and 5′-AA**G ATA TC**A GGA AGT TCA GGC CTT ATC ACC-3′, in which an *Eco*RV site replaced the hexamer half-site. This mutation was called RARc. The double mutation was produced using the HSc mutation as template and the primer 5′-GGC C**CC GCG G**TC CT**G ATA TC**T TCA GAG ACC-3′, together with the second primer from the RARc mutant. This mutation was called double and contained *Sac*II and *Eco*RV restriction endonuclease sites replacing the hexamer half-sites. Finally, mutating the 3′ half-site (AGGGCA) into a canonical half-site (AGTTCA) was achieved using the primers 5′-CTT CCT TG**A A**CT TTC AGA GAC CGA GCC AA-3′ and 5′-AAA GTT CAA GGA AG**T T**CA GGC CTT ATC ACC-3′. This mutation was designated perfect. All constructs were verified by sequencing.

Cloning of the Mouse Phenobarbital-responsive Enhancer Module (PBREM)—The mouse 51-bp PBREM from *Cyp2b10* and the corresponding 51-bp fragment from *Cyp2b9* (28) were synthesized by Intron (Kaltbrunn, Switzerland) with *Xba*I restriction sites at their ends to facilitate ligation into the pBLCAT5 vector. For both the *Cyp2b10* and the *Cyp2b9* 51-bp fragments, two copies were ligated head to tail into the reporter vector, and these constructs were used in all experiments. All constructs were verified by sequencing.

Analysis of Reporter Gene Expression—16 h after drug treatment, the cells were harvested and nonradioactive chloramphenicol acetyltransferase (CAT) assays were performed using the CAT-ELISA kit according to the manual of the supplier (Roche Molecular Biochemicals). Cell extracts were also used for the determination of protein concentration using the ESL protein assay for normalization of specific CAT expression to total protein content (Roche Molecular Biochemicals).

RNA Isolation and Taqman Analysis—RNA from LMH cells was isolated with the RNeasy kit (Qiagen AG, Basel, Switzerland). One μg of total RNA was reverse-transcribed with the Moloney murine leukemia virus reverse transcriptase assay (Roche Molecular Biochemicals). PCR was performed using the Taqman PCR core reagent kit (PE Applied Biosystems), and the transcript level quantitated with an ABI PRISM 7700 sequence detection system (PE Applied Biosystems) according to the manufacturer's protocol. Briefly, relative transcript levels in induced cells and nontreated control cells were determined using the relative quantitation method measuring the $\Delta\Delta$Ct. The following primers and fluorescent probes were used in these PCRs. *CYP2H1*: probe, 5′-TCG CAG TTG CCT CCA GGT CTC CC-3′; forward primer, 5′-AGG GTG GTG AGG GCA AAT C-3′; reverse primer, 5′-ACA GGC ATT GTG ACC AGC AA-3′. *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*): probe, 5′-TGG CGT GCC CAT TGA TCA CAA GTT T-3′; forward primer, 5′-GGT CAC GCT CCT GGA AGA TAG T-3′; reverse primer, 5′-GGG CAC TGT CAA GGC TGA GA-3′. *CYP2H1* and *GAPDH* transcript levels were measured in separate tubes, and *GAPDH* was used for normalization of the *CYP2H1* values.

RESULTS

We sequenced 5.9 kb of 5′-flanking region and used this sequence for further isolation and analyses of PB-responsive elements (Fig. 1*A*). The subfragments derived from a previously reported 4.8-kb enhancer element (25) were inserted into the pBLCAT5 vector containing a heterologous tk promoter. Inducibility was measured in transfected LMH cells after 16 h of incubation with 400 μM PB and compared with control values (Fig. 1*B*). The results revealed two major inducible regions, a 1376-bp fragment located at the 5′ end of the 5.9-kb flanking region (–5913/–4537) that was induced about 5-fold and a 264-bp fragment located at bp –1657/–1393 mediating a 6–7-

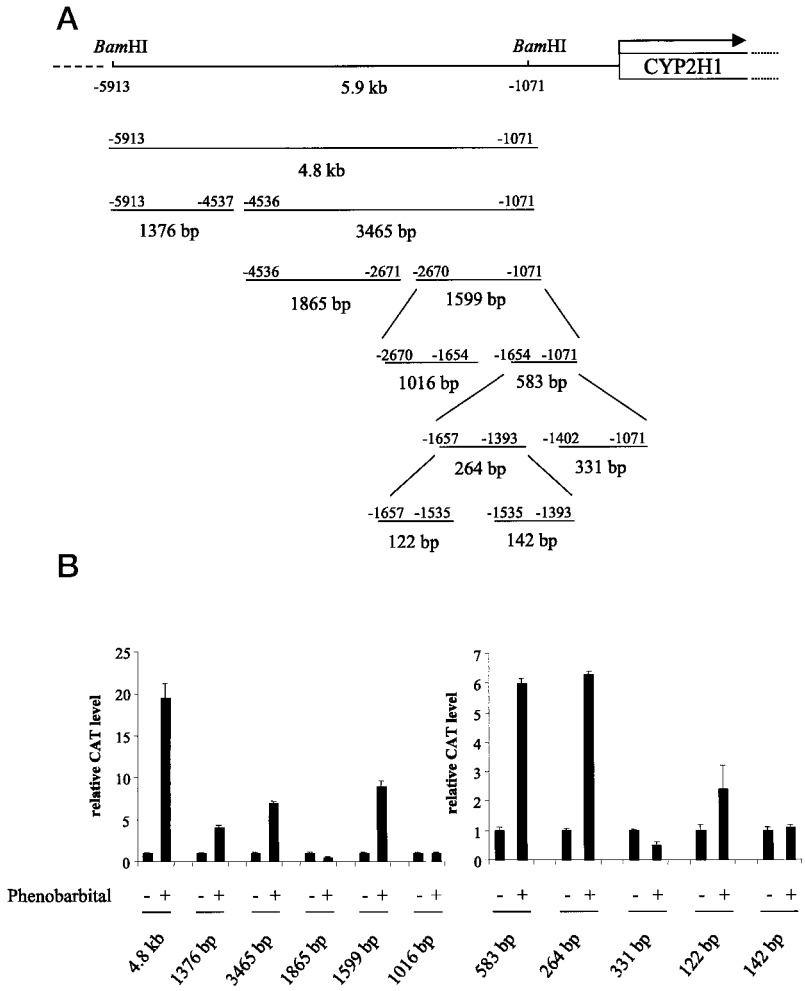

FIG. 1. **Analysis of the 5′-flanking region of *CYP2H1*.** *A,* generation of subfragments of the 4.8-kb enhancer fragment of the chicken *CYP2H1* 5′-flanking region by restriction fragmentation and PCR amplification as described under "Materials and Methods." These fragments were cloned into the pBLCAT5 reporter vector containing a heterologous tk promoter. *B,* reporter gene assays of the fragments. The constructs were transfected into the chicken hepatoma cell line LMH in suspension before plating them on gelatin-coated 6-well dishes to ensure equal transfection efficiencies for control and treated cells. The cells were treated with 400 μM phenobarbital for 16 h and lysed, and a CAT-ELISA was performed. The relative CAT expression was standardized against untreated control cells and expressed in fold induction. Data represent the average of three independent experiments, and *error bars* represent S.D.

fold induction. We further dissected this 264-bp fragment and observed inducibility on the 5′ 122 bp piece (Fig. 1*B*). Because inducibility of this 122-bp fragment was only 2–3-fold under the standard conditions, we used the 264-bp PB-responsive element for subsequent analyses and called it the phenobarbital-responsive unit (PBRU), similarly to the 163-bp PB response unit in rat *CYP2B2* (14). The identification of two response elements of 1376 and 264 bp in length from 5.9 kb of the chicken *CYP2H1* 5′-flanking region allowed us compare the 264-bp PBRU to the described 51-bp PBREM derived from mouse *Cyp2b10* (28) in reporter gene assays.

The 264-bp PBRU from chicken *CYP2H1* was transfected into LMH cells, and reporter gene assays were performed after applying different inducers for 16 h. In parallel, the mouse 51-bp PBREM from *Cyp2b10* and its noninducible mutated counterpart from *Cyp2b9* (28) were transfected into LMH cells as a trans-species comparison. We used PB (400 μM) and the prototypical PB-like inducers propylisopropylacetamide (250 μM) and glutethimide (500 μM), all of which have predominant effects on *CYP2B* genes. Furthermore, dexamethasone (50 μM) and metyrapone (400 μM), which are two common *CYP3A* inducers, and the prototypical *CYP1A1* inducer β-naphthoflavone (10 μM) were also tested. In addition, we were interested in the effects of PCN (50 μM) and rifampicin (100 μM) due to their species-specific effects on *CYP3A*s. The mouse PBREM from *Cyp2b10* was inducible in the chicken LMH cell line, whereas the analogous *Cyp2b9* 51-bp fragment was not inducible (Fig. 2). Those findings are in agreement with previous reports for mouse primary hepatocytes (18, 28). Moreover, the spectrum of inducers activating the chicken 264-bp PBRU closely matched the pattern of the 51-bp *Cyp2b10* PBREM (Fig. 2). The *CYP2H1* PBRU was 12-fold induced by PB, whereas 22-fold induction was observed with metyrapone and glutethimide. The *Cyp2b10* PBREM conferred slightly higher induction levels, particularly 15-fold induction by PB, 21-fold induction by glutethimide, and 30-fold induction by metyrapone. Dexamethasone and β-naphthoflavone exhibited only minor effects, generating about 4-fold induction of the 264-bp PBRU and between 5- and 7-fold induction of the 51-bp PBREM (Fig. 2). PCN and rifampicin activated neither the *CYP2H1* PBRU nor the *Cyp2b10* PBREM significantly (Fig. 2). None of the inducers significantly altered *Cyp2b9* levels. These results show that the chicken 264-bp PBRU and the mouse 51-bp PBREM react in the same way to different chemicals when transfected into LMH cells. Therefore, analysis of the sequence of the 264-bp PBRU and comparison to rodent and human PB-responsive element was attempted.

The MatInspector program was used to analyze the 264-bp PBRU for putative regulatory elements and transcription factor binding sites (accessible on the World Wide Web). This algorithm scans DNA sequences for matches to nucleotide distribution matrices that define putative transcription factor binding sites accessible in the TRANSFAC data base (29). Using a core similarity of 0.75 and an optimized matrix similarity, a total of 45 matches were found. Those of potential interest are depicted in Fig. 3: two putative activator protein-1 sites at –1539/–1533 and –1440/–1434, two E-box family binding sites at –1445/–1433, and a putative nuclear factor-1 (NF-1) binding site at –1607/–1604. These findings are in agreement with previous reports by Dogra *et al.* (26) who assigned the site

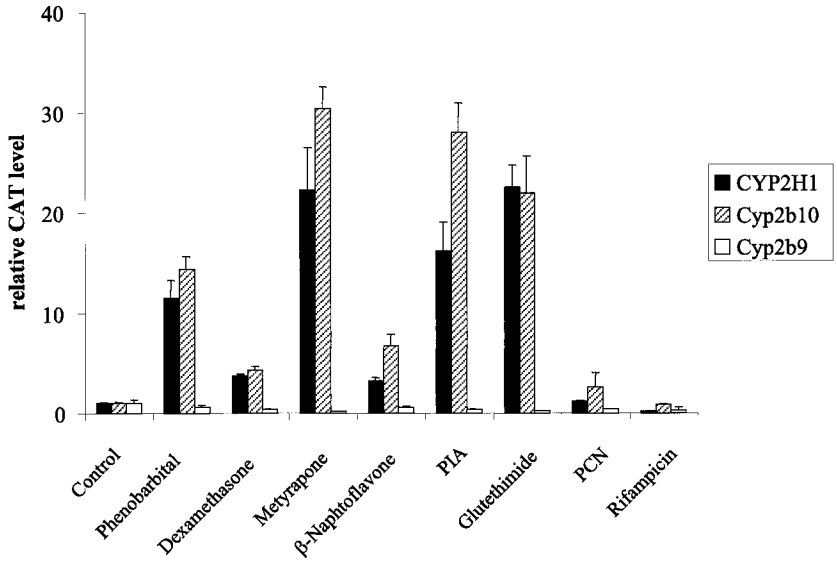

FIG. 2. **Trans-species comparison of the chicken *CYP2H1* 264-bp PB response unit to the mouse *Cyp2b10* 51-bp PB-responsive enhancer module and the corresponding mouse *Cyp2b9* 51-bp fragment in LMH cells.** The PB-responsive elements from the chicken *CYP2H1* and the mouse *Cyp2b10* and *Cyp2b9* were cloned into the pBLCAT5 reporter vector containing a tk promoter as described under "Materials and Methods." The constructs were transfected into LMH cells in suspension, and the cells were induced for 16 h with PB (400 μM), dexamethasone (50 μM), metyrapone (400 μM), β-naphthoflavone (10 μM), propylisopropylacetamide (*PIA*) (250 μM), glutethimide (500 μM), PCN (50 μM), or rifampicin (100 μM). Cells were harvested, and a CAT-ELISA was performed. The relative CAT expression was standardized against untreated control cells and expressed in fold induction. Values represent the average of three independent experiments, and *error bars* represent S.D.

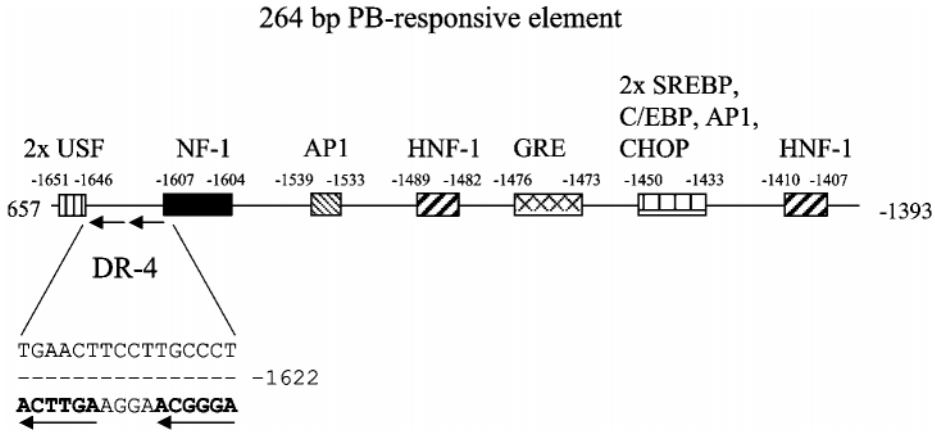

FIG. 3. **Putative transcription factor binding sites on the 264-bp PB response unit and on the *CYP2H1* promoter.** The 264-bp PBRU from the *CYP2H1* 5′-flanking region was analyzed using the MatInspector program for putative transcription factor binding sites defined in the TRANSFAC data base. A core similarity threshold of 0.75 and an optimized matrix similarity were used in the MatInspector program. Hexamer half-sites were searched manually by looking for half-site repeats found in known nuclear receptor binding sites.

at –1607/–1604 to be a CCAAT box binding site, either for CCAAT/enhancer-binding protein (C/EBP) or for NF-1. Our analysis provided a number of additional recognition sites of potential importance for induction of CYPs. The two E-box family sites mentioned above were identified by MatInspector as sterol response element-binding protein consensus elements. Two additional E-box family binding sites at the 5′ end of the 264-bp fragment (–1651/–1646) potentially bind upstream stimulating factor, a basic helix-loop-helix-leucine zipper DNA-binding protein (30). A C/EBP site (–1449/–1446) and a C/EBP homologous protein site (–1450/–1447) are clustered around the activator protein-1 and the sterol response element-binding protein sites, flanked by two hepatic nuclear factor-1 sites (–1489/–1482 and –1410/–1407) and a glucocorticoid receptor consensus site (–1476/–1473). C/EBP homologous protein and C/EBPα heterodimerize and trigger induction after stress stimuli, such as nutrient deprivation, acute-phase response, or toxin exposure (31), whereas hepatic nuclear factor-1 is a member of the family of hepatic transcription regulatory proteins (32). Sterol response element-binding proteins are involved in cholesterol homeostasis by inhibiting the *de novo* synthesis of cholesterol in a negative feedback signaling pathway (33). Mutational analysis of both sterol response element-binding protein sites alone and in combination showed no major effect on PB induction of the 264-bp PBRU (data not shown).

Because nuclear receptors have been demonstrated to be involved in PB induction (6, 11, 12), we searched for hexamer half-sites (34) by defining patterns in the GCG sequence analysis program package and scanning the 264-bp fragment. Two canonical hexamer half-sites (AG(T/G)TCA) and one imperfect hexamer half-site (AGTTGA) were identified at –1571/–1566, –1507/–1502, and –1535/–1530, respectively. None of these sites is in close proximity to each other to form a repeat typical for nuclear receptor binding sites. However, two additional sites at –1637/–1632 and –1627/–1622 form a direct repeat with a spacing of four nucleotides (DR-4) on the complementary strand (Fig. 3). The 5′ hexamer constitutes a canonical nuclear half-site (AGTTCA), whereas the 3′ hexamer forms an imperfect half-site (AGGGCA).

This DR-4 element was found directly upstream of the putative NF-1 binding site, forming a structure that is similar to the ones in rat *CYP2B1/2,* mouse *Cyp2b10,* and human *CYP2B6* PB-responsive elements (Fig. 4*A*). Sequence comparison of the PB-responsive fragments of *CYP2H1*, *Cyp2b10* (28), *CYP2B2*

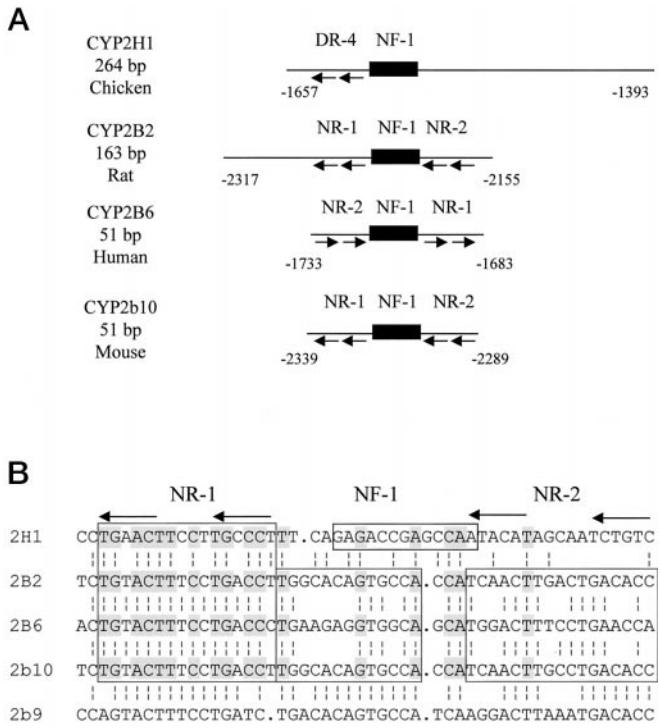

FIG. 4. **Comparison of the structures of the chicken *CYP2H1*, the rat *CYP2B2*, the human *CYP2B6*, and the murine *Cyp2b10* PB-responsive elements.** *A,* structure and localization of the different PB-responsive elements from chicken *CYP2H1,* rat *CYP2B1/2,* human *CYP2B6,* and mouse *Cyp2b10* on their respective PB-responsive elements. The hexamer half-site repeats and the putative NF-1 binding sites reveal the conserved DR-4/NF-1 and NR-1/NF-1 units, respectively. *B,* DNA sequence comparison of the chicken *CYP2H1*, the rat *CYP2B2,* the human *CYP2B6*, the mouse *Cyp2b10,* and the noninducible mouse *Cyp2b9* 51-bp containing the hexamer half-site/NF-1 units. The NR-1, the putative NF-1, and the NR-2 elements are *boxed,* and nucleotides conserved in the four PB-inducible CYPs are highlighted in *gray*.

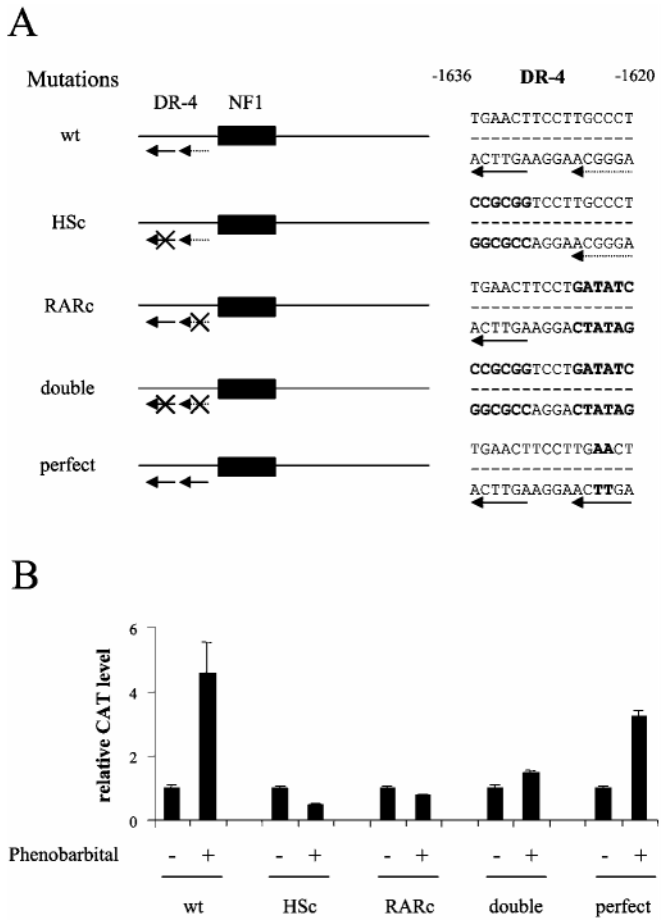

FIG. 5. **Site-directed mutagenesis of the DR-4 element within the 264-bp PB response unit from *CYP2H1*.** *A,* mutations of half-sites were introduced by site-directed mutagenesis using a PCR-based approach as described under "Materials and Methods." Mutations are depicted in *boldface*. The constructs were subcloned into the pBLCAT5 vector containing a tk promoter. *B,* LMH cells were transfected in suspension and treated with 400 μM phenobarbital for 16 h. The cells were harvested, and a CAT-ELISA was performed. The *columns* show relative induction values of expressed CAT protein standardized against nontreated control cells. Values are the average of three independent experiments, and *error bars* represent S.D.

(13, 14), *CYP2B6* (19), and *Cyp2b9* (28) revealed that the NR-1 site is conserved among the inducible CYPs. One mismatch is present in each half-site in the *CYP2H1* when compared with the rodent and human CYPs. A spacing of four nucleotides is common in chicken *CYP2H1*, rat *CYP2B2*, mouse *Cyp2b10,* and human *CYP2B6* (Fig. 4*B*). On the 3′ side of the NF-1 binding site, no NR-2-like element was observed in the chicken *CYP2H1* sequence, in contrast to the rodent and human response elements. Several putative transcription factor binding sites possibly involved in CYP regulation were identified on the 264-bp PBRU. Of these, a hexamer repeat was chosen for further analysis in this report, as this arrangement of binding sites is found in several PB-responsive elements of rodent and human CYPs.

The significance of the DR-4 element found in the 264-bp PBRU from chicken *CYP2H1* on PB induction was tested by site-directed mutagenesis of the hexamer half-sites. Single knock-outs of the 5′ hexamer half site (AGTTCA to CCGCGG, designated HSc), the 3′ hexamer half-site (AGGGCA to GGTATC, designated RARc), the double knock-out of both half-sites (designated double) and mutation of the more proximal half-site into a canonical hexamer half-site (AGGGCA to AGTTCA, designated perfect) were performed by PCR (Fig. 5*A*). Mutagenized fragments were cloned into the pBLCAT5 reporter vector and these constructs were transfected into LMH cells. Inducibility was measured after treating the cells for 16 h with 400 μM PB. Mutation of either half-site alone or both together eliminated PB induction of the 264-bp PBRU (Fig. 5*B*). When the more proximal half-site was mutated into a canonical hexamer half-site, 3-fold induction was observed; this level was lower than that detected (5-fold induction) in the wild-type construct. These findings are similar to those reported for the *Cyp2b10* PBREM in mouse primary hepatocytes (23). Thus, the DR-4 element found within the 264-bp PBRU plays a major role in mediating PB induction of this enhancer element.

Protein phosphorylation and dephosphorylation has a major effect on PB induction (for a review, see Ref. 10, and for more recent reports, see Refs. 35–37). Inhibition of protein phosphatases by okadaic acid (OA) has been linked to decreased PB induction of rat *CYP2B1/2* (38) and mouse *Cyp2b10* (39), *Cyp2a5,* and *Cyp1A* (37), as well as to an increase in mRNA of rat UDP-glucoronosyl transferase, another PB-inducible enzyme (40). Additionally, OA has been proposed to inhibit PB-triggered nuclear translocation of the orphan nuclear receptor CAR in mouse liver *in vivo* (41). In our experiments, OA increased transcript levels of δ-aminolevulinate synthase, the first and rate-limiting enzyme in heme synthesis, but inhibited induction of chicken *CYP3A37* in LMH cells.[2]

Induction of *CYP2H1* was measured after 16 h of exposure of

[2] C. Handschin and U. A. Meyer, unpublished observations.

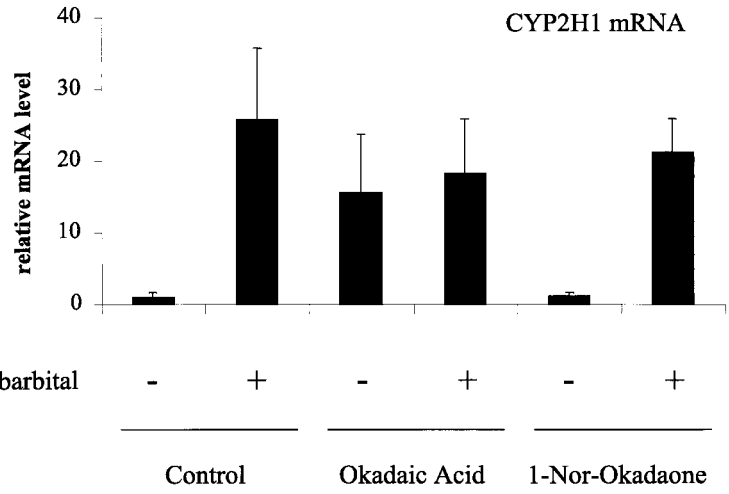

FIG. 6. **Inhibition of protein phosphatases by okadaic acid induces *CYP2H1* mRNA.** LMH cells were treated with 400 μM PB and/or 1 μM OA for 16 h, and total RNA was isolated and reverse transcribed. Relative mRNA levels of *CYP2H1* from treated cells against control cells was determined with a Taqman ABI PRISM 7700 sequence detection system and normalized against GAPDH mRNA level as described under "Materials and Methods." Values are the average of three independent experiments, and the *error bars* represent S.D.

LMH cells to 1 μM OA or 1 μM 1-nor-okadaone either in the absence or presence of 400 μM PB. The structural analog 1-nor-okadaone of OA that does not inhibit protein phosphatases and was therefore used as negative control. Quantification of *CYP2H1* mRNA was performed using the Taqman real-time PCR quantification system as described under "Materials and Methods." OA alone increased *CYP2H1* mRNA levels 15-fold, whereas the negative analog 1-nor-okadaone had no effect (Fig. 6). The extent of induction by OA was in general lower than induction of *CYP2H1* mRNA by PB that triggered a 25-fold increase. When adding PB to OA-treated cells, no additive effect on transcript levels were observed and a 17-fold induction was obtained.

Surprisingly, when testing the effect of OA with the 4.8-kb enhancer fragment of the 5′-flanking region of *CYP2H1* after 16 h of incubation, 1 μM OA inhibited the 20-fold PB induction (Fig. 7*A*). In contrast, 1 μM OA activated the more proximal 1.1-kb fragment (–1057/–1) that contains the *CYP2H1* promoter 8-fold after 16 h of incubation. This fragment was not inducible by 400 μM PB after 16 h (Fig. 7*A*). To further localize the effects exerted by OA, we tested the 264-bp PBRU and the previously characterized *CYP2H1* promoter (–205/–1) in reporter gene assays (27). The promoter construct was 5-fold induced by OA comparable to the 8-fold induction of the 1.1-kb fragment after 16 h treatment with 1 μM OA. The 10-fold 264-bp PBRU induction by PB was abolished by 1 μM OA after 16 h. Inhibition of dephosphorylation by OA blocks PB induction of the 264-bp PBRU, which is in agreement with the hypothesis that OA blocks PB-triggered nuclear translocation of the orphan nuclear receptor CAR in mouse liver (41). OA further directly activates transcription from the *CYP2H1* promoter presumably by inhibiting dephosphorylation of as yet unknown proteins. Nevertheless, our results show the similarity between the chicken 264-bp PBRU and the rodent 51-bp PBREM in regard of regulation and modulation of PB-type induction mechanisms.

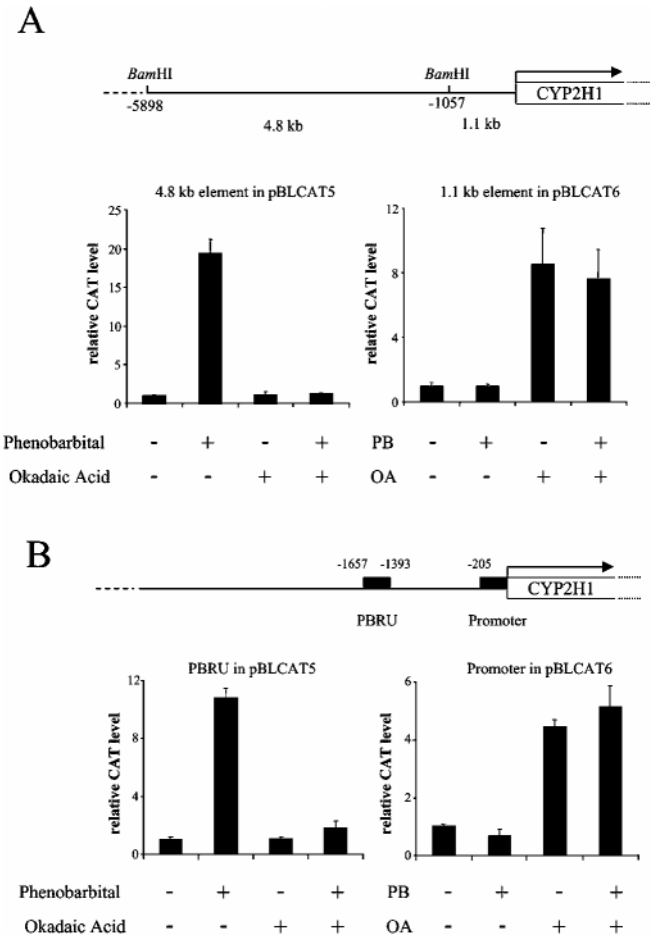

FIG. 7. **Localization of the okadaic acid effect on the 5′-flanking region of *CYP2H1*.** *A,* LMH cells were transfected in suspension either with the pBLCAT5 vector containing a tk promoter and the 4.8-kb enhancer fragment from *CYP2H1* or the pBLCAT6 vector lacking a heterologous promoter but containing the 1.1-kb proximal 5′-flanking region of *CYP2H1*. Cells were treated for 16 h with 400 μM PB and/or 1 μM OA and harvested, and a CAT-ELISA was performed. The *columns* represent the relative induction of expressed CAT protein normalized to nontreated control cells. Values are the average of three independent experiments, and the *error bars* represent S.D. *B,* the same protocol was followed as described in *A* but using the constructs of the 264-bp PB-responsive unit in pBLCAT5 and the 205-bp promoter in pBLCAT6. Values are the average of three independent experiments, and the *error bars* represent S.D.

DISCUSSION

In this report, we describe a 264-bp fragment in the 5′-flanking region of the chicken *CYP2H1* gene that responds to phenobarbital. Sequence analysis of this fragment revealed a conserved nuclear receptor consensus sequence arranged as a direct hexamer repeat with 4-bp spacing, a so-called DR-4 element. Mutation of this DR-4 sequence eliminated PB response under the chosen conditions. However, in experiments using serum-deprived medium, we observed some residual PB induction on the mutated DR-4 constructs corresponding to about 10% of PB induction of the wild-type 264-bp PBRU,

indicating that other elements on the 264-bp PBRU may also contribute to total PB induction.[2] The DR-4 element of the *CYP2H1* gene described here resembles PBRUs or PBREMs in the 5′-flanking region of rat *CYP2B1* and *CYP2B2* (13, 14), mouse *Cyp2b10* (18), and human *CYP2B6* (19), indicating evolutionary conservation of this enhancer sequence.

A mouse 51-bp PBREM from *Cyp2b10* transfected into avian hepatoma cells responded to drugs and chemicals in fashion similar to the *CYP2H1* PBRU. For example, PB and metyrapone strongly induced both the mouse 51-bp and the chicken 264-bp element, whereas dexamethasone and β-naphthoflavone had only weak effects on both response elements. The results with the 51-bp mouse PBREM in avian hepatoma cells closely resemble data from mouse hepatocyte primary cultures (28). High induction pattern similarity between the avian 264-bp enhancer element and the mouse 51-bp PBREM in the chicken hepatoma cells suggests similar or identical transcription factors to be responsible for activation of these enhancers in chicken and mouse.

Different conclusions were reached by Dogra *et al.* (26), who analyzed a 240-bp element that overlaps with the 264-bp sequence reported here with exception of the most 5′ 17 bp and the most 3′ 7 bp. They interpreted their sequence analysis as revealing no common protein binding sites with the rodent enhancers and suggested that the proteins binding to the chicken enhancer must be different from those required for the activity of the PBRUs of mouse and rat CYP genes (26). One reason for this discrepancy may be the limited PB inducibility of the 240-bp fragment 2–3-fold after 48 h using 500 μM PB in chicken primary hepatocytes (26). Moreover, employing a larger fragment of 556 bp containing the 240-bp enhancer resulted in 7-fold induction of CAT expression (26). In comparison to these results obtained in the primary hepatocyte cultures, inductions of the 264-bp PBRU in LMH cells were 6–10-fold after 16 h treatment with 400 μM PB. It appears that either LMH cells are more responsive to PB-effects or that other factors such as easier transfection or higher cell homogeneity contribute to the discrepancy. Furthermore, the additional bp of our fragment may have an enhancing effect on PB inducibility compared with the shorter 240-bp fragment of Dogra *et al.* (26).

We applied the MatInspector program that refers to the TRANSFAC data base for identifying putative transcription factor binding sites (29). Of 45 recorded hits, we chose candidates likely to modify CYP regulation. A weak consensus sequence of the glucocorticoid receptor binding site at bp –2243/–2225 was observed to be essential for conferring maximal PB inducibility in the rat *CYP2B1/2* 163-bp PBRU (14). Furthermore, glucocorticoid-responsive elements have been noted in other PB-responsive fragments in *CYP2B* genes at –1.4/–1.2 kb outside the PBRU or PBREM (reviewed in Ref. 42). The arrangement of a DR-4 element upstream of a putative NF-1 site in the chicken *CYP2H1* PBRU is repeated in the mouse 51-bp PBREM and the rat *CYP2B2* PBRU (14, 23). The putative NF-1 binding in the chicken PBRU is more distant from the NR-1 site and on the opposite strand compared with the rat and mouse elements. Moreover, the 5′ part of the bipartite NF-1 motif is not a canonical one in the chicken. Nevertheless, the chicken putative NF-1 site is more closely related to the NF-1 consensus than the putative NF-1 site on the human *CYP2B6* 51-bp PBREM (see Fig. 4*B*), and comparison of the chicken sequence to the TRANSFAC matrix for NF-1 reveals high conservation. Mutational analysis of the NF-1 site *in vitro* in reporter gene assays reduced PB inducibility of the mouse 51-bp PBREM (23), whereas mutations of the NF-1 site of the rat PBRU in transgenic mice had no effect on inducibility (43). Therefore, the role of the NF-1 site in PB induction remains unclear. In the mouse 51-bp PBREM, the NR-1 site has been shown to be sufficient for mediating PB induction (23). Therefore, the lack of a clear NR-2 site in the chicken 264-bp PBRU may be of no consequence. A more in-depth analysis of the different putative binding sites is currently being attempted.

Honkakoski *et al.* (23), in a series of elegant experiments, have demonstrated that the orphan nuclear receptor CAR forms heterodimers with the retinoid X receptor and binds to the mouse *Cyp2b10* 51-bp PBREM in response to drug treatment (23). CAR was originally found to constitutively activate gene transcription in human (44) and mouse cells (45). A mechanism by which CAR mediates drug induction was recently proposed by Kawamoto *et al.* (41), who postulated that CAR is located in the cytoplasm in the noninduced state and that PB or PB-like inducers lead to nuclear translocation with subsequent activation of CYPs (41). The protein phosphatase inhibitor OA could inhibit this nuclear translocation by unidentified mechanisms (41).

Moreover, OA and several other protein phosphatase or protein kinase inhibitors have been shown to affect PB induction in mouse, rat, and chicken (for a review, see Ref. 10). Induction of both rat *CYP2B1/2* (38) and mouse *Cyp2b10* mRNA (39) by PB was inhibited by OA, whereas the chicken *CYP2H1* transcript level was elevated after exposure to OA alone. Inhibition of protein phosphatases by OA leading to increased phosphorylation of different transcription factors such as C/EBP (46) and hyperphosphorylation of transcription factors or regulatory proteins have been associated with increased gene transcription (47). However, down-regulation of genes such as the glucose-induced transcription of the *S14* gene by OA has also been observed (48). Moreover, OA has been reported to increase the PB-inducible phase II enzyme UDP-glucoronosyl transferase in rat (40). Thus OA exhibits a variety of gene-specific effects on transcription by influencing protein interaction with different regulatory elements. In this study, we show that OA predominantly activates transcription via the proximal promoter of *CYP2H1* and blocks PB induction at the level of the PBRU. These findings explain why *CYP2H1* mRNA is induced by OA to a certain level and that this OA induction is not changed when adding PB. Our results may explain why in the rat, OA inhibits *CYP2B1/2* mRNA induction by PB but induces a *CYP2B1/2* minigene containing the proximal sequence of the 5′-flanking region in reporter gene assays (39, 49). The similarity of these data to the inhibition of the transfer of CAR to the nucleus in mouse liver suggests that a CAR-like receptor protein is involved in induction mediated by the 264-bp PBRU in the *CYP2H1* 5′-flanking region of the chicken.

Despite its considerable activation by PB, it is suspected that the PBRU described here does not account for all of the transcriptional activation of the *CYP2H1* gene expression *in vivo*. Additional elements, including the 1376-bp fragment in the more distal 5′-flanking region of *CYP2H1,* have been shown to be PB-inducible. Comprehensive analysis of this element is currently being carried out. Also, the induction of the 264-bp PBRU in reporter gene assays with different drugs does not fully explain the induction pattern of *CYP2H1* mRNA. For example, PCN exhibits only minor effects on the 264-bp PBRU, whereas the same drug markedly induced *CYP2H1* gene expression in LMH cells (50). Receptor cross-talk and multiple response elements are observed in rodent and human PB-inducible CYP flanking regions. To date, the mouse 51-bp PBREM is the only well characterized PB-responsive element in the *Cyp2b10* 5′-flanking region, and the rat *CYP2B*s clearly contain more than one enhancer element (reviewed in Refs. 7 and 42). In another recent report, the flanking region of the

human *CYP3A4* was shown to contain at least three different hexamer half-site repeats, all contributing to the total response to rifampicin and PB (51), supporting the concept of multiple drug response elements in these genes. On the basis of these observations, we hypothesize that a variety of different response elements and several nuclear receptors may be responsible for the overall response of *CYP2H1* to PB and PB-type inducers. Candidate proteins include nuclear receptors, such as CAR or pregnane X receptor, which are the predominant regulators of *CYP3A* genes (21, 22), because *CYP2H1* induction was similar to the chicken *CYP3A37* response after treatment with different drugs (50).

We conclude from the present investigation that the chicken hepatoma cell line LMH is a valuable tool for the study of the regulatory mechanism of PB-type induction. LMH cells are the first PB-inducible cell line showing both qualitative and quantitative responses comparable to *in vivo* findings. They offer all of the advantages inherent to a continuously dividing cell line in the investigation of the transcription factors and response elements for drug-inducible genes. Therefore, our current investigations focus on identifying chicken nuclear receptors involved in PB induction and the subsequent study of the interactions and redundancies of receptors and response elements mediating PB response in LMH cells. Moreover, comparative mapping of homologous genes in different organisms surprisingly revealed that the organization of the human genome is closer to that of the chicken than that of the mouse (52). These findings and the results presented in this report suggest the chicken to be a potentially invaluable model system for comparison studies of molecular mechanisms of PB induction in chicken, rodents, and humans.

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A Conserved Nuclear Receptor Consensus Sequence (DR-4) Mediates Transcriptional Activation of the Chicken *CYP2H1* Gene by Phenobarbital in a Hepatoma Cell Line

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